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Revisiting the antifibrinolytic effect of carboxypeptidase N:
novel structure and regulation

by

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Dedication

To Mom, Dad, and Alexx:

Thank you for your endless love and support.

Abstract

Carboxypeptidase N (CPN) is a plasma carboxypeptidase that was discovered in the 1960s as a regulator of inflammation and vascular tone. Through the removal of carboxy-terminal basic residues, CPN alters the activity or binding specificity of inflammatory mediators and vasoactive peptides. CPN shares significant homology with carboxypeptidases known to mediate antifibrinolysis through the removal of basic residues from fibrin clots, which would otherwise stimulate fibrinolysis. Despite the similarity of these enzymes, CPN is generally regarded as lacking a role in fibrinolysis. This thesis demonstrates that CPN is indeed a capable antifibrinolytic enzyme, and that the antifibrinolytic activity of CPN was previously undisclosed due to the presence of a circulating CPN inhibitor, which is likely the free CPN2 subunit. This inhibitor is described for the first time here. Furthermore, potential mechanisms of inhibition and mechanisms of enhancing activity of CPN are proposed based upon the additional structural characterization of CPN presented here.

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List of Symbols, Nomenclature, and Abbreviations

εACA: ε-amino caproic acid

AIIt: annexin II heterotetramer

ACE: angiotensin I converting enzyme

AMPSA: aminopropylmercaptosuccinic acid

BSA: bovine serum albumin

CIP: calf intestinal alkaline phosphatase

CK: creatine kinase (also CK-MM, CK-MB, and CK-BB are isoforms of CK)

CP: carboxypeptidase (e.g. CPA, CPB, CPD, CPH, CPE, CPM, CPZ)

CPN1: CPN catalytic subunit

CPN2: CPN regulatory subunit

CPNc: plasmin-cleaved CPN (including two-chain CPN)

CPN2c: plasmin-cleaved CPN2

DMEM: Dulbecco's modified Eagle medium

dn/dc: refractive index increment

DTT: dithiothreitol

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

FA-Ala-Lys: furylacryloyl-alaninyl-lysine

FBS: fetal bovine serum

GEMSA: 2-guanidinoethylmercaptosuccinic acid

GFP: green fluorescence protein

GPSA: guanidinopropylsucininc acid

HRP: horseradish peroxidase

K_M: Michaelis constant

k_{cat}: catalytic rate constant

K_d: dissociation constant

K_i: inhibitory constant

k_{cat} /K_M: catalytic efficiency

LRR: leucine rich repeat

MALLS: multi-angle laser light scattering

MEM: minimal essential media

MERGETPA: DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid

NHP: normal human plasma

NHPdCPN: CPN-depleted normal human plasma

P_1: residue on the amino-terminal side of a proteolytic cleavage site

P_1': residue on the carboxy-terminal side of a proteolytic cleavage site

PAI-1: plasminogen activator inhibitor-1

PDB: protein databank

pI: isoelectric point

ITS+: insulin-transferrin-selenium

PCR: polymerase chain reaction

PPACK: D-phenylalanylprolylarginyl cloromethyl ketone

PTCI: potato tuber carboxypeptidase inhibitor

rCPN2: recombinant CPN2

R_M: retention value

S-2251: HD-valyl-L-leucyl-L-lysine-p-nitroaniline dihydrochloride

SDF-1: stromal cell-derived factor-1

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

Serpin: serine protease inhibitor

TAFI(a): (activated) thrombin activatable fibrinolysis inhibitor

tPA: tissue-type plasminogen activator

TT: transthyretin

uPA: urokinase-type plasminogen activator (also, sc- (single-chain) and tc- (two-chain) uPA)

VFKCK: d-valylphenylalanyllysyl chloromethyl ketone

1. INTRODUCTION

1.1. Literature review

1.1.1. The fibrinolytic system

The primary protein within blood clots is an extensive network of fibrin. When clots form inappropriately within the vasculature they restrict blood flow and can cause lethal conditions such as a heart attack or stroke. The fibrinolytic system comprises a cascade of enzymatic reactions leading to the breakdown of fibrin clots to re-establish blood flow, as shown in Figure 1.1. Ultimately, the enzyme responsible for clot lysis is plasmin, a serine protease produced by the proteolytic activation of the zymogen plasminogen. The activation of plasminogen is the primary target for many of the regulatory mechanisms influencing fibrinolysis. To gain a full appreciation of fibrinolysis and the systems that regulate it, it is necessary to first examine the formation and structure of a fibrin clot.

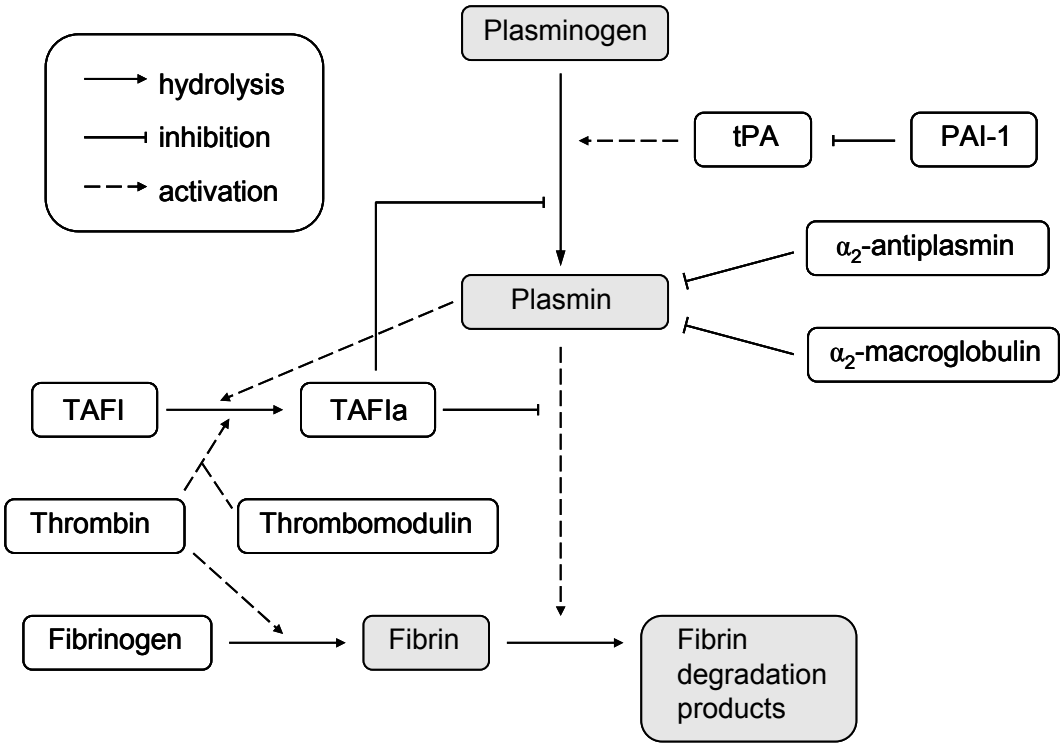


Figure 1.1. The fibrinolytic system. Shaded boxes represent the main steps to fibrinolysis, the white boxes represent the regulators of fibrinolysis. The actions of inhibitors are denoted by blunt-ended arrows; the actions of activators are denoted by dashed arrows; and protein conversion is denoted by solid arrows. tPA is tissue-type plasminogen activator; PAI-1 is plasminogen activator inhibitor-1; and TAFI is thrombin activatable fibrinolysis inhibitor. Some crossover with the coagulation cascade is also depicted, including the conversion of fibrinogen to fibrin.

1.1.1.1. Formation of fibrin clots

The result of the complex multi-component coagulation cascade is the activation of prothrombin to thrombin (EC 3.4.21.5). Thrombin is the enzyme responsible for cleaving fibrinogen precursor molecules into fibrin monomers; and these polymerize to form the fibrin meshwork of blood clots.

Fibrinogen is a 340 kDa glycoprotein composed of six polypeptide chains, two each of Aα, Bβ and γ (*1*). These polypeptide chains are linked by 27 intra- and inter-chain disulfide bonds into a symmetrical tertiary structure, shown in Figure 1.2. The amino-termini of the six chains bundle together to form a central globular domain, known as the E region. Two α-helical coiled coil regions (each comprising three chains: Aα, Bβ and γ) extend out in opposite directions from the central E region. The carboxy-termini of the Bβ and γ chains cluster to form two identical peripheral globular domains, called the D regions, while the carboxy-terminus of the Aα chain ($A\alpha^{220}$-$A\alpha^{610}$) bends back towards the central E region in a structure known as the αC region (*2-5*).

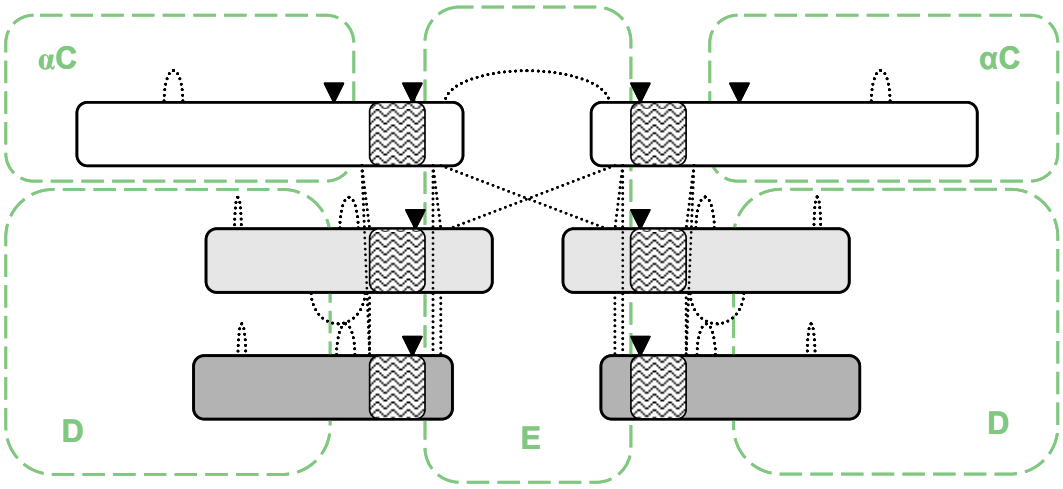


Figure 1.2: The structure of fibrinogen. The Aα chains are coloured white, the Bβ chains are coloured light grey and the γ chains are coloured dark grey. Disulfide bonds are represented as dotted lines; of the 51 sites, important plasmin cleavage sites are indicated with black triangles; and the coiled-coil regions are represented by patterned areas. The D, E and αC regions are labelled by green dashed boxes. The αC region should curl back upon itself, towards the E region, but for simplicity the entire Aα is depicted as a linear chain.

Fifteen inter-chain disulfides link the Aα, Bβ and γ polypeptide chains by forming disulfide rings on the outer edges of each D and E region (*5*). These disulfides link the Aα and Aα chains, the Aα and Bβ chains, the Bβ and γ chains and the γ and Aα chains (*6;7*). These disulfide rings hold the chains together throughout the major structural rearrangements that occur during fibrin clot formation and its subsequent dissolution.

Thrombin, formed as a consequence of the coagulation cascade, rapidly cleaves fibrinogen at the amino-termini of the Aα chain, releasing a small peptide (residues 1-16) known as fibrinopeptide A from the large remaining α chain, and exposes the cryptic polymerization site E_A (named for its presence in the E domain and for its display following fibrinopeptide A release) (*7-11*). The E_A polymerization site contains an exposed sequence known as the A-knob (beginning with the residues Gly-Pro-Arg-…) which can associate with a constitutive complementary A-hole in the D_a polymerization site (named for its presence in the D domain and for its interactions following fibrinopeptide A release), which is located on one of the outer D regions of a neighbouring molecule (*9;10;12-15*). Association of the A-hole of the D_a polymerization site with the A-knob of the E_A polymerization site creates what are known as the D:E interactions, and constitutes a major mechanism of lateral association of fibrin monomers.

Thrombin also releases fibrinopeptide B (residues 1-14) from the amino-termini of the Bβ chain of fibrinogen, albeit at a much slower rate (*3;9*). Analogous to fibrinopeptide A release, proteolytic cleavage of fibrinopeptide B exposes the B-knob (with a sequence beginning with the residues Gly-His-Arg-…) in the E_B polymerization site. This B-knob has a constitutive complementary B-hole on the D_b polymerization site located in the D domain of a neighbouring molecule (*12;16-18*). Association of the B-hole of the D_b polymerization site with the B-knob of the E_B polymerization site creates further D:E interactions.

The thrombin-catalyzed release of fibrinopeptides A and B from fibrinogen generates fibrin monomers. These spontaneously polymerize due to the interactions at the complementary E_A-D_a and E_B-D_b sites (*19*). The newly formed D:E interactions create a half-staggered, end-to-middle domain pairing of neighbouring molecules, which ultimately results in the formation of elongated twisting double-stranded protofibrils as

additional fibrin monomers associate (Figure 1.3) (*19-30*). Protofibrils interact laterally with other protofibrils, and coalesce into fibrin fibers (*31*).

In addition to the D:E interactions, further interactions known as D:D and γ_{XL}:γ_{XL} occur between adjacent fibrin monomers through interactions of the D domains. Both D:D and γ_{XL}:γ_{XL} interactions stabilize the formation of the fibrin polymer by facilitating the end-to-end alignment of the fibrin monomers. Moreover, transverse γ_{XL}:γ_{XL} interactions aid in the association of γ chain carboxy-termini of neighbouring molecules, promoting fibrin cross-linking by factor XIIIa (EC 2.3.2.13) (it is from this role that the name γ_{XL} arises: cross (X)-linking (L) between γ chains) (*11;19;32*).

Factor XIIIa is a transglutaminase, which is activated from its zymogen form (factor XIII) by thrombin. Factor XIII is a heterotetramer of two catalytic subunits and two carrier subunits. Proteolytic cleavage by thrombin at Arg^{37}-Gly^{38} activates the catalytic subunits and following dissociation of the carrier subunits, factor XIIIa becomes fully active (*3*).

Stabilization and cross-linking of fibrin molecules first occurs at the γ_{XL}:γ_{XL} interaction sites of adjacent fibrin monomers. Factor XIIIa forms intermolecular ε-amino(γ-glutamyl) lysine isopeptide bonds between Lys^{406} of one γ chain and Glu^{398} or Glu^{399} of the neighbouring γ chain (*3;19;33-38*). This produces two antiparallel isopeptide bonds between the two molecules. Factor XIIIa also forms intermolecular cross-links between α chains, but at a much slower rate. These secondary links help create oligomers and may later play a role in retarding plasmin cleavage during fibrinolysis (*33;36;37*).

Factor XIIIa also cross-links several plasma proteins into the fibrin network. Of particular importance is the inclusion of α_2-antiplasmin. This serine protease inhibitor (serpin) is the primary physiological inhibitor of the clot lysing enzyme plasmin (*39;40*). Factor XIIIa cross-links Gln^2 of α_2-antiplasmin to Lys^{303} of the fibrin α chain (*41-43*). Similarly, factor XIIIa cross-links thrombin activatable fibrinolysis inhibitor (TAFI; another inhibitor of the fibrinolytic system) to the fibrin meshwork at TAFI residues Gln^2, Gln^5 or Gln^{252} (*44*).

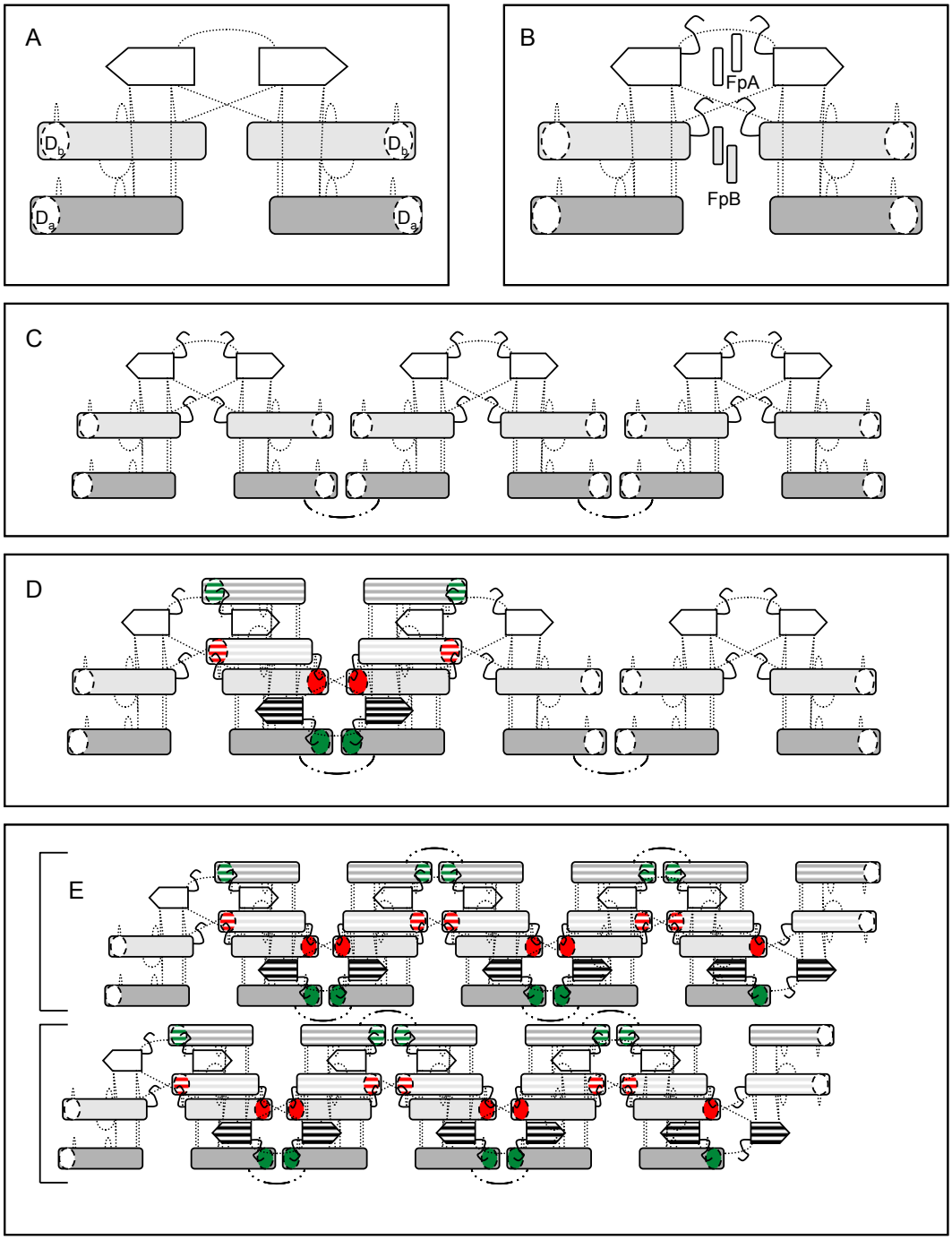

A
D_b
D_b
D_a
D_a
B
FpA
FpB
C
D
E

Figure 1.3: The association of fibrin monomers. Panel A: a single fibrinogen molecule, with D_a and D_b polymerization sites labelled. The αC region folds back onto itself, forming a separate globular domain which cannot be accurately represented in this two dimensional figure, therefore the αC regions have been omitted and the α chain has been replaced with an arrowhead to represent the missing portion of the chain. Panel B: thrombin-mediated cleavage of fibrinogen removes fibrinopeptides A and B, resulting in a fibrin monomer. Removal of fibrinopeptides exposes the A- and B-knobs of the E_A and E_B polymerization sites. The A- and B-knobs are depicted as "S" shapes. Panel C: fibrin monomers spontaneously associate laterally through D:D and γ_{XL}:γ_{XL} interactions, depicted as patterned lines. Panel D: fibrin monomers associate with inverted monomers in a half-staggered, overlapping pattern due to E_A-D_a and E_B-D_b interactions. For clarity, E_A-D_a associations are coloured in green and E_B-D_b associations are coloured in red. All fibrin molecules of one orientation are solid in colour, while those fibrin molecules of the inverse orientation are striped. Panel E: as more fibrin monomers associate, twisted double-stranded protofibrils are generated. These then associate laterally with other protofibrils creating larger fibers and the fibrin meshwork. Two protofibrils are depicted in panel E; they are demarcated by brackets on the left side. This figure is a composite of data from references 1-38.

Other proteins which are incorporated into the fibrin clot by factor XIIIa include plasminogen activator inhibitor-2 (*45;46*), fibronectin (*47;48*), collagen (*47;49*), von Willebrant factor (*50;51*), vitronectin (*52;53*), thrombospondin (*54*), coagulation factor V (*55;56*), actin (*57;58*), myosin (*59*), vinculin (*60*) and platelet integrin $\alpha_{IIb}\beta_3$ (*61*). Cross-linking of coagulation or fibrinolytic components into the fibrin meshwork can alter the activities of these enzymes: it localizes the components to the fibrin clot thereby increasing their effective local concentrations and increasing their apparent activities, but it can also prevent the enzymes from accessing other sites on the clot, thereby decreasing their effective activities. The other commonly cross-linked proteins are involved in extracellular matrix formation, structure and cell attachment. The role of fibrin in binding to these proteins may be related to its role in tissue remodelling and therefore these proteins may be of less importance in coagulation and fibrinolysis.

1.1.1.2. Dissolution of fibrin clots

The breakdown of the fibrin meshwork is achieved by the activated fibrinolytic system. Plasminogen, a single-chain glycoprotein of 92 kDa that circulates at a concentration of 2 μM in plasma, is the precursor to the fibrin-dissolving enzyme plasmin (EC 3.4.21.7) (*62;63*). Plasminogen can be activated to plasmin through proteolytic cleavage at its Arg^{560}-Val^{561} peptide bond by tissue-type plasminogen activator (tPA; EC 3.4.21.31) or by urokinase-type plasminogen activator (uPA; EC 3.4.21.73) (*63*).

Plasmin is a serine protease with trypsin-like specificity; therefore it cleaves substrates following basic residues: arginine or lysine. Aside from this P_1 residue restriction, plasmin has a broad substrate specificity (*64*). It readily proteolyzes fibrin, exposing carboxy-terminal basic residues in its wake. Plasmin must cleave six peptide bonds to traverse the full thickness of a single fibrin protofibril due to the presence of three disulfide-linked polypeptide chains (α, β and γ) in each alpha helical coiled coil, and two coiled coils per protofibril depth, as shown in Figure 1.4. Depending on the frequency of plasmin cleavage sites, soluble fibrin degradation products are released (*65*). Plasmin does not always cleave the full thickness of the protofibril; therefore non-productive nicks are also created during plasmic cleavage. However, as nicks accumulate, the chances of a productive cleavage resulting in the release of a soluble product increases, therefore this stochastic process can be interpreted as positive cooperativity.

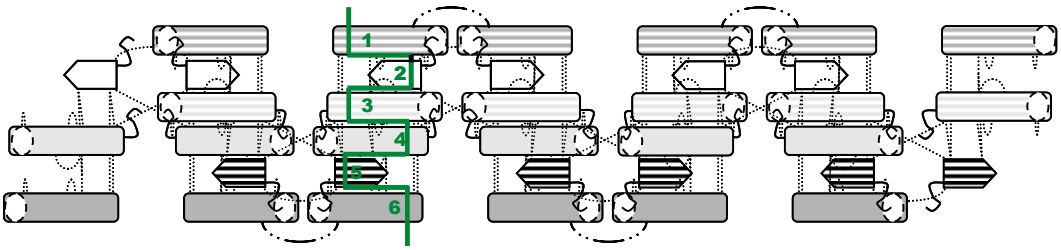
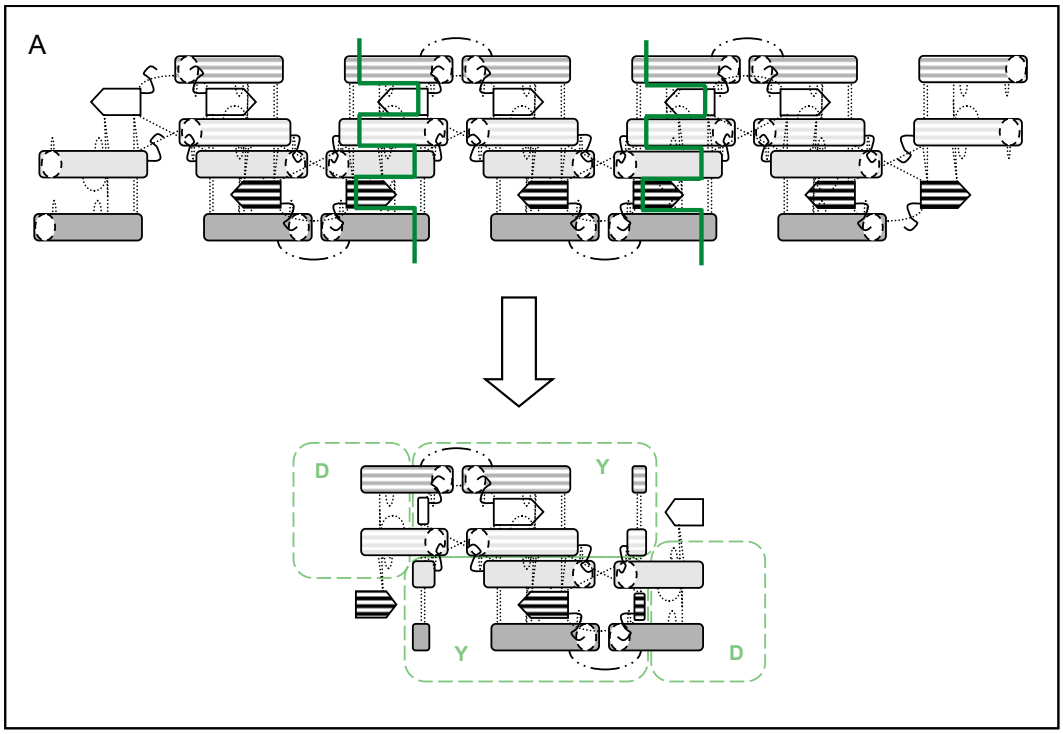


Figure 1.4: Plasmic cleavage of fibrin. Plasmin must cleave six polypeptide chains to traverse the entire thickness of a protofibril, as depicted by the solid black line with numbered cleavage sites.

The terminal fibrin degradation product resulting from plasmic digestion is known as (DD)E, shown in Figure 1.5 (*65-68*). This 260 kDa product comprises two D regions (originating from two distinct fibrinogen molecules that were covalently linked end-to-end by factor XIIIa) and a non-covalently attached E region (originating from a third distinct fibrinogen molecule) (*12*). Soluble (DD)E arises as a result of minimally 14 plasmic cleavages: 4 α chains are cleaved at Lys^{78}-Asn^{79}, 4 β chains are cleaved at Lys^{122}-Asp^{123}, 4 γ chains are cleaved at Lys^{62}-Ala^{63}, and two 2 α chains (one per D region) are cleaved in the αC region at Lys^{219}-Ser^{220} (*2;69-71*). Several modified versions of (DD)E caused by excessive plasmic digestion also exist, including $(DD)E_3$, which is cleaved at the Lys^{53}-Thr^{54} peptide bond of the γ chain, which releases it from the covalent DD molecule (*68-72*).

(DD)E is not the only soluble fibrin degradation product, several others, which are shown in Figure 1.5, can exist depending on the distance between each set of full protofibril-thickness plasmic cleavages. Common small soluble fibrin degradation products include the 520 kDa (DY)YD, and the 780 kDa (DXD)YY (*5;73;74*). These products are named after the fragments which they contain: the fragment X consists of covalently linked D-E-D domains from a single fibrinogen precursor (i.e. a single fibrinogen molecule lacking its αC regions), Y fragments consist of covalently linked D-E domains arising from a single fibrinogen precursor, and the D fragment has previously been defined. Regardless of the size of the fibrin degradation product, the vast majority of αC regions are removed; plasmin rapidly cleaves the α chain Lys^{219}-Ser^{220} peptide bond to release the αC region and gain access to the other major cleavage sites within the α, β and γ chains (*5*). Plasmin continues to digest the αC region following its removal from the fibrin polymer, producing small molecular mass (< 25 kDa) fragments (*65*).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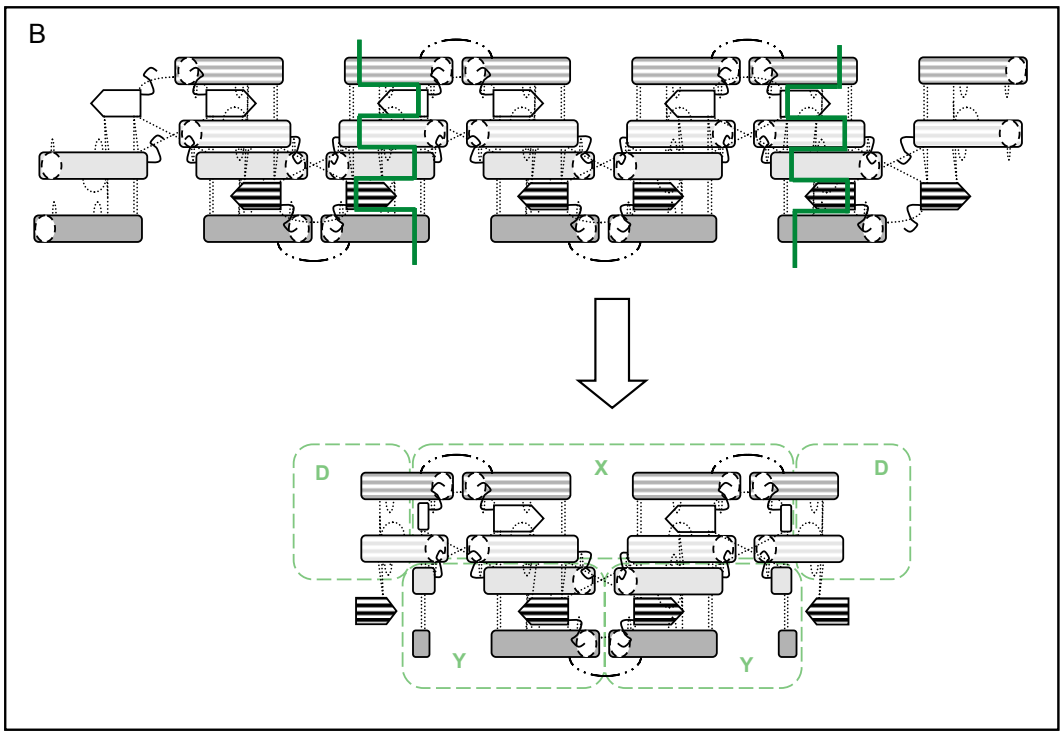
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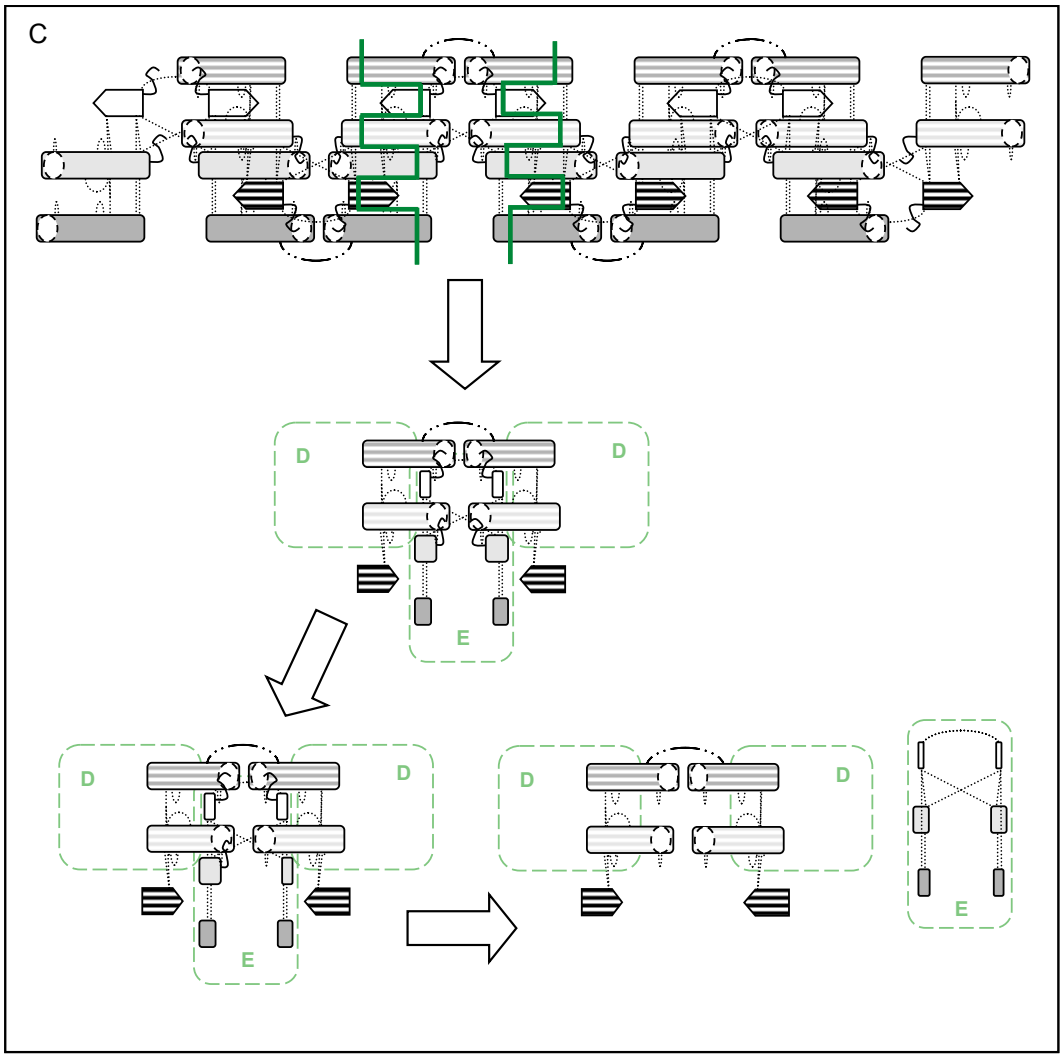


Figure 1.5: The structure of low molecular mass fibrin degradation products. Panel A: plasmin-mediated digestion of fibrin to form (DXD)YY. D, X and Y regions are indicated by green dashed boxes. The αC region is also cleaved by plasmin, but for simplicity, the white arrowhead has been left unchanged (consistent with Figure 1.3). Solid green lines indicate locations of fibrin cleavage. Panel B: plasmin-mediated digestion of fibrin to form (DY)YD. Panel C: plasmin-mediated digestion of fibrin to form (DD)E. Further digestion of (DD)E in the E region produces $(DD)E_2$. Excess digestion of (DD)E in the E region produces $(DD)E_3$, which dissociates due to removal of the A- and B-knobs.

An interesting feature of the fibrinolytic system is that fibrin is not only a substrate for plasmin, but it also acts as a cofactor for the activation of plasminogen to plasmin. Plasminogen, as well as its physiological plasma activator tPA, bind to the surface of fibrin and partially degraded fibrin. This binding is mediated by lysine residues naturally exposed on the fibrin molecule, and those exposed by plasmic digestion (*75*). Binding to fibrin produces a scaffold effect, effectively increasing the local concentrations of tPA and plasminogen, thereby increasing the rate of plasminogen activation (*76-80*). This rate enhancement is an approximately 100- to 1 000-fold increase over solution-phase plasminogen activation (*77;81-83*). This effect is often referred to as the cofactor activity of fibrin, and it restricts plasminogen activation temporally and spatially to the fibrin polymer.

Minute catalytic amounts of active plasmin initiate the fibrinolytic process. Plasmin proteolyzes the fibrin to which it is bound, thereby exposing an increased number of carboxy-terminal lysine residues (as described above). This leads to an upregulation of plasminogen activation due to increased plasminogen binding to these newly exposed basic residues (*84*). The affinity of plasminogen to partially degraded fibrin and fibrin degradation products is greater than its affinity to intact fibrin, which further increases the rate of plasminogen activation (*85*). This fibrin cofactor activity provides the basis for the majority of the regulation of the fibrinolytic system and is detailed in section 1.1.2..

The binding of plasminogen to fibrin surfaces is further affected by the form of the plasminogen. Native plasminogen, also called Glu^{1}-plasminogen (named for its amino-terminal residue), exists in a closed conformation. Glu-plasminogen undergoes limited amino-terminal proteolysis by plasmin at the Arg^{67}-Met^{68}, Lys^{76}-Lys^{77} or Lys^{77}-Val^{78} peptide bonds to produce Met^{68}-plasminogen, Lys^{77}-plasminogen or Val^{78}-plasminogen, respectively (*63*). The most common cleaved form is Lys-plasminogen, which has an open conformation. This open conformation facilitates binding to fibrin, as demonstrated by the dissociation constant (K_d) of 13 μM and 0.13 μM for Glu-plasminogen and Lys-plasminogen, respectively (*81*). The open conformation of Lys-plasminogen also contributes to its enhanced rate of activation by tPA (*75;76;86-89*). The catalytic efficiency (k_{cat}/K_M) of tPA-catalyzed conversion of plasminogen to the active enzyme plasmin in the presence of fibrin is 0.63 $\mu M^{-1}s^{-1}$ for Glu-plasminogen and 10 $\mu M^{-1}s^{-1}$ for Lys-plasminogen (*62;77*).

An extra layer of complexity is added to the fibrinolytic system as plasminogen activation affects and is affected by the structure of its plasminogen activator. tPA is a 527-residue serine protease of tryptic specificity that is secreted as a single-chain molecule (*62;63*). tPA demonstrates low activity in its single-chain form (*62*); however, limited hydrolysis by plasmin (the product of tPA's own action) at the Arg^{275}-Ile^{276} peptide bond yields the highly active, disulfide-linked two-chain form of tPA (*62;63;75*). Thus plasmin provides positive feedback for its own activation. tPA is also regulated by secretion, circulating inhibitors and fibrin-binding; together these are the primary mechanisms of regulating tPA activity.

It is on the fibrin surface that the majority of the plasminogen activaton occurs; and fibrin is not only the substrate for plasmin, but it also participates as an active component contributing to its own degradation. The structure of the fibrin clot (thickness, mass-to-length ratio, degree of cross-linking), as well as the availability of specific plasmin/plasminogen and tPA binding sites, greatly influences the ability of plasmin to degrade the clot, and constitutes the major form of regulation of fibrinolysis.

1.1.2. Regulation of the fibrinolytic system

Although the components of the fibrinolytic cascade are far fewer than those of the coagulation cascade, due to its multifaceted regulation, the system retains a high level of complexity to properly balance its counterpart. The primary basis of the regulation of fibrinolysis is the fibrin cofactor activity (*76-78*), which itself affects the other modes of regulation including inhibition by α_2-antiplasmin, and the open/closed conformation of plasminogen. Each differentially contributes to the positive and negative regulation of the fibrinolytic system; and each role is considered separately below.

1.1.2.1. Regulation of fibrinolysis by fibrin cofactor

The 791-residue plasminogen molecule contains 7 independently folded domains: an amino-terminal growth-factor-like domain, 5 lysine binding kringle domains, and a carboxy-terminal catalytic domain (*63;75*). The kringle domains remain intact in active Glu- and Lys-plasmin and mediate the binding of both the zymogen and the enzyme forms to lysine residues of fibrin, partially degraded fibrin, and fibrin degradation products (*90*). These lysine residues exist internally or at the newly formed carboxy-termini following limited plasmic digestion (*91-97*).

Due to the template mechanism describing the cofactor activity of fibrin, surface-bound plasminogen is a better substrate for its primary activator, tPA (Figure 1.6) (*76-80*). In the absence of cofactor, the Michaelis constant (K_M) for plasminogen activation by tPA is 65 μM for Glu-plasminogen, and 19 μM for Lys-plasminogen (*77*). In the presence of partially degraded fibrin, the K_M decreases to 0.16 μM and 0.02 μM for Glu-plasminogen and Lys-plasminogen, respectively (*77*). The catalytic rates are also increased, such that the catalytic efficiency increases from 0.001 $\mu M^{-1}s^{-1}$ to 0.63 $\mu M^{-1}s^{-1}$ for Glu-plasminogen and from 0.011 $\mu M^{-1}s^{-1}$ to 10 $\mu M^{-1}s^{-1}$ for Lys-plasminogen (*77*). The binding of plasminogen to fibrin is via the high affinity lysine binding sites as demonstrated by the elimination of fibrin enhancing effects in the presence of the lysine analogues tranexamic acid or ε-amino-caproic acid (εACA) (*67;77;98*). Furthermore, the binding specificity for carboxy-terminal lysine residues of fibrin is also abolished in the presence of lysine analogues (*67;77*), but is unchanged with active site blocked plasmin, indicating that the active site is not required for fibrin binding (*67*).

In the presence of non-polymerized, native fibrinogen, tPA-mediated activation of Glu-plasminogen proceeds with a K_M of 28 μM and a catalytic efficiency of 0.011 $\mu M^{-1}s^{-1}$, which are nearly identical to the rate constants of Lys-plasminogen in the absence of cofactor. This is consistent with Glu-plasminogen being in an open conformation. Lys-plasminogen is promoted minimally in the presence of fibrinogen, exhibiting a catalytic efficiency of 0.017 $\mu M^{-1}s^{-1}$ (*72;77*). The very small stimulatory effect was not altered by the presence of tranexamic acid, indicating that the minimal plasminogen-fibrinogen association was not mediated by lysine-kringle interactions (*77*).

With respect to plasminogen binding, it is not the polymerization of fibrin that causes the greatest rate enhancement, but rather the exposure of carboxy-terminal lysine residues. The affinity of plasminogen for intact fibrin is similar to that of fibrinogen (*76;85*). However, during the initial stages of plasmic digestion of fibrin, the cofactor activity increases by up to an order of magnitude. This enhancement is due to the generation of additional binding sites, as lysine residues are exposed (*84;92;99-101*). The effect of plasmin-mediated fibrin degradation on the stimulation of plasminogen activation is more pronounced with Glu-plasminogen than it is with Lys-plasminogen. This is due to the closed, less accessible conformation of Glu-plasminogen which converts to an open

conformation upon binding, and also because Lys-plasminogen is a naturally better substrate for tPA (in the absence of cofactor) (*84;96;98;102;103*).

Plasmin and plasminogen also bind to fibrin degradation products, including the terminally digested product (DD)E. Plasminogen binds to these products with an affinity comparable to that of partially digested fibrin, and with a K_d (for Glu-plasminogen) of approximately 0.2 μM for all common fibrin degradation products (*97;98*). This interaction is also mediated through the kringle domains as evidenced by the interruption of binding in the presence of lysine analogues (*67;81*). The K_d of plasminogen interaction with fibrin degradation products is independent of the molecular mass of the product (*98*). However, excessive plasmic digestion of (DD)E resulting in dissociation of the DD and E regions abolishes any cofactor activity (*69;71;84;89;104*).

The fibrin-mediated enhancement in plasminogen activation depends not only on plasminogen binding, but also the binding of tPA; and the binding of tPA to fibrin and degraded fibrin is a doubly complex situation. tPA can interact with fibrin through two modes; a fibronectin finger-like domain and a kringle domain (Figure 1.6). The mode of tPA binding is influenced by the degree of fibrin cross-linking, as well as the extent of fibrin degradation: tPA predominantly binds intact fibrin through its high affinity finger domain (with a K_d of 0.053 μM) and to a lesser extent through its low affinity kringle domain (with a K_d of 2.6 μM) (*81;98;103;105-110*); while binding with a high affinity to the terminally degraded fibrin product (DD)E uniquely through its lysine-dependent kringle domain (K_d of 0.029 μM) (*66;72;81;103;105*). Although the primary mode of interaction of tPA with fibrin or (DD)E shifts, ultimately the affinity of interaction is comparable (*67;81*).

The shift in the binding mode of tPA can be explained by its requirement for two adjacent, intact D:E domain connections for successful finger-dependent binding (*97;105*). (DD)E, the smallest fibrin degradation product does not fulfill this requirement, and therefore tPA is required to bind through its kringle domain (*105*). Although both plasminogen and tPA bind degraded fibrin through kringle-dependent interactions, they do not compete for (DD)E binding, which indicates the presence of distinct binding sites (*76;77;81;103*).

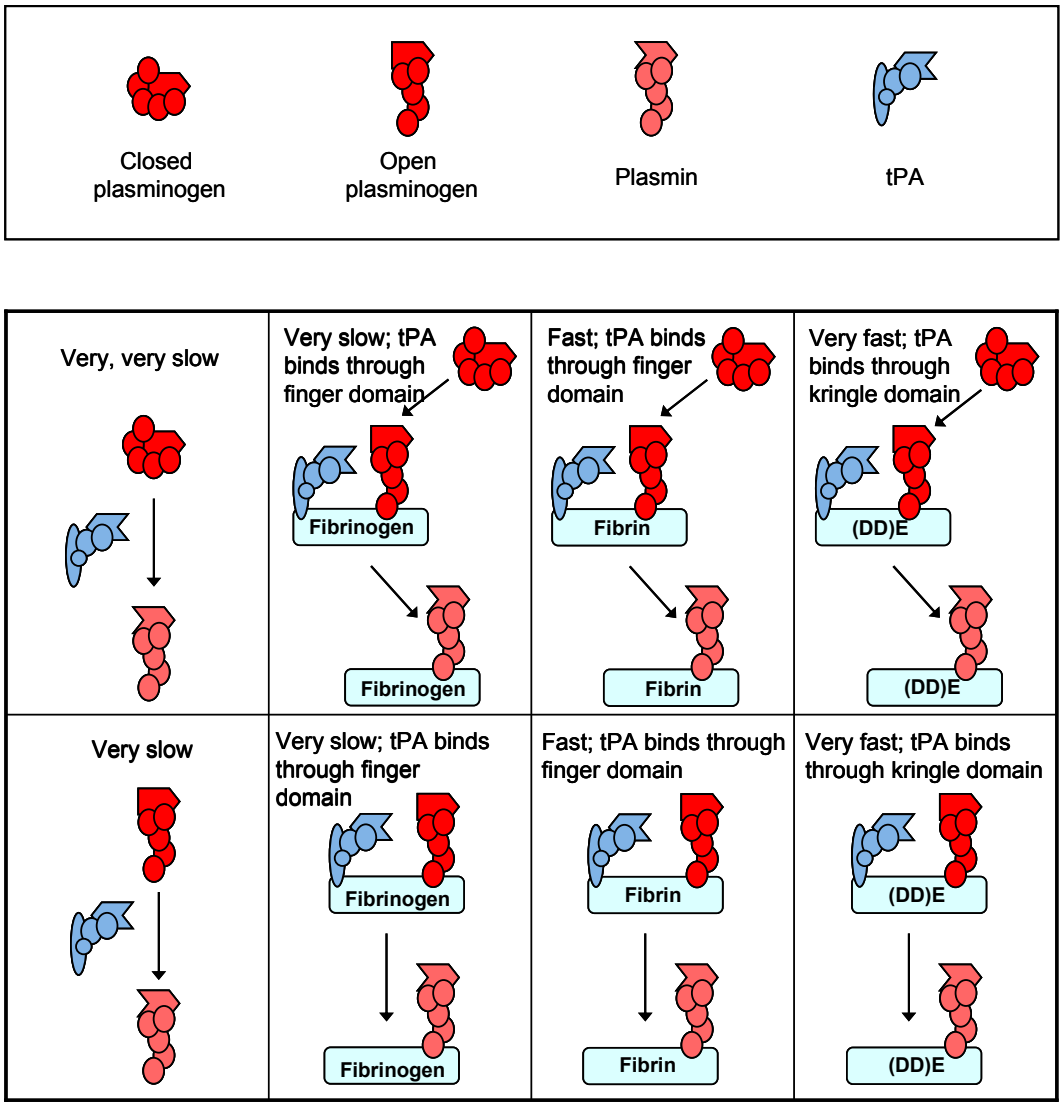


Figure 1.6: The relative rates of plasminogen activation by tPA in the presence of various cofactors. Glu-plasminogen is present in the circulation in the closed conformation. Binding to cofactor converts it to the open conformation. Lys-plasminogen exists in the open conformation in the presence or absence of cofactor. tPA binds through its finger or kringle domain depending on the type of cofactor.

Due to the predominantly finger-dependent binding mode of tPA, its binding to intact fibrin is stimulated by the polymerization event and not by the exposure of carboxy-terminal lysine residues, as is the case with plasminogen binding. Fibrinogen molecules (which, unlike fibrin monomers, retain fibrinopeptides A and B and therefore lack exposed polymerization sites and are deficient in D:E interactions) cross-linked at γ_{XL}:γ_{XL} sites by factor XIIIa form polymeric structures, and are sufficient to cause some minor tPA binding (*11*). However, the most potent stimulation of tPA binding is due to D:E interactions (*11*). The cryptic sites that become exposed upon conversion of

fibrinogen to fibrin are $\alpha^{148-160}$ and $\gamma^{312-324}$ (or $\gamma^{311-379}$) (*84;96;111-117*). Inhibition of fibrin polymerization by the peptide Gly-Pro-Arg-Pro (which mimics the A-knob and interferes with A-knob to A-hole interactions) abolishes the potentiating effect of fibrin (*87*).

Together the various binding interactions serve to regulate fibrinolysis through plasmin production. Plasminogen activation, the primary event of fibrinolysis, proceeds via three phases. The first is a lag phase characterized by very slow tPA-catalyzed plasminogen activation due to the lack of sufficient plasminogen binding sites for full scale activation (*97*). The second is a period of rapid plasminogen activation due to the exposure of carboxy-terminal lysine residues at the initial fibrin cleavage sites by the minimal plasmin created during the first phase. This acts as a positive feedback loop, and these residues act as additional plasminogen binding sites, which continuously up-regulate plasminogen activation (*7;84;90-97*). The final phase is the stop phase, at the completion of fibrin degradation (*97*).

1.1.2.2. Regulation of fibrinolysis by α_2-antiplasmin

In addition to the positive feedback loop caused by the binding of plasmin to freshly exposed carboxy-terminal lysine residues, this binding event has major consequences on the inhibition of plasmin by its primary plasma inhibitor, α_2-antiplasmin. Plasmin bound to fibrin or its degradation products is protected from inhibition by α_2-antiplasmin (*109;118-121*). In solution, α_2-antiplasmin is an incredibly efficient inhibitor of plasmin, with an initial binding rate among the fastest known (*109*). However, binding to fibrin or its degradation products shields plasmin from α_2-antiplasmin, because both the active site and the kringle domain of plasmin seem to be required for proper inhibition to occur (Figure 1.7) (*62;67;110;118;122*).

α_2-antiplasmin is a single-chain 70 kDa glycoprotein of the serpin family of inhibitors that circulates at a concentration of 1 μM in human plasma (*109;119*). It reacts extremely rapidly with plasmin to form a stable 1:1 stoichiometric complex, which ultimately irreversibly inhibits plasmin (*39;40;109*). The inactivation reaction occurs via two steps: rapid reversible binding, followed by slower irreversible inhibition.

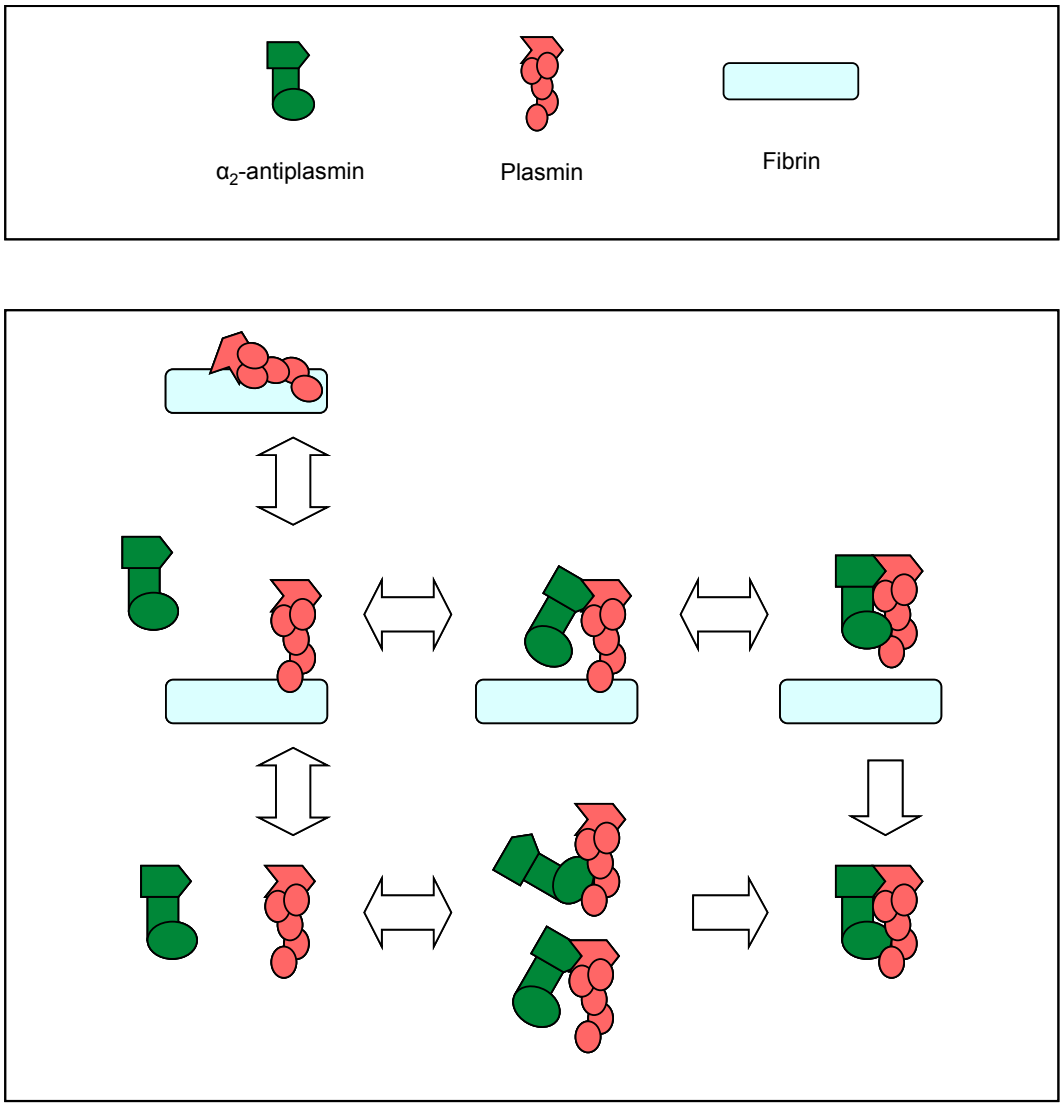


Figure 1.7: Inhibition of plasmin by α_2-antiplasmin in the presence and absence of fibrin cofactor. Free plasmin is rapidly inhibited by α_2-antiplasmin. Fibrin-bound plasmin that is actively involved in digesting the fibrin to which it is bound is inaccessible to inhibition by α_2-antiplasmin. Fibrin-bound plasmin with a free active site slowly binds to α_2-antiplasmin, and may become irreversibly inhibited when released from the fibrin polymer.

The initial binding rate for the plasmin:α_2-antiplasmin complex approaches the theoretical limit for a diffusion-controlled process (*123*). This first step involves interaction of the kringle domain of plasmin with a complementary lysine on α_2-antiplasmin (*67;109;110;118;124;125*). There is a carboxy-terminal lysine residue on the α_2-antiplasmin molecule (Lys^{452}); however, despite original reports that this residue was involved in plasmin binding (*126*), mutational studies have demonstrated that it is rather the internal residue Lys^{436} that is responsible for the interaction with plasmin (*125*). The

environment surrounding Lys^{436} likely also plays a role in the plasmin:α_2-antiplasmin interaction (*125*). Incubation of the plasmin with the lysine analogue εACA dramatically reduces the rate this reversible binding step (*109;119*).

In contrast, εACA has no effect on the second, irreversible inactivation step, in which α_2-antiplasmin acts as a suicide substrate (*119*). Following cleavage of the scissile peptide bond in the reactive center loop of α_2-antiplasmin, an ester bond is formed between the carboxyl group of the newly cleaved peptide bond and the hydroxyl group of the active site serine in plasmin (*125*). This second step alone is sufficient to cause inhibition of plasmin activity: a low molecular weight form of plasmin that lacks lysine binding sites has been created (*110;127*), and it also forms a stable irreversible complex with α_2-antiplasmin, albeit at a significantly slower rate. This reaction is unaffected by εACA as the interaction is mediated solely through the active site (*109;110*).

The difference in the reaction rate of α_2-antiplasmin with plasmin versus the low molecular weight form of plasmin demonstrates the protection afforded by fibrin binding. When plasmin is bound to fibrin or its degradation products, its kringle domains are inaccessible to α_2-antiplasmin, which causes a dramatic reduction in the rate of inactivation (*109;119;128;129*). Furthermore, fibrin-bound plasmin may be actively involved in fibrin degradation; therefore the active site of plasmin may also be blocked, essentially preventing inhibition altogether. Free plasmin in the presence of α_2-antiplasmin has a half-life of 0.1 seconds, whereas the half-life of fibrin-bound plasmin is in the range of 10-100 seconds (*63*). This competition of fibrin and α_2-antiplasmin for the kringle domains of plasmin represents a major form of regulation of fibrinolysis (*130*). Binding has the complementary function of increasing the rate of plasmin formation and increasing the amount of work that plasmin can perform prior to inhibition.

1.1.2.3. Other regulators of fibrinolysis

Several other minor regulatory mechanisms contribute to the up- and down-regulation of fibrinolysis; among these is α_2-macroglobulin. This tetrameric plasma protein is a non-specific inhibitor of serine, cysteine, aspartic and metalloproteases, and counts plasmin among its substrates (*131-136*). α_2-macroglobulin is considered to be the secondary

physiological inhibitor of plasmin, behind α_2-antiplasmin (*85*). This inhibitor traps its target proteases by presenting a bait sequence containing their cleavage sequences. Upon attempted cleavage by the protease, conformational changes occur within α_2-macroglobulin which result in physical capture of the protease (*131;132;137-140*).

Analogous to inhibition with α_2-antiplasmin, fibrin and its degradation products provide protection of plasmin from inhibition by α_2-macroglobulin (*119-121*). Fibrin binding decreases the second-order rate constant for the interaction of plasmin with α_2-macroglobulin by 300- to 500-fold (*120;121*). Unlike α_2-antiplasmin, the mechanism of inhibition by α_2-macroglobulin does not depend on the presence of an unoccupied kringle domain; therefore the protection afforded by fibrin and its degradation products is likely due to the steric constraints associated with the large plasmin-fibrin complex (compared to the small size of free plasmin). This potentially limits access of the active site of plasmin to the bait region of α_2-macroglobulin and thereby results in protection from inhibition (*121*). This is further supported by the continued hydrolysis of small chromogenic substrates by plasmin that is 'inhibited' by α_2-macroglobulin, because these substrates do not offer the same degree of steric interference as does fibrin or its degradation products.

α_2-macroglobulin becomes an important inhibitor of plasmin in situations of quantitative plasminogen activation. Plasminogen exists at a concentration of 2 μM in plasma, while its primary inhibitor α_2-antiplasmin circulates at only 1 μM. This serpin may therefore be consumed during cases of extreme plasmin generation (*85*). In contrast, α_2-macroglobulin is present in the circulation at a concentration of 3 μM, and when α_2-antiplasmin is consumed, α_2-macroglobulin becomes the primary inhibitor of plasmin (*85*). The effect of the inhibitors is dramatically increased in the absence of adequate plasmin binding sites on fibrin, such as in the presence of basic carboxypeptidases that remove the carboxy-terminal lysine residues from fibrin (see section 1.1.3.)

An additional regulator of fibrinolysis is plasminogen activator inhibitor-1 (PAI-1), a 50 kDa single-chain glycoprotein and another member of the serpin family of inhibitors. It is the primary inhibitor of the generation of plasmin, as it inactivates both tPA and uPA by forming irreversible 1:1 stoichiometric complexes (*40;62;141-144*). The second order rate constant for the inhibition reaction is measured at 10^6 to 10^7 $M^{-1}s^{-1}$ (*143*). PAI-1

circulates at variable concentrations in the active inhibitor form, but then spontaneously converts, with a half-life of 1-2 hours, to a latent state and then an inactive state (*143;144*). Only the active state is a functional plasminogen activator inhibitor.

Inhibition of tPA and uPA by PAI-1 proceeds through a series of steps, beginning with reversible binding of the plasminogen activator to PAI-1. Following this, a covalent enzyme-inhibitor complex is formed by reaction of the active site serine of tPA or uPA with the mock P_1-P_1' peptide bond of PAI-1. Upon creation of the covalent complex, a major structural rearrangement occurs in both tPA and PAI-1, distorting the active site of the plasminogen activator and preventing completion of the catalytic cycle (*145-149*). The plasminogen activator-PAI-1 complex binds and is endocytosed by members of the low density lipoprotein receptor family (*150-160*). The rate of clearance of plasminogen activator-PAI-1 complexes is approximately 100-fold faster than that of free PAI-1 or plasminogen activator (*157*).

All major forms of regulation of fibrinolysis are influenced by the binding of tPA, plasminogen, and plasmin to the fibrin clot. This binding relies on the presence of lysine residues, particularly the high concentration of carboxy-terminal lysine residues produced by plasmic digestion. Therefore, arguably the most important mechanism of fibrinolytic control is the presence, production, and removal of lysine residues from partially degraded fibrin: this results in decreased plasminogen binding, leading to decreased plasminogen activation and increased plasmin inhibition.

1.1.3. The antifibrinolytic role of basic plasma carboxypeptidases

One final important mechanism of fibrinolytic regulation is the action of basic plasma carboxypeptidases. Basic carboxypeptidases remove the carboxy-terminal lysine or arginine residue from their substrate proteins and peptides; and those found in plasma count partially degraded fibrin and fibrin degradation products among their substrates. During fibrinolysis, plasmin-mediated cleavage generates new carboxy-terminal lysine residues and thus new plasminogen binding sites on fibrin to maintain plasminogen activation, while carboxypeptidases remove these residues and effectively suppress plasminogen activation and enhance the effects of plasma inhibitors such as α_2-antiplasmin and α_2-macroglobulin (*72;85;109;118-121;161;162*). The primary basic carboxypeptidase in plasma is presumed to be activated thrombin activatable fibrinolysis

inhibitor (TAFIa; EC 3.4.17.20), although *in vitro* experiments have also demonstrated the antifibrinolytic role of carboxypeptidase B (CPB; EC 3.4.17.2), a pancreatic enzyme which can be released into the bloodstream in the course of disease, such as pancreatitis (*85;163-167*).

Both TAFIa and CPB are zinc metalloproteases that are released in zymogen form (TAFI and pro-CPB, respectively) and are activated by proteases in their environment. Pro-CPB is activated by trypsin in the stomach (*168*), while TAFI is activated primarily by the thrombin-thrombomodulin complex throughout the vasculature (*169*). TAFIa has been called plasma CPB due to its similarities to pancreatic CPB; carboxypeptidase U, due to its intrinsic instability (U indicates unstable); and carboxypeptidase R, due to its preference for cleaving carboxy-terminal arginine residues (*163;170-172*).

The primary structure of TAFI comprises an activation peptide of 92 residues and a catalytic domain of 309 residues (*171*); similar to the activation peptide of 95 residues and the 306 residue long catalytic domain of pancreatic pro-CPB (*173*). In fact, there is 50% amino acid sequence identity between these two enzymes (*174*), and TAFI and pro-CPB also have very similar three-dimensional structures, as shown in Figure 1.8. They share a common globular fold throughout the catalytic domain, with the exception of a differently positioned activation peptide (*175*). The residues important for catalytic activity, zinc ion coordination and substrate binding are conserved between the two carboxypeptidases (*174*).

TAFI demonstrates its distinctiveness from pancreatic pro-CPB in regard to its activation and inactivation. The primary physiological activator of TAFI is thrombin (of the coagulation cascade) with cofactor thrombomodulin, an integral membrane protein localized to endothelial cells; activation may also occur by plasmin (of the fibrinolytic cascade) (*171;174*). The identity of these activators suggests a physiological role for TAFIa in fibrinolysis. TAFIa is intrinsically unstable and is rapidly inactivated, with a half-life of only 5-10 minutes at 37°C (*172;176-179*). This limits the duration of physiological TAFIa activity in plasma, and affects its regulation of fibrinolysis.

The effect of basic carboxypeptidase activity (i.e. the reduction of plasminogen binding to fibrin) on the rate of fibrinolysis has been demonstrated in whole blood clot lysis assays.

The addition of purified TAFI significantly inhibits the lysis of whole blood clots (*90*). This inhibition is overcome by the addition of potato tuber carboxypeptidase inhibitor (PTCI), a specific inhibitor of TAFIa (*90;180*). Similarly, addition of PTCI to plasma (without addition of any exogenous TAFIa) causes a marked acceleration of clot lysis. The specificity of this latter experiment was verified by use of TAFI-depleted plasma, which demonstrates a similar clot lysis time to PTCI-treated plasma (*90*).

Although both CPB and TAFIa prolong clot lysis time, their overall effect differs. Aside from the general absence of pancreatic CPB from the circulation, the main difference is that CPB is a stable enzyme, while TAFIa is rapidly and spontaneously inactivated at 37°C (*172;176-179*). The dose-response curves (a plot of the concentration of the carboxypeptidase versus the lysis time), for *in vitro* clots formed from TAFI-depleted plasma increase exponentially with increasing CPB concentration, but display saturation with the addition of TAFIa (Figure 1.9) (*85;181*).

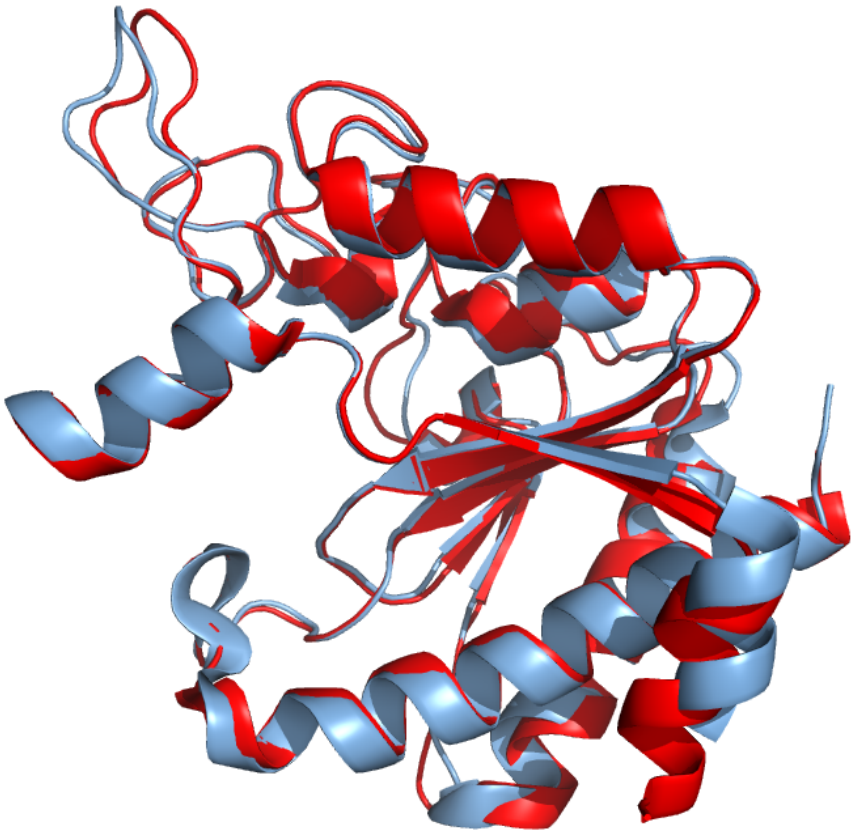


Figure 1.8: A comparison of the crystal structures of TAFI and pro-CPB. TAFI is coloured in red and proCPB in blue. This figure was generated with PyMOL software. The TAFI structure was obtained from PDB accession ID 3DGV, and the pro-CPB structure was obtained from PDB accession ID 1KWM.

TAFIa is antifibrinolytic when present at a concentration above the threshold value; however, as it spontaneously decays and falls below this threshold, clot lysis is no longer attenuated (*182;183*). During ongoing fibrinolysis, there would be ongoing TAFI activation by plasmin, or any other activator present, and the concentration of TAFIa could remain above the threshold until the circulating TAFI precursor is depleted. The threshold of TAFIa is dependent upon the relative rates and concentrations of the fibrinolytic components (*90*). *In vitro* experiments are complicated by the instability of TAFIa, because the result of continuous activation of TAFI and therefore continuous presence of activated TAFIa, on fibrinolysis cannot be easily measured (Figure 1.10).

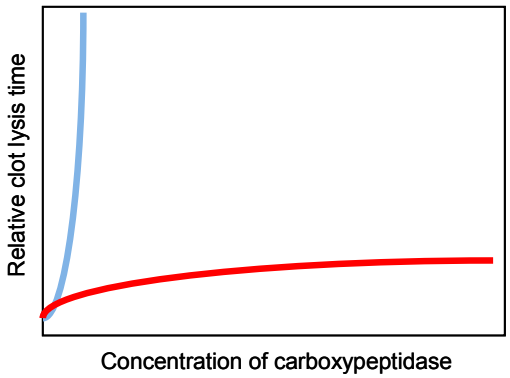


Figure 1.9: Dose-response curves for the lysis times of plasma clots supplemented with CPB or TAFIa. TAFIa shows saturation, while CPB can completely halt fibrinolysis. The dose-response curve for CPB is coloured in blue, and the dose-response curve for TAFIa is coloured in red. This diagram represents the general effects of these enzymes and does not represent any specific experiment.

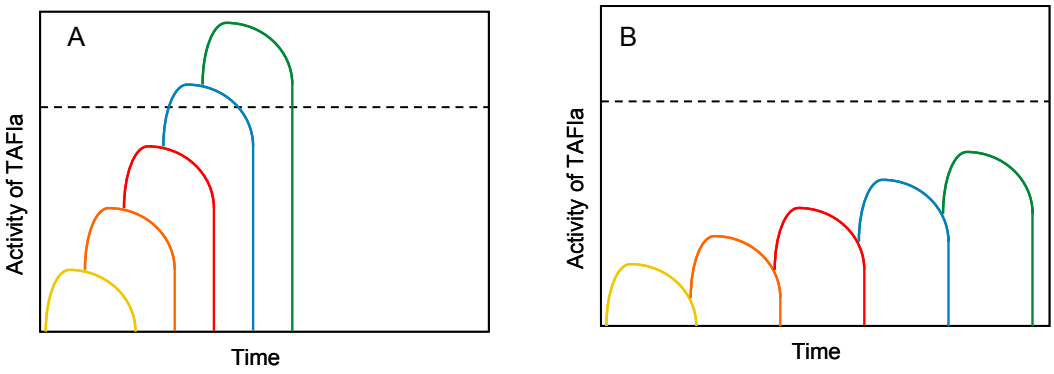


Figure 1.10: The threshold mechanism of TAFIa antifibrinolytic activity. Panel A: when TAFI is activated at a high rate, and the concentrations of fibrinolytic inhibitors are high, TAFIa activity is above the threshold level (dashed line), and fibrinolysis is halted. Panel B: when TAFI is activated at a low rate, or the concentrations of fibrinolytic inhibitors are low, TAFIa activity is below the threshold level, and fibrinolysis continues. Each coloured curve represents successive stepwise activation of TAFI.

The antifibrinolytic effect caused by any basic carboxypeptidase is altered by the pool of inhibitors present. In a system of purified components, in the absence of plasmin inhibitors, the antifibrinolytic effect of CPB or TAFIa is nearly unmeasurable (*85*). However, in the presence of plasmin inhibitors normally present in the circulation, such as α_2-antiplasmin and α_2-macroglobulin, the antifibrinolytic effect of the carboxypeptidases becomes significant (*85*). Therefore alone, basic carboxypeptidases do not halt fibrinolysis, because the rate of plasminogen activation and the work performed by the generated plasmin exceed the inhibitory capacity of the carboxypeptidase. A necessary combination of a basic carboxypeptidase to remove plasminogen binding sites and plasmin inhibitors to inhibit the free plasmin is required to completely arrest fibrinolysis. Therefore, many components must work in concert to significantly, consistently and continuously halt fibrinolysis.

One final component which has not yet been discussed is carboxypeptidase N (CPN). CPN is a basic plasma carboxypeptidase, similar to TAFIa, but it reportedly plays no role in antifibrinolysis (see section 1.1.5.). Unlike TAFIa, CPN is present at a high concentration in the circulation, and is constitutively active (does not require the removal of an activation peptide from a zymogen form). This enzyme could be an important plasma regulator of fibrinolysis and is therefore considered in isolation.

1.1.4. Carboxypeptidase N

Carboxypeptidase N (CPN; kininase I, carboxypeptidase K, anaphylatoxin inactivator, protaminase, creatine kinase conversion factor, peptidyl-L-arginine hydrolase, arginine carboxypeptidase, lysine carboxypeptidase; EC 3.4.17.3) is a basic plasma zinc metallocarboxypeptidase related to TAFIa and CPB. CPN was discovered in the early 1960s as a regulator of inflammation, but since then it has been found to modulate the activity of multiple systems due to its varied substrate specificity (*184-188*). CPN is believed to be essential for life, playing a role in the protection against systemic dissemination of inflammatory mediators and vasoactive peptides (*189*). A wide substrate specificity, a unique structure, and an apparent lack of regulation make CPN a very interesting enzyme.

1.1.4.1. Background

CPN is related to the metalloproteases TAFIa and CPB, but is more distantly related to these enzymes than they are to each other (Figure 1.11). CPN is a member of the regulatory CPN/E family of carboxypeptidases, a family which partially takes its name from CPN, the first discovered member of the family (*166;190*). This basic carboxypeptidase is synthesized in the liver and circulates in plasma at a concentration of approximately 100 nM (30 μg/mL) (*191-198*). Despite the hepatic origin of CPN, the enzyme has not be isolated from the organ, nor has its mRNA be identified; CPN is most likely immediately secreted upon synthesis (*191;195;199;200*). In culture, HepG2 cells, which are derived from a human hepatoma line and have retained the capacity to synthesize and secrete typical hepatic glycoproteins, can produce CPN, but only under a narrow range of culture conditions (*193*). The intracellular and membrane fraction-associated carboxypeptidase activity of these cells is only 15% that of the secreted activity, further demonstrating the unlikelihood of CPN storage in the liver (*193*).

CPN isolated from plasma is a heteromer composed of 55 kDa catalytic subunits (termed CPN1) and 83 kDa regulatory subunits (termed CPN2). The generally accepted structure of CPN is of a heterotetramer, containing two each of the regulatory and catalytic subunits, for a total molecular mass of 280 kDa (*188;192;195;201-205*). (For a more detailed description of the structure of CPN, see section 1.1.4.5.). This multi-subunit structure is a unique attribute among the human carboxypeptidases (*188;189;203*). The additional, atypical regulatory subunits are thought to play a role in stabilizing the catalytic subunit by preventing its removal from the bloodstream by glomerular filtration, and by reducing the degradation of enzymatic activity at 37ºC (*195;201*). The small size and lack of carbohydrate on CPN1 would lead to rapid clearance from the circulation if not complexed to CPN2 (*194;201;206*). CPN2 is also suggested to modulate the activity of CPN1 toward certain larger substrates and inhibitors, as is the case with kallikrein versus its isolated active light chain (*189;203;206;207*). However, of all the known substrates of CPN, the only difference in activity of intact multimeric CPN versus the isolated catalytic CPN1 subunit proved to be with the anaphylatoxin C3a and its carboxy-terminal octapeptide, both of which were proteolyzed faster by the tetrameric enzyme (*203*).

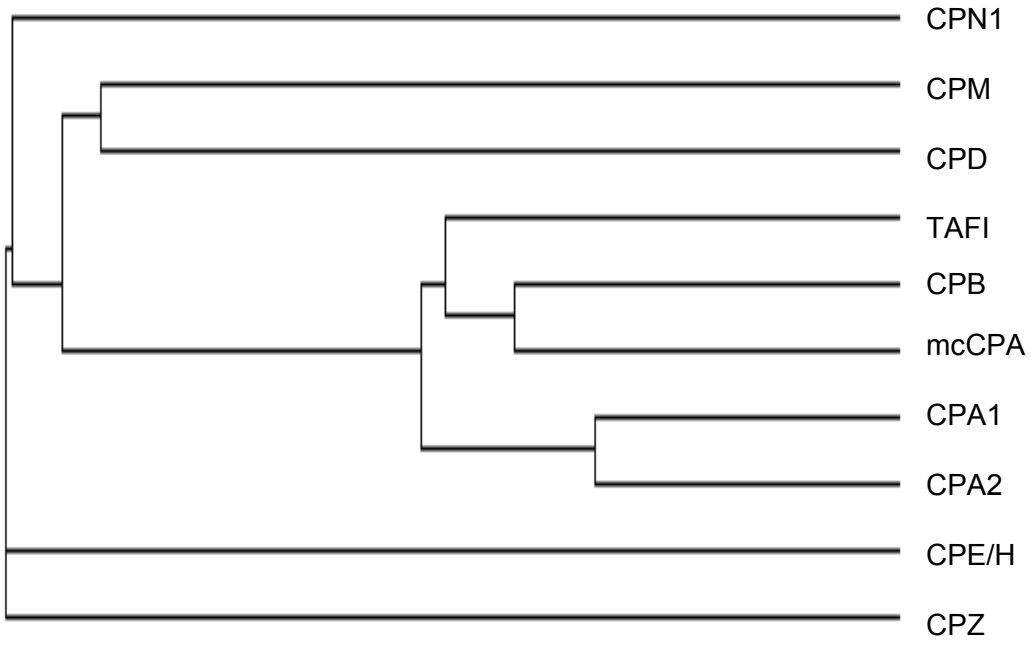


Figure 1.11: The carboxypeptidase family. Cladogram depicts relative relationships between the clan MC carboxypeptidases. Only the catalytic subunit of CPN is used for alignment purposes, therefore in the diagram it is termed CPN1. This figure was generated with the CLUSTALW sequence alignment program.

The structure of CPN appears to be sensitive to proteolysis. The serine proteases plasmin and trypsin cleave both the catalytic and regulatory subunits, which results in a very moderate increase in peptidase activity (Figure 1.12) (*166;188;198;201;208*). Cleavage of the 83 kDa regulatory subunit releases a 13 kDa 'docking peptide' and reduces the major product to 72 kDa; the 55 kDa catalytic subunit is cleaved to 48 kDa, and prolonged digestion produces a non-covalently linked pair of 21 kDa and 27 kDa chains (*188;198*). The role of the two-chain form of CPN1 is not well understood, and it has yet to be found in normal human plasma or preparations or purified CPN. However, one hypothesis is that in certain pathological conditions characterized by the activation of proteases, CPN1 may be converted to the two-chain form and play a unique and important role (*199*). CPN isolated from fresh blood (even in the presence of high concentrations of protease inhibitors) and CPN isolated from HepG2 secretions (without purification) contain nearly equal concentrations of the 55 kDa and the 48 kDa CPN1 subunits (*193;199;201;206*). These two forms are therefore thought to exist mutually and simultaneously in the circulation; this is likely due to constitutive proteolysis during processing and secretion from the liver (*199*).

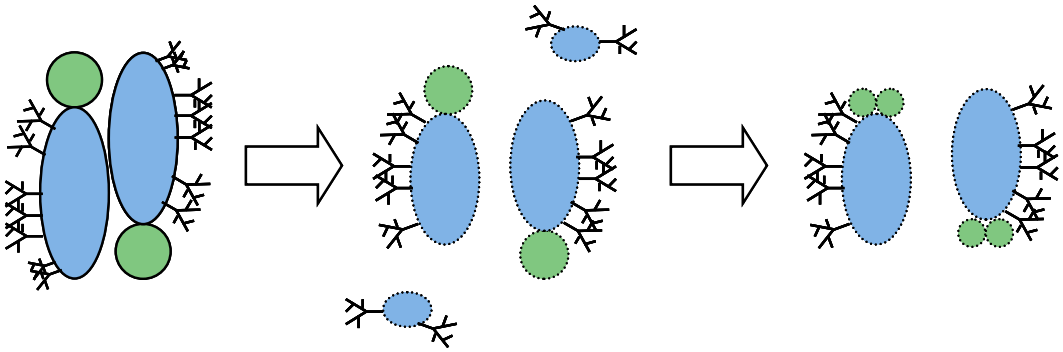


Figure 1.12: The effects of plasmic cleavage on the structure of CPN. CPN1 subunits are coloured in light grey and CPN2 subunits in dark grey. Glycosylation is shown as stick figures. Initial digestion with plasmin cleaves both the CPN1 and CPN2 subunits. Prolonged digestion produces the two-chain form of CPN, which has a higher catalytic activity that single-chain CPN.

The unprocessed, circulating form of CPN is thought to be fully and constitutively active. There are only a few plasma proteins reported to date which possesses a carboxy-terminal lysine or arginine residue in their circulating form (*192*); and freshly drawn heparinized blood and serum demonstrate full CPN activity (toward hippuryl-arginine and hippuryl-lysine), and this activity does not increase upon storage (*209*). CPN contains both a peptidase and an esterase activity (*205;209;210*). The esterase activity peaks at pH 8.7, while the peptidase activity is maximal at pH 7.5 (*210*). The activity of CPN decreases significantly below pH 7.0, and this is thought to be due to loss of the active site zinc ion (*199*).

In vitro this zinc ion can be replaced by a cobalt ion, which, at neutral pH, results in an increase in CPN activity of 2- to 6-fold, depending on the substrate. This increased activity can be attributed to an increase in the catalytic rate constant (*187;199;206;209;211;212*). Cobalt is also able to activate CPN at low pH when zinc-containing CPN is essentially inactive. Cobalt-containing CPN at pH 5.5 has 156% of the activity of zinc-containing CPN at pH 7.5 (*212*). Note, however, that the term 'replacement' has been used synonymously with addition of cobalt to the reaction solution; true replacement has never been shown conclusively, and the increased activity may in fact be due to the binding of cobalt to an allosteric site. Nickel and 1-butanol also provide some moderate activation of serum CPN (*209*). Cadmium inhibits CPN (*209;210*).

The activity of CPN toward many natural and synthetic substrates has been studied. The catalytic rate constants of these substrates are given in Table 1.1 (*187;195;205;206;210;211;213-216*). These substrates include the commonly used chromogenic substrates benzoyl-Ala-Lys and furylacryloyl-Ala-Lys (FA-Ala-Lys), as well as the physiological substrates bradykinin and the enkephalins.

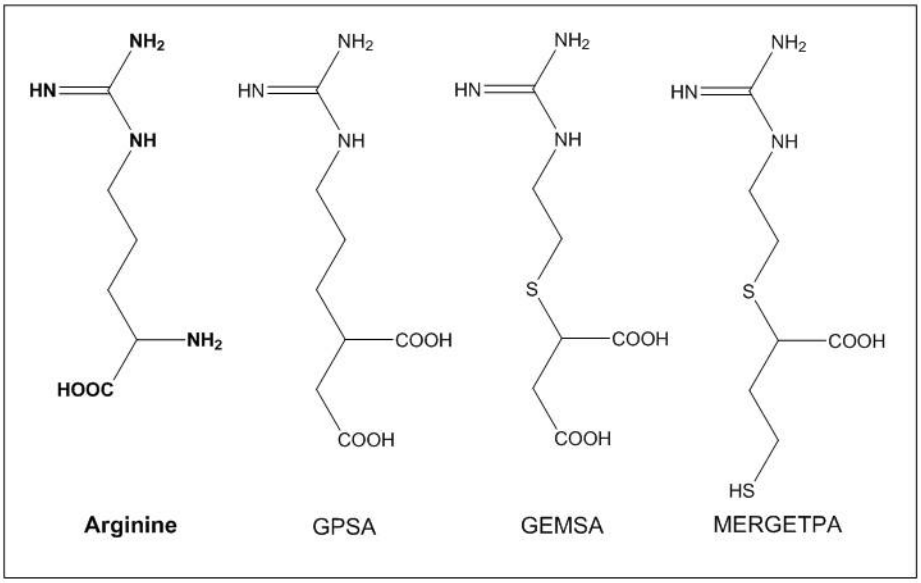
Based on the relative catalytic efficiencies of various synthetic substrates, the substrate preference of CPN can be predicted (*187;195;205;206;210;211;213;215-217*). Although the carboxy-terminal residue, be it lysine or arginine, does not affect the binding affinity of the substrate, it does significantly affect the rate of catalysis. Lysine is the preferred carboxy-terminal residue: the catalytic rate constants for benzoyl-Gly-Lys versus benzoyl-Gly-Arg are 0.012 $\mu M^{-1}s^{-1}$ and 0.006 $\mu M^{-1}s^{-1}$, respectively. Similarly, the catalytic rate constant for the preferred substrate benzoyl-Ala-Lys is 1.005 $\mu M^{-1}s^{-1}$, while it is only 0.654 $\mu M^{-1}s^{-1}$ for benzoyl-Ala-Arg (*206*).

It is the penultimate amino acid which determines binding affinity (while also influencing the rate of catalysis in some cases). When a substrate terminates in lysine, the preferred penultimate residue is alanine, and a distinctly unfavourable residue is glycine (*199;205*). This is exemplified by the disparity in catalytic efficiency between benzoyl-Gly-Lys (0.012 $\mu M^{-1}s^{-1}$) and benzoyl-Ala-Lys (1.005 $\mu M^{-1}s^{-1}$) (*206*). Similarly, with a carboxy-terminal arginine residue, the most favourable penultimate amino acids are alanine (the catalytic efficiency for benzoyl-Ala-Arg is 0.654 $\mu M^{-1}s^{-1}$) and methionine (the catalytic efficiency for benzoyl-Met-Arg is 0.865 $\mu M^{-1}s^{-1}$), while the least favourable residue is glycine (the catalytic efficiency for benzoyl-Gly-Arg is 0.006 $\mu M^{-1}s^{-1}$) (*216*). CPN cannot cleave substrates with a penultimate proline residue. The preference of CPN for penultimate alanine over glycine residues may help explain the increased activity of CPN toward the anaphylatoxin C3a in comparison to C5a, as C3a terminates in …-Leu-Ala-Arg, whereas C5a terminates in …-Leu-Gly-Arg (*195;197;203;206;213;216*).

Table 1.1: CPN kinetic constants toward physiological and synthetic substrates.

Substrate	K_M *(μM)*	k_{cat} *(s^{-1})*	k_{cat}/K_M *($\mu M^{-1}s^{-1}$)*
[Leu^5]enkephalin-Arg^6	57	6	0.110
[Met^5]enkephalin-Arg^6	49	17	0.348
[Met^5]enkephalin-Lys^6	216	103	0.478
Bradykinin	19	1	0.052
FA-Ala-Lys	340	97	0.285
FA-Ala-Arg	260	31	0.118
Benzoyl-Met-Arg	288	249	0.865
Benzoyl-Leu-Arg	220	49	0.223
Benzoyl-Trp-Arg	390	13	0.33
Benzoyl-Tyr-Arg	320	56	0.175
Benzoyl-Phe-Arg	300	37	0.123
Benzoyl-Thr-Arg	320	68	0.213
Benzoyl-Gln-Arg	240	46	0.192
Benzoyl-His-Arg	480	32	0.067
Benzoyl-Val-Arg	410	13	0.032
Benzoyl-Ser-Arg	490	103	0.210
Benzoyl-Asn-Arg	270	61	0.226
Benzoyl-Ala-Arg	228	149	0.654
Benzoyl-Gly-Arg	1573	10	0.006
Benzoyl-Pro-Arg	N/A	N/A	N/A
Benzoyl-Gly-Lys	1400	16	0.012
Benzoyl-Ala-Lys	350	352	1.005

Several inhibitors of CPN activity have been discovered. Heavy metals inhibit the activity of CPN (including CPN previously activated by cobalt); these include $HgCl_2$, $ZnSO_4$, $CdSO_4$, and $MnCl_2$ (*209;218*). Along similar lines, chelating agents, such as 1,10-phenanthroline or ethylenediaminetetraacetic acid (EDTA), inhibit CPN by removing the active site zinc ion (*192;218-220*). Glutathione, cysteine, ornithine, histidine, β-alanine, ovomucoid trypsin inhibitor and trasylol slightly inhibit CPN; and 6 M urea abolishes all activity (*218;219;221*). CPN is inhibited by lysine with an inhibition constant (K_i) of 82 μM and by arginine with a K_i of 190 μM; note that the plasma concentration of lysine is 80-230 μM and arginine is 20-140 μM (*221*). No other physiological inhibitors of CPN activity have been discovered.



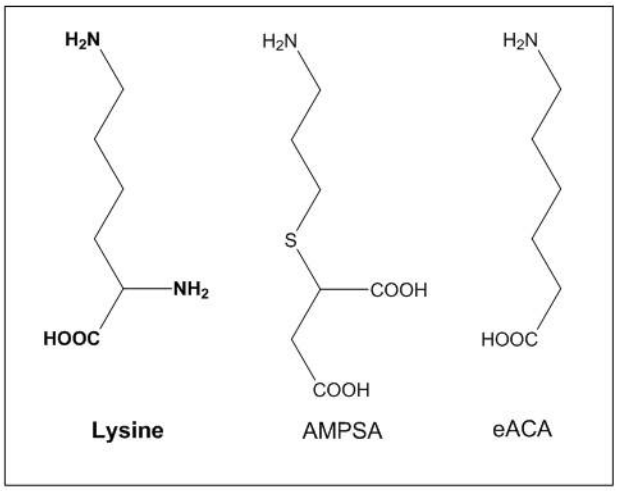


Figure 1.13: The structures of several CPN inhibitors. The chemical structures of several high affinity CPN inhibitors and the amino acids from which they were derived.

Based on the inhibitory activity of lysine and arginine, several synthetic CPN inhibitors have been developed, which are analogues of these residues containing additional sulfhydryl or carboxylate groups, (Figure 1.13) (*213;222-226*). Guanidinopropylsuccinic acid (GPSA) is an arginine analogue that inhibits CPN with a K_i of 0.9 μM (*225*). 2-guanidinoethylmercaptosuccinic acid (GEMSA) has a similar structure to GPSA but with a sulfur in place of one methylene group; it inhibits CPN with a K_i of 1.5 μM (*205;222;225*). Aminopropylmercaptosuccinic acid (AMPSA), which inhibits CPN with a K_i of 52 μM, mimics lysine, but is otherwise similar to GEMSA (*225*). DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MERGETPA), a potent CPN inhibitor with a K_i of 2 nM, is an arginine analogue with a mercaptomethyl group in place of the methyl carboxylate (*206;222;223;225;227*). A series of MERGETPA related inhibitors were synthesized (including acetyl-, Phe-, Boc-Phe-, Ala-, Boc-Ala-, and benzoyl-Ala- derivatives), all with similar inhibitory strengths (*228*). Arginic acid, δ-amino-*n*-valeric acid, ε-amino-*n*-caproic acid (εACA), N^{α}-formyl-Arg, N^{α}-carbamoyl-Arg, D-penicillamine-Arg, biotinyl-Arg, cinnamoyl-Arg, maleoyl-Arg, and acetylsalicylyl-Arg all poorly inhibit CPN (K_i in the millimolar range) (*218;220;225;228*). Several non-specific inhibitors exist, such as 2-mercaptoethanol, 3-mercaptopropionic acid, and 2,3-dimercaptopropanol (*219*). Unfortunately this entire range of CPN inhibitors is either poorly inhibitory or not particularly specific for CPN.

Protamine is an interesting CPN inhibitor. It is not a single compound, but rather a mixture of fish sperm proteins, each with four carboxy-terminal arginine residues (*207;229*). Protamine is used as a therapeutic in patients following extracorporeal circulation, in order to reverse the anticoagulant effect of heparin (*207*). *In vitro*, the activity of CPN (toward small synthetic substrates, such as FA-Ala-Lys, as well as the physiological proteins bradykinin and anaphylatoxin C3a) is partially inhibited (60-70%) by protamine (*204;207*). CPN removes all four carboxy-terminal arginine residues from protamine, but this does not abolish inhibition by protamine: desArg-protamine retains 30-35% of its inhibitory activity toward CPN, indicating that it has a mechanism of inhibition not entirely dependent on these residues (*207*). The so-called protamine reversal syndrome occurs in a small percentage of patients undergoing cardiac bypass. Following administration of protamine after extracorporeal circulation, these patients suffer pulmonary hypertension and bronchoconstriction with subsequent systemic hypotension. It is believed that the catastrophic syndrome is due to release of

anaphylatoxins and bradykinin, which cannot be adequately controlled due to protamine-induced inhibition of CPN (*207;230*). The exact mechanism is still under debate.

1.1.4.2. The carboxypeptidase family

In most mammalian species, including humans, there are thirteen known members of the metallocarboxypeptidase gene family (*190*). These members are grouped into subfamilies based on substrate specificity (the CPA subfamily preferentially cleaves aromatic amino acids, while the CPB subfamily preferentially cleaves basic residues) or amino acid sequence similarities (CPA/B subfamily and CPN/E subfamily). The latter is the more commonly used classification system. Subfamilies typically have 25-63% amino acid sequence identity between members, but this falls to only 15-25% identity for comparison across subfamilies (*231*).

The CPA/B subfamily includes the pancreatic enzymes CPA1, CPA2, the related mast cell CPA (mc-CPA), CPB, and the plasma enzyme TAFIa. These enzymes generally have a molecular mass in the range of 34-36 kDa and are secreted as inactive zymogens, requiring proteolytic activation through removal of a pro-peptide segment (*174;190;232-235*). The CPN/E subfamily counts CPN, CPE (also known as CPH), CPM, CPD, and CPZ among its members; all of which have CPB-type specificity. This subfamily is also known as the 'regulatory' carboxypeptidase subfamily due to the roles of its members in activating or inactivating biological mediators and peptide hormones (*236-239*). Several other members of the metallocarboxypeptidase gene family exist that are inactive and unrelated to either the CPA/B or CPN/E subfamilies, these include AEBP1, CPX1, CPX2, and the third domain of CPD (*189*).

Members of the CPN/E subfamily differ widely in function, size, enzymatic properties (such as pH optima) and localization (*198;236-240*). These enzymes, unlike their CPA/B counterparts, are formed as active carboxypeptidases and do not require the removal of a pro-peptide. Inappropriate activity is prevented by reliance on substrate specificity and sub-cellular compartmentalization (*190*). The structure of CPN/E enzymes also differs from CPA/B enzymes due to the presence of an additional domain of approximately 80 residues, which bears resemblance to the β-barrel fold of transthyretin and is therefore called the transthyretin-like domain (Figure 1.14) (*231;241;242*).

Unlike any other mammalian carboxypeptidase, CPN contains a regulatory subunit. The catalytic and regulatory subunits of CPN are unrelated functionally, structurally and immunogenically (*196;201*). However, the catalytic subunit bears strong sequence similarity to its subfamily members, specifically CPH/E, which is described as its closest ancestor (*196;197;243*). These two members differ mainly at their carboxy-termini, with CPN being extremely basic, while the CPE/H is acidic (*242*). CPN also has strong sequence similarity with CPM, a membrane-bound carboxypeptidase (*197*).

The human CPN1 subunit also has a strong sequence similarity to CPN1 of other species, including chimpanzee, monkey, cow, dog, rat, and mouse (Figure 1.15) (*191;195;200*). These species also have multimeric CPN. For example, porcine CPN is a 315 kDa protein composed of a 90 kDa glycoyslated regulatory subunit and a 30 kDa catalytic subunit (*208*). Despite the high degree of similarity (greater than 77% for the species listed in Figure 1.15), there are some differences in substrate specificities and catalytic rates (*191*). Porcine CPN removes carboxy-terminal arginine residues from substrates faster than lysine residues when the penultimate residue is an alanine, which is the inverse of the human CPN specificity (*195*). CPN from all species appear to have similar susceptibilities to human CPN inhibitors.

1.1.4.3. Activity of CPN

Prior to the 1960s, carboxypeptidases were not thought to exist in the blood (*209;244;245*). However, these impressions changed with the discovery of CPN as a regulator of the kinins in 1962 (*218*). CPN is now accepted to be an important regulator of potent peptide hormones released into the circulation (*197;206*). CPN exerts its regulation through hydrolysis of the carboxy-terminal basic residues of its substrates, which changes their activity or binding specificity (*192;218;246*).

Figure 1.14: Structure of CPA/B enzymes versus CPN/E enzymes. Carboxypeptidase B (of the CPA/B family) is shown in blue and the catalytic subunit of carboxypeptidase N (of the CPN/E family) is shown in red. The β-barrel fold transthyretin-like domain is clearly shown at the bottom of CPN1. This figure was generated with PyMOL software. The CPB structure was obtained from PDB 1KWM, and the CPN1 structure from 2NSM.


Dog MSGLLLVSFHLLLLFKLVAP VTFRHHRYDDLVRTLYKVHNE C PYITRVYSIGRSVKGRHLYVLEFSDYPGIHEPLEPEVK 80
Cow MSDLVSIFLH-LLLFKLVAP VTFRHHRYDDLVRMLYKVHNE C PHITRVYSIGRSVKGRHLYVLEFSDYPGIHEPLEPEVK 79
Human MSDLLSVFLHLLLLFKLVAP VTFRHHRYDDLVRTLYKVQNE C PGITRVYSIGRSVEGRHLYVLEFSDHPGIHEPLEPEVK 80
Chimp MSDLLSVFLHLLLLFKLVAP VTFRHHRYDDLVRTLYKVQNE C PGITRVYSIGRSVEGRHLYVLEFSDHPGIHEPLEPEVK 80
Monkey MSDLLSVFLHLLLLFKLVAP VTFRHHRYDDLVRTLYKVQNE C PSITRVYSIGRSVEGRHLYVLEFSDHPGIHELLEPDVK 80
Rat MPDLPSAFLPLLLLSKFVTP VTFRHHRYDDLVRTLYKVHNQ C PDITRLYNIGRSVKGRYLYVLEFSDYPGTHEPLEPEVK 80
Mouse MPDLPSAFLPLLLLSKFVTP VTFRHHRYDDLVRTLYKVHNQ C PDITRLYNIGRSVKGRYLYVLEFSDYPGIHEPLEPEVK 80
Opossum MSEVPSVLCRLLLLLGGVAS VTFHHHRYDGLMRTLYKVHNE C PHITRVYSIGRSVKGRHLYALEFSDSPGIHELLEPEFK 80
Dog YVGNM H GNEVLGR E LLLQLAEFL C EEFRNGNQRIVRLVEGTRVHILPSMNPDGYEVAVAQG-ADSSGYLVG R NNANGVDL 159
Cow YVGNM H GNEVLGR E LLLQLSEFL C EEFRNRNQRIVRLVEDTRIHIMPSMNPDGYEVAAAAQERDISGYLVG R NNANGVDL 159
Human YVGNM H GNEALGR E LMLQLSEFL C EEFRNRNQRIVQLIQDTRIHILPSMNPDGYEVAAAQG-PNKPGYLVG R NNANGVDL 159
Chimp YVGNM H GNEALGR E LMLQLSEFL C EEFRNRNQRIVQLIQDTRIHILPSMNPDGYEVAAAQG-PNKPGYLVG R NNANGVDL 159
Monkey YVGNM H GNEALGR E LMLQLSEFL C EEFRNRNQRIVRLIQDTRIHILPSMNPDGYEVAAAQG-PNKHGYLVG R NNANGVDL 159
Rat YVGNM H GNEVLGR E LLLQLSEFL C EEFRNRNQRILRLIQDTRIHILPSMNPDGYEVAAAQG-PNTSGYLVG R NNANGVDL 159
Mouse YVGNM H GNEVLGR E LLLQLSEFL C EEFRNRNQRILRLIQDTRIHILPSMNPDGYEVAAAQG-PNMSGYLVG R NNANGVDL 159
Opossum YVGNM H GNEVLGR E LLIQLCEFL C EEYRQRNERIVRLIHNTRIHILPSMNPDGYEVAADQG-PDSNGYLVG R NNANGIDL 159
Dog NRN FPDLNTYIYYNEKHGGPNHHLPLPDNWKSQVEPETQAVIHWIRSFNFVLSANL H GGAVVANYPY D KSLEHRVRGF RR 239
Cow NRN FPDLNTYIYYNEKNGGPNHHFPLPDNWKSQVEPETQAVIQWIRSFNFVLSANL H GGAVVANYPY D KSLGHRVRGF RR 239
Human NRN FPDLNTYIYYNEKYGGPNHHLPLPDNWKSQVEPETRAVIRWMHSFNFVLSANL H GGAVVANYPY D KSFEHRVRGV RR 239
Chimp NRN FPDLNTYIYYNEKNGGPNHHLPLPDNWKSQVEPETRAVIRWMHSFNFVLSANL H GGAVVANYPY D KSFEHRVRGV RR 239
Monkey NRN FPDLNTYIYYNEKYGGPNHHLPLPDNWKSQVEPETRAVIRWMHSFNFVLSANL H GGAVVANYPY D KSFEHRVRGV RR 239
Rat NRN FPDLNTYFYYNSKYGGPNHHLPLPDNWKSQVEPETRAVIQWIRSLNFVLSANM H GGAVVANYPY D KSLEHRFRSP HR 239
Mouse NRN FPDLNTYFYYNSKNGGPNHHLPLPDNWKSQVEPETRAVIQWIRSLNFVLSANM H GGAVVANYPY D KSLEHRFRGP HR 239
Opossum NRN FPDLNTYMYYNDKHGGPNHHIPLPDNWRNQVEPETEAVIKWMDSINFILSANL H GGAVVANYPY D KSREHRVRGF RR 239

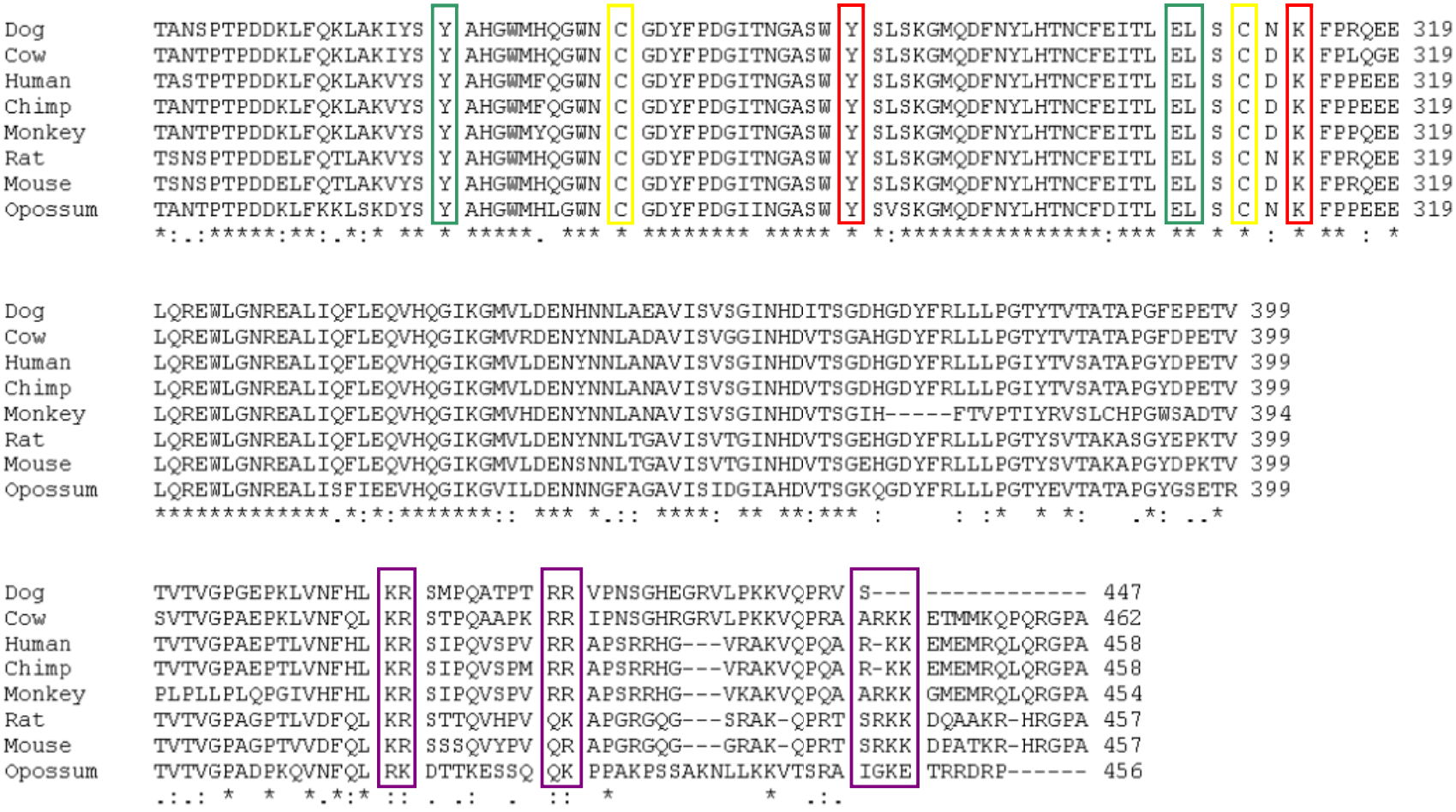

Dog TANSPTPDDKLFQKLAKIYS Y AHGWMHQGWN C GDYFPDGITNGASW Y SLSKGMQDFNYLHTNCFEITL EL S C N K FPRQEE 319
Cow TANTPTPDDKLFQKLAKIYS Y AHGWMHQGWN C GDYFPDGITNGASW Y SLSKGMQDFNYLHTNCFEITL EL S C D K FPLQGE 319
Human TASTPTPDDKLFQKLAKVYS Y AHGWMFQGWN C GDYFPDGITNGASW Y SLSKGMQDFNYLHTNCFEITL EL S C D K FPPEEE 319
Chimp TANTPTPDDKLFQKLAKVYS Y AHGWMFQGWN C GDYFPDGITNGASW Y SLSKGMQDFNYLHTNCFEITL EL S C D K FPPEEE 319
Monkey TANTPTPDDKLFQKLAKVYS Y AHGWMYQGWN C GDYFPDGITNGASW Y SLSKGMQDFNYLHTNCFEITL EL S C D K FPPQEE 319
Rat TSNSPTPDDELFQTLAKVYS Y AHGWMHQGWN C GDYFPDGITNGASW Y SLSKGMQDFNYLHTNCFEITL EL S C N K FPRQEE 319
Mouse TSNSPTPDDELFQTLAKVYS Y AHGWMHQGWN C GDYFPDGITNGASW Y SLSKGMQDFNYLHTNCFEITL EL S C D K FPRQEE 319
Opossum TANTPTPDDKLFKKLSKDYS Y AHGWMHLGWN C GDYFPDGIINGASW Y SVSKGMQDFNYLHTNCFDITL EL S C N K FPPEEE 319
LQREWLGNREALIQFLEQVHQGIKGMVLDENHNNLAEAVISVSGINHDITSGDHGDYFRLLLPGTYTVTATAPGFEPETV 399
LQREWLGNREALIQFLEQVHQGIKGMVRDENYNNLADAVISVGGINHDVTSGAHGDYFRLLLPGTYTVTATAPGFDPETV 399
LQREWLGNREALIQFLEQVHQGIKGMVLDENYNNLANAVISVSGINHDVTSGDHGDYFRLLLPGIYTVSATAPGYDPETV 399
LQREWLGNREALIQFLEQVHQGIKGMVLDENYNNLANAVISVSGINHDVTSGDHGDYFRLLLPGIYTVSATAPGYDPETV 399
LQREWLGNREALIQFLEQVHQGIKGMVHDENYNNLANAVISVSGINHDVTSGIH-----FTVPTIYRVSLCHPGWSADTV 394
LQREWLGNREALIQFLEQVHQGIKGMVLDENYNNLTGAVISVTGINHDVTSGEHGDYFRLLLPGTYSVTAKASGYEPKTV 399
LQREWLGNREALIQFLEQVHQGIKGMVLDENSNNLTGAVISVTGINHDVTSGEHGDYFRLLLPGTYSVTAKAPGYDPKTV 399
LQREWLGNREALISFIEEVHQGIKGVILDENNNGFAGAVISIDGIAHDVTSGKQGDYFRLLLPGTYEVTATAPGYGSETR 399
TVTVGPGEPKLVNFHL KR SMPQATPT RR VPNSGHEGRVLPKKVQPRV S--- ---------- 447
SVTVGPAEPKLVNFQL KR STPQAAPK RR IPNSGHRGRVLPKKVQPRA ARKK ETMMKQPQRGPA 462
TVTVGPAEPTLVNFHL KR SIPQVSPV RR APSRRHG---VRAKVQPQA R-KK EMEMRQLQRGPA 458
TVTVGPAEPTLVNFHL KR SIPQVSPM RR APSRRHG---VRAKVQPQA R-KK EMEMRQLQRGPA 458
PLPLLPLQPGIVHFHL KR SIPQVSPV RR APSRRHG---VKAKVQPQA ARKK GMEMRQLQRGPA 454
TVTVGPAGPTLVDFQL KR STTQVHPV QK APGRGQG---SRAK-QPRT SRKK DQAAKR-HRGPA 457
TVTVGPAGPTVVDFQL KR SSSQVYPV QR APGRGQG---GRAK-QPRT SRKK DPATKR-HRGPA 457
TVTVGPADPKQVNFQL RK DTTKESSQ QK PPAKPSSAKNLLKKVTSRA IGKE TRRDRP------ 456

Figure 1.15: Alignment of primary sequence of CPN1 from various species. The alignment includes the CPN1 sequence from human, chimpanzee, monkey, cow, dog, opossum, rat, and mouse. Residues that are identical across all species are indicated with an asterisk (*), conservative changes are indicated with a colon (:), and semi-conservative changes are indicated with a full-stop (.). Important residues for catalysis (green), zinc coordination (blue) and substrate binding (red) are outlined. Cysteines (yellow) are outlined. Potential dibasic plasmin cleavage sites (purple) are also outlined. The amino-terminal signal sequence (black) is included in the alignment and comprises residues 1-20, therefore Val^{1} is described as Val^{21} in this alignment. This figure was generated with the CLUSTALW sequence alignment program.

In the years since the discovery of CPN, numerous substrates have been identified, including: bradykinin (*218;219*), kallidin (*218;219*), the complement-derived anaphylatoxins (*192;247;248*), hexapeptide enkephalins (*187*), fibrinopeptides and fibrin degradation products (*166;181;184*), protamine (*249*), creatine kinase MM- and MB-isoenzymes (*186;250;251*), stromal cell derived factor-1 α (*185*), AIIt receptor (*90;252*), the α-chain of hemoglobin (*253*), and most recently chemerin (*254*). These proteins and peptides are involved in a wide range of (patho)physiological pathways, which suggests a role for CPN in the modulation of many varied processes, such as inflammation, fibrinolysis, sepsis, cell homing, cancer metastasis, and pain regulation. Furthermore, although the 48 kDa cleavage product of CPN1 has a similar substrate specificity as the full-length 55 kDa CPN1, it might in fact be slightly more active toward certain substrates (*166;188;198;201;203;207*). The two forms of CPN1 are thought to co-exist, although the role of the two forms has yet to be fully explained.

A CPN1 knock-out mouse model was recently developed to examine the role and importance of CPN in the circulation (*255*). Interestingly, although CPN1 is expressed early on during embryonic development (*256*), the absence of CPN1 did not lead to embryonic lethality. In fact, $CPN1^{-/-}$ mice developed normally, had no gross abnormalities and produced viable offspring. They did not overcompensate by upregulating any alternate active carboxypeptidase, as the plasma of these mice was unable to cleave the carboxy-terminal residue from C3a, C5a or FA-Ala-Lys. $CPN1^{+/-}$ mice demonstrated intermediate cleavage of these substrates. The primary difference between the wild type and CPN1 knock-out mice is the increased susceptibility of the knock-out to injection with cobra venom factor, a protein which initiates complement activation. The increased mortality of cobra venom factor-treated $CPN1^{-/-}$ mice appears to be due to the effects of histamine release as a result of uncontrolled C5a activity.

1.1.4.3.1. Bradykinin and kallidin

Bradykinin is a nonapeptide of the sequence Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, and kallidin (Lys-bradykinin) is a related decapeptide with an additional amino-terminal lysine residue (*257-259*). These peptide hormones act in an autocrine and paracrine fashion to produce numerous effects such as hypotension, edema formation, pain sensation, smooth muscle contraction, cell proliferation, increased vascular permeability, and trafficking of leukocytes (*260-265*). Kinins are produced by proteolytic cleavage of

kininogens by kallikrein; cleavage of high molecular weight kininogen yields bradykinin, and cleavage of low molecular weight kininogen yields kallidin (*260*).

Kinins act on the G-protein coupled receptors B_1 and B_2. The B_2 receptor is constitutively expressed, and bradykinin and kallidin have relatively similar binding affinities. In contrast, the B_1 receptor is generally absent (or undetectable) on the cellular surface of most cell types, but its expression can be induced during instances of tissue injury and inflammation. The B_1 receptor responds primarily to partially digested kinins, which have lost their carboxy-terminal arginine residues (desArg-kallidin and desArg-bradykinin) (*266;267*). The kinin receptors differ in their degree of receptor desensitization, ligand binding kinetics and response time (*268-276*). The B_1 kinin receptor, with its inducible nature, agonist-induced up-regulation and persistent response is well suited for periods of chronic inflammation, while the B_2 receptor, with its fast ligand dissociation, transient activity and desensitization, is primarily associated with acute inflammation (*274*).

There are at least nine enzymes in the circulation which degrade the kinins, and these are collectively termed kininases (Figure 1.16). This activity was first discovered when researchers noticed that the effects of bradykinin and kallidin were significantly diminished upon standing in plasma (*277;278*). When bradykinin is pre-incubated with the IV-1 fraction of plasma, its activity (as measured by rat uterus contraction and blood pressure) is diminished as a function of incubation time (*218*). The enzyme responsible for this effect was isolated and named kininase I (and later renamed CPN). Incubation of bradykinin with purified CPN results in a similar destruction of bradykinin activity (*279-282*).

Of all the kininases, only CPN and the related CPM (of lung tissues) are responsible for altering the kinin receptor specificity from B_2 to B_1 (*246;262*). The other enzymes, including angiotensin I converting enzyme (ACE), cleave bradykinin and kallidin at alternate peptide bonds, and this completely abolishes receptor binding (*283-294*). In human blood, the concentration of desArg-bradykinin is three-fold higher than that of full-length bradykinin (*199;280*). In fact, desArg-bradykinin is the most abundant bradykinin degradation product, therefore CPN is thought to be the major kininase in blood (*280-282*).

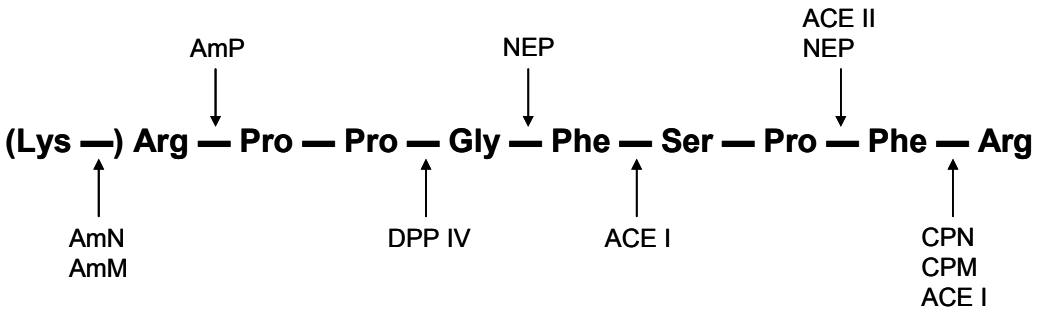


Figure 1.16: The location in bradykinin and kallidin of the peptide bonds cleaved by kininases. Kallidin (Lys-bradykinin) begins at Lys^1, while bradykinin begins at Arg^1. The kininases which act on these peptides cleave the same bonds.

In the absence of CPN activity, bradykinin and kallidin act on the B_2 receptor, resulting in vasodilatation. Kinin action on the endothelium causes nitric oxide, prostacyclin and hyperpolarizing factor release (which act on smooth muscle cells causing relaxation), while action directly on smooth muscle cells can cause vasodilatation or vasoconstriction depending on the tissue source (*295*). In the presence of CPN activity, bradykinin and kallidin are converted to their desArg forms. These peptides act on the B_1 receptor if it has been upregulated, such as during instances of inflammation. The effects of the desArg peptides on the B_1 receptor are debatable. Depending on the species, types of tissues, and variety of other drugs administered, the effects vary significantly. Whether B_1 receptor agonism induces vasodilatation in humans in the absence of cardiovascular disease or ACE inhibitor therapy is questionable, but if it does, it is a smaller response than that seen by the action of bradykinin or kallidin on B_2 receptors. However, in addition to any response that may be generated, during times of high B_1 receptor expression this pathway may act as a sink to prevent B_2 receptor-dependent vasodilatation.

1.1.4.3.2. Anaphylatoxins

The complement cascade is a series of biochemical reactions which creates, among other products, the anaphylatoxins C3a, C4a and C5a. These peptides promote the inflammatory response by inducing the release of histamine from cells, which results in smooth muscle contraction, increased capillary permeability, and chemotaxis of selective bone marrow-derived myeloid cells (*192;296*). C5a is the most potent peptide; it causes guinea pig ileum contraction at low picomolar concentrations, and it enhances vascular permeability at sub-nanomolar concentrations (*227;297*). The anaphylatoxins induce inflammation through binding to their receptors: the C3a receptor and the C5a receptor.

These G-protein coupled receptors are 10- to 100-fold more sensitive to the full-length anaphylatoxins than the desArg-forms produced by the action of CPN (*296*).

Human C3a and C5a are rapidly inactivated, and therefore lose the ability to contract guinea pig ileum, when pre-incubated in serum from a human, a guinea pig, a rat or a rabbit (*192*). The enzyme responsible was isolated from human serum and termed anaphylatoxin inactivator (later renamed CPN). Incubation of anaphylatoxins with purified CPN results in a similar destruction of anaphylatoxin activity (*192*). The CPN-cleaved form of C3a was examined electrophoretically and found to migrate less rapidly towards the cathode; and free arginine was also detected. Additionally, amino acid analysis indicated one mole of arginine was released per mole of C3a; no other amino acids were released (*192*).

Cobra venom factor activates complement factor C3 and is used to induce complement activation in the research setting. Cobra venom factor was administered to normal guinea pigs and those that had been pre-injected with the (non-specific but effective) CPN inhibitor MERGETPA. No untreated guinea pigs experienced adverse reactions, while all MERGETPA-treated animals died very rapidly (*227*). These animals had distended lungs, increased wall thickness of their small muscular arteries and an interstitial infiltrate of mononuclear cells (*227*). Similarly, when purified C3a was given to normal and MERGETPA-treated guinea pigs, C3a proved lethal only in the MERGETPA-treated animals (*227;298*).

In plasma, the anaphylatoxins (at physiological concentrations) are inactivated within seconds. Human serum removes 1.15 μmol Arg from C3a per minute per nanomole of CPN and 0.508 μmol Arg from C5a per minute per nanomole of CPN (*188;227*). Purified CPN also rapidly inactivates the anaphylatoxins. At room temperature, a single molecule of CPN inactivates 150 to 200 molecules of C3a in per minute (*192*).

CPN may not be the only plasma carboxypeptidase responsible for the inactivation of the anaphylatoxins. The kinetics of C3a and C5a cleavage by CPN and TAFIa have been compared *in vitro*; TAFIa is more efficient at removing the carboxy-terminal arginine from C5a than is CPN, while CPN is the more efficient enzyme with C3a (*296;297*). However, the magnitude of the role of TAFIa *in vivo* is unknown, because TAFI

activation may not occur to an adequate degree in the course of inflammatory diseases (*296;297;299*). Furthermore, constitutive low levels of C3a are present in plasma due to minimal activation by the alternative pathway; in contrast, C5a is not generated in the absence of any foreign microorganisms. As CPN is the preferred enzyme inactivation of C3a, it follows that CPN is responsible for controlling inflammation in the absence of infection (*297*).

1.1.4.3.3. Creatine kinase

Creatine kinase (CK; EC 2.7.3.2.) is an intracellular cytosolic enzyme that catalyzes the reversible transfer of a phosphate group from adenosine triphosphate to creatinine (*185;186;296;300*). CK is a dimeric enzyme composed of two possible subunits which form three possible isoenzymes: CK-MM, CK-MB, CK-BB (*186*). Although generally an intracellular enzyme, CK can be released into the circulation during muscular damage or heart attack (*186*). The CK-M subunit has a single carboxy-terminal lysine residue, which is cleaved from CK when it is released into plasma. This results in several possible circulating CK forms: the three original enzymes CK-MM, CK-MB, CK-BB, as well as CK-MM1 (a single lysine released from one M subunit), CK-MM2 (two released lysine residues, one from each subunit) and CK-MB1 (one lysine released from the single M subunit) (*185;250;301-307*). The cleaved forms appear to have identical activity to the native enzymes, and they may not have any physiologically relevant extracellular activity. The ratio of native to cleaved CK isoforms is a marker for acute myocardial infarction; increased concentrations of intact CK-MM or CK-MB correlates to increased tissue damage following myocardial infarction (*251;296;301;308-311*).

Addition of the CPN inhibitors GEMSA and MERGETPA to plasma prior to the addition of purified CK-MM abolished the ability of plasma to cleave CK, and therefore CK-MM1 and CK-MM2 were not produced (*186;311*). Similarly, the addition of an antiCPN antibody inhibited CK-MM cleavage (*311*). Furthermore, purified CPN cleaves purified CK-MM and CK-MB *in vitro* and amino acid analysis indicates the release of lysine residues (*250;251;312*).

Although CPN activity in patients with angina or myocardial infarction are similar to normal patients at the time of admission to the hospital, CPN activity begins to increase approximately 48 hours after onset of chest pain, and peaks after 96 hours, at

approximately 140% of baseline CPN activity (*301;311*). However, these peaks do not correlate to infarct size, severity, or CK-MM or CK-MB ratios. It is unclear whether the increase in CPN is significant, given the large variability in the study measurements. If the increase is real, it is hypothesized to be due to induction of CPN activity caused by the increase in endogenous substrate (*301*). This theory has yet to be vigorously investigated.

1.1.4.3.4. Chemerin

Chemerin (also known as tazarotene-induced gene 2 and retinoic acid receptor responder 2) is a chemoattractant molecule for CMKLR-1 expressing cells. It circulates as prochemerin (residues 21-163) prior to proteolytic activation by factor XIIa, factor VIIa, plasmin, neutrophil elastase, mast cell chymase or mast cell tryptase (*254;313-316*). The carboxy-terminal cleavage sites of the various activators are not conserved, and the resultant products demonstrate diverse potencies *in vitro* (*254*). The shortest product (residues 21-152) is formed by elastase cleavage, and the longest (residue 21-158) by plasmin or mast cell tryptase (*254;313-316*). Active chemerin induces leukocyte migration and intracellular calcium mobilization, while mature prochemerin has minimal chemotactic activity (*317*).

The chemerin nonapeptide Tyr-Phe-Pro-Gly-Gln-Phe-Ala-Phe-Ser (residues 149-157) retains most of the activity of chemerin and is commonly used in *in vitro* assays (*254;317*). The decapeptide Tyr-Phe-Pro-Gly-Gln-Phe-Ala-Phe-Ser-Lys (residues 149-158) which contains the additional carboxy-terminal lysine residue is as inactive as the intact prochemerin molecule. There is no significant chemotactic activity of the decapeptide at up to 10 μM concentrations, while the nonapeptide demonstrates a peak response at 10-100 nM (*254*). When the decapeptide (which mimics the product formed by plasmic activation) is treated with purified CPN, it induces migration of CMKLR-1-expressing cells to a similar degree as the nonapeptide (*254*). A similar result was found when either the pentadecapeptide Tyr-Phe-Pro-Gly-Gln-Phe-Ala-Phe-Ser-Lys-Ala-Leu-Pro-Arg-Ser or full prochemerin was digested with plasmin, followed by CPN. Moreover, when plasmin-cleaved prochemerin was incubated in platelet-poor plasma, endogenous CPN cleaved the carboxy-terminal lysine residue and generated full chemerin activity (*254*). This is a unique role for CPN, as an up-regulator of activity, since the majority of its activities cause down-regulation.

1.1.4.3.5. Stromal cell-derived factor-1α

Stromal cell-derived factor-1 (SDF-1; also known as chemokine C-X-C motif ligand 12: CXCL12) is a chemokine of the family CXC. It has six splice variants, of which SDF-1α is the most predominant (*318-321*). The primary sequence of SDF-1 is highly conserved, with only a single residue differing between mouse and human sequences, which suggests an important biological role for SDF-1 (*322*). SDF-1 binds primarily to its G protein-coupled receptor CXCR4 (also known as fusin), which is expressed by most leukocytes, endothelial cells, epithelial cells and carcinomatous cells (it may also bind to a secondary receptor, CXCR7) (*323-327*). SDF-1 is strongly chemotactic for CXCR4-expressing cells, resulting in its important role in B-cell lymphopoiesis, hematopoiesis, cardiogenesis, neuronal development, immune cell homing and trafficking, tumorigenesis and metastasis (*323;326-333*).

SDF-1α (residues 1-68) is rapidly proteolyzed in plasma; initial rapid cleavage at the carboxy-terminus produces SDF-1α (residues 1-67) and this is followed more slowly by hydrolysis at the amino-terminus to form SDF-1α (residues 3-67) (*185*). SDF-1α loses its ability to stimulate pre-B-cell proliferation and chemotaxis following release of its carboxy-terminal lysine residue; similarly, when the undecapeptide Ile-Gln-Glu-Tyr-Leu-Glu-Lys-Ala-Leu-Asn-Lys (representing SDF-1α residues 58-68) is incubated with plasma, serum or purified CPN, the carboxy-terminal lysine residue is released (*185*). CPN was determined to be the plasma carboxypeptidase responsible for SDF-1α cleavage based on immunoprecipitation experiments, ruling out a contribution by TAFIa (*185*).

Unlike most chemokines, which are regulated by inducible expression, SDF-1α is constitutively expressed. Therefore, stepwise degradation, as a method to control the activity of SDF-1α, is a crucial regulatory mechanism (*185;318;319;334*). SDF-1α isolated from plasma or serum lacks a carboxy-terminal lysine residue, indicating constitutive proteolysis of the chemokine by CPN in the circulation (*185*).

1.1.4.3.6. Other CPN substrates

CPN has been implicated in the reduced binding affinity of plasminogen to cell surface receptors. The binding of plasminogen to its cellular receptors is mediated through its lysine binding kringle domains (*335;336*). Analogous to the binding of plasminogen to fibrin, cell surface binding reduces the susceptibility of plasmin to its plasma inhibitors,

controls the localization of plasmin activity, and since uPA and plasminogen receptors are present on the same cells, it enhances the rate of plasminogen activation (*129;335;337*).

One endothelial cell surface plasminogen receptor is the annexin II heterotetramer (AIIt). This receptor is composed of two annexin II monomers and two monomers of the calmodulin-related protein S100A10; this latter protein has lysine as the ultimate and penultimate carboxy-terminal residues (*338*). The ability of this receptor to stimulate plasminogen activation was dramatically decreased (to less than 20%) by site-directed mutagenesis of the S100A10 subunits to eliminate the terminal lysine residues (*339*). Similarly, when AIIt was treated with CPN, its ability to enhance plasminogen activation was decreased to the rate of desLys-AIIt. Additionally, CPN was demonstrated to release 4 moles of lysine per mole of AIIt, indicating that all four ultimate and penultimate lysine residues were removed (*90;252*).

CPN is one of the enzymes implicated in the processing of carboxy-terminally extended enkephalins (*187*). There are two forms of enkephalins in humans, Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu), which compete with and mimic the effects of opiate drugs (*340*). Met-enkephalin is derived from the proenkephalin and proendorphin precursors, while Leu-enkephalin is derived from the proenkephalin and prodynorphin precursors (*341-345*).

The primary sequence of each precursor molecule contains several copies of the Met- and/or Leu-enkephalin peptide sequence, each one flanked by dibasic sites: Lys-Arg, Arg-Arg, or Lys-Lys (*340;341;346;347*). Various peptidases of tryptic specificity cleave between the dibasic sites and free the enkephalin peptides (*341;348*). This results in free carboxy-terminally extended hexapeptide enkephalins, with an arginine or lysine residue remaining in the final position. Additionally, some highly extended enkephalins can be released, such as [Met-enkephalin]-Arg-Phe, [Met-enkephalin]-Arg-Gly-Leu, and [Leu-enkephalin]-Arg-Lys-Tyr-Pro, which require further proteolysis prior to attaining full activity (*341;349*).

The hexapeptide enkephalins (carboxy-terminally extended by arginine or lysine) are not particularly specific toward binding the μ-, δ-, or κ-opioid receptors; and compared to all

other enkephalin peptides they demonstrate the highest binding rates toward the lesser used κ-opioid receptor. In contrast, the fully truncated, pentapeptide Met- and Leu-enkephalins are very specific for the δ-opioid receptors and have almost non-existent binding at the κ-opioid receptor (*187;189;194;349*). CPN, as well as its isolated catalytic subunit, remove the carboxy-terminal basic residue from hexapeptide enkephalins (*187*). The kinetic constants related to this hydrolysis reaction are presented in Table 1.1, (although not listed, the kinetic constants for the isolated catalytic subunit are nearly identical to those found with the tetrameric enzyme). CPN may therefore play a major role in altering the binding specificity of enkephalins.

In vitro CPN appears to be involved in altering the binding affinity of oxygen to hemoglobin (*253*). Hemoglobin is a heterotetrameric protein (two α subunits and two β subunits) which transports oxygen from the lungs to the tissues (*350*). The 141-residue α subunit has a carboxy-terminal arginine residue which forms salt bridges to the amino group of the amino-terminal valine and to the carbonyl group of Asp^{126} of the other α subunit. These salt bridges stabilize the deoxyhemoglobin form, which results in a lowered affinity of hemoglobin for oxygen (*351*).

CPN cleaves the carboxy-terminal arginine residue from the hemoglobin α subunit with a K_M of 105 μM, and a catalytic efficiency of 0.00047 $\mu M^{-1}s^{-1}$ (*253*). The isolated catalytic subunit of CPN also removes this arginine residue with nearly identical efficiency (*253*). This cleavage appears to dramatically increase the dissociation of hemoglobin from tetramers into dimers: deoxyhemoglobin dissociates into dimers with a half-life of 13.9 hours, while desArg-deoxyhemoglobin dissociates with a half-life of only 19.9 seconds (*253*). Therefore *in vitro* CPN is implicated in the enhanced dissociation of hemoglobin, the increase in its affinity for oxygen, and an overall decrease in the amount of oxygen delivered to tissues; however, the physiological effects of CPN toward hemoglobin are unclear. Hemoglobin is an intracellular protein, and CPN is an extracellular enzyme, therefore the ability of CPN to cleave hemoglobin is restricted by compartmentalization. However, CPN may prevent oxygen scavenging of released hemoglobin.

Several other substrates have been suggested for CPN, but have yet to be definitively proven or are not physiologically relevant. These include the enolase α-α isoform, albumin propeptide and the complement component Ba. Enolase (EC 4.2.1.11) is an

intracellular dimeric protein made up of 3 possible subunits derived from different genes: α, β, and γ. The α-α enolase isoform is found in the adrenals, red blood cells, and brain-, thyroid-, liver-, kidney-, and adipose-tissues. In the circulation, the α subunit is modified to a form with a higher anodal mobility in electrophoresis, and the α-α isoform produces three distinct forms: an unmodified form, a form with a single subunit modification, and a doubly modified form (*352*). This activity can be inhibited by the chelator EDTA, and the plasma-purified CK-MM modifying enzyme (later renamed CPN) can modify α-α enolase. Unfortunately, at the time of discovery of this modifying enzyme, TAFI had not yet been discovered. Although the researchers claim CPN is the serum protein responsible, it is possible that TAFIa plays a role, particularly because the modifying enzyme was found to be heat labile, and the activity is lost following a 30 min incubation at 60°C. Since the original discovery, the potential of α-α enolase as a substrate for CPN has not been pursued, possibly because even as the authors state, it is highly possible that α-α enolase is just an innocent bystander and that the reaction is not physiologically relevant.

Another potential CPN substrate which has dubious physiologically relevance is the albumin propeptide. In the Golgi, immediately prior to secretion proalbumin is converted to albumin by proteolytic cleavage of the amino-terminal propeptide. This propeptide has the sequence Arg-Gly-Val-Phe-Arg-Arg. This propeptide is secreted along with albumin, but is degraded in plasma, beginning with cleavage of the two carboxy-terminal arginine residues (*353*). CPN is suspected to be the responsible enzyme; however the relevance of this reaction is questionable, as the propeptide serves no known function once released from albumin. This may be another case of CPN action on an innocent bystander. Similarly, the complement component Ba has the carboxy-terminal sequence …-Gln-Lys-Arg, which is degraded in the circulation through loss of its terminal basic residues (*354*). CPN inhibitors added to serum inhibit this action, and therefore CPN is suspected to play a role; however, the relevance of this reaction is unknown. Note that although these are not likely relevant reactions in themselves, these types of innocent bystanders may act as competitive inhibitors of CPN, preventing or slowing its action toward its physiological substrates.

1.1.4.4. Clinical aspects of CPN activity

Despite the significant amount of published research regarding CPN, its physiological role is not entirely clear. CPN is thought be necessary for life, as no person who completely lacks CPN has been discovered, yet CPN1-knockout mice appeared relatively normal except during anaphylatoxin challenge (*255*), and the concentration of CPN in various diseased states appears to remain relatively unchanged (*195;218;227;230;355-365*). Plasma samples collected from the United States of America, the Soviet Union, and Sweden demonstrate nearly identical average CPN activities (*204;210;366*). However, the situation is not so straightforward; the complexities relating to CPN activity and function and a poor understanding of the mechanism of action of the enzyme cause significant errors in the functional determination of CPN.

1.1.4.4.1. Measurement of CPN activity

Complicating the research into the physiological relevance of CPN and its clinical utility (as a diagnostic or therapeutic target) is the method of CPN determination, with the preferred method having dramatically varied over the years. Several of the methods used are complex, unreliable, poorly performed or have wide variances. Furthermore, the vast majority of the studies were performed before the discovery of the other basic plasma carboxypeptidase, TAFI; therefore the problem may be compounded by the presence of an additional carboxypeptidase of similar specificity which may have been activated to varyious degrees during CPN analysis (*367*). Further inconsistencies include the use of plasma versus serum samples, the methods of blood collection, the temperature, pH, and buffer conditions of the assay, the use of native versus cobalt-activated CPN, and the type and characteristics of the substrate. A summary of the methods and conclusions of the major CPN determinations are briefly outlined below.

One of the most commonly used assays was that of Schweisfurth *et al.* (*368*). This assay measures the activity of CPN toward two synthetic substrates, one with a carboxy-terminal arginine residue (hippuryl-L-arginine) to measure what they term CN_1 activity, and one with a carboxy-terminal lysine residue (hippuryl-L-lysine) to measure the so-called CN_2 activity. Substrates are added to serum and after a set incubation period, the reaction by-product, hippuric acid, is extracted and its absorbance is read at 228 nm. Unlike most other assays, this method includes detailed blood collection procedures.

Using the Schweisfurth method, CPN activity was measured in patients with various lung conditions (*361;368*). The researchers found that CN_1 and CN_2 activities were elevated in patients with sarcoidosis, lung cancer and chronic bronchitis (compared to control patients); CN_2 activity was also elevated in patients with asthma and pneumonia but CN_1 activity was not. The inconsistency in CN_1 and CN_2 activities might be explained by the varying specificity of CPN toward the synthetic and endogenous substrates (if there is a large concentration of endogenous substrates in the sample toward which CPN has a higher affinity than it does toward the CN_1 substrate but a lower affinity than it does toward the CN_2 substrate, it will produce inconsistent conclusions for CPN activity), as well as by a contribution of the enzyme CPM. The membrane-bound CPM is present in many tissues and has a particularly high concentration in the lungs (*238*). CPM demonstrates a nearly identical pH optimum and activator and inhibitor profiles as CPN. However, unlike CPN, CPM prefers to hydrolyze substrates with carboxy-terminal arginine residues over those with carboxy-terminal lysine residues, as demonstrated by the kinetic constants in Table 1.2 (*369*). If CPM is released into the blood during instances of tissue injury, it could falsely inflate the results for CN_2 activity (which is measured with a carboxy-terminal arginine containing substrate).

Table 1.2: CPM and CPN kinetic constants toward hexapeptide enkephalins.

Substrate	*Enzyme*	K_M *(μM)*	k_{cat} *(min^{-1})*	k_{cat}/K_M *($\mu M^{-1}min^{-1}$)*
[Met^5]enkephalin-Arg^6	CPM	46	934	20.3
	CPN	49	1020	20.9
[Met^5]enkephalin-Lys^6	CPM	375	663	1.8
	CPN	216	6180	28.7
[Leu^5]enkephalin-Arg^6	CPM	63	106	1.7
	CPN	57	360	6.6

Schweisfurth *et al.* also found that neither CN_1 nor CN_2 activity was changed in patients with Hodgkin's disease, non-Hodgkin's lymphoma, or plasmacytoma; but that CPN activity was raised in patients with carcinomas. Interestingly, in patients that responded to treatment with chemo- and/or radiation-therapy, the elevated CN_1 and CN_2 activities decreased to the normal range (*361;368*). Again, the increased CPN activity may be explained by factors other than increased CPN concentration. The fibrinolytic system has extensive links to cancer. Specifically, the plasminogen activator, uPA, is upregulated in

some forms of cancer; this leads to increased plasmin generation, which degrades the extracellular matrix and aids in tumour invasion and metastasis (*370*). Plasmin is also known to cleave CPN to a more active form, as described in section 1.1.4.5. Therefore the increase in CPN described by Schweisfurth *et al.* may be due to an increase in CPN activity, rather than an increase in CPN concentration.

Using this same method, Sommer *et al.* analyzed the activity of CPN in various forms of bowel disorders (*371*). CPN activity was decreased in patients with inflammatory bowel disease and raised in those with Crohn's disease and ulcerative colitis. However, in all cases, the values determined for individual patients were quite widely scattered, and no significant changes in enzyme concentration could be concluded. Furthermore, there was no correlation in CPN activity with respect to location, extent, or severity of the diseases, nor following treatment with corticosteroids.

Although long and technically demanding, the assay used by Schweisfurth *et al.* has many benefits over other commonly used methods. The hydrolysis reaction is performed under optimal conditions, the assay variability is low, and the contamination by TAFIa activity is probably low. Unfortunately, this experimental method precludes multiple simultaneous analyses and the measurement comes as only a single end-point. Curiously, these researchers comment that EDTA, 1,10-phenanthroline and various other inhibitors affect the CN_1 and CN_2 activities differently. This result is unexpected because CN_1 and CN_2 are both supposed to represent CPN activity. Also, Schweisfurth *et al.* comment that an increase in endogenous CPN substrates causes a decrease in the CN_1 activity (but has no effect on CN_2 activity). Any endogenous CPN substrates present in the samples can alter the measured activity toward the exogenous synthetic substrates; therefore if careful controls are not made to account for endogenous substrates, the measurements of CPN activity would be inaccurate. These types of controls were not performed in any of the sudies mentioned above (nor in any of the remaining studies mentioned in this section). This assay therefore doesn't measure CPN concentration as a function of CPN activity, but rather CPN activity toward a specific substrate, which may be altered under various circumstances. These issues cast some doubt on the conclusions of these researchers.

Another group of researchers, Erdos *et al.*, developed a continuous spectrophotometric assay for the determination of CPN activity (*372*). Serum samples are activated with

cobalt and the substrate hippuryl-L-lysine is added to diluted serum. The splitting of hippuric acid is followed continuously at 254 nm; and the rate of hydrolysis is calculated from the initial rate. Using this method, Erdos *et al.* found CPN activity to be unchanged (compared to controls) in patients with cardiac disorders, varied infectious and inflammatory diseases, arthritic conditions, gastrointestinal disorders, uremia, renal diseases, diabetes, uticaria, angioneurotic edema, bronchial asthma, non-infectious rhinitis and pancreatitis (*209;210;361;373*). Their additional findings of raised CPN activity in patients with neoplastic conditions agrees with those of Schweisfurth *et al*. They also found CPN activity to be lowered in patients with liver cirrhosis. This decrease may be due to decreased CPN synthesis, as CPN is synthesized in the liver. Finally, they also detected enhanced CPN activity in pregnant women in their third trimester, which returned to normal following birth.

Unlike other CPN assays, it is likely that TAFIa would be entirely inactivated by the method of Erdos *et al.* due to a prolonged incubation at 37°C to activate CPN with cobalt. This assay is quick and continuous, therefore kinetic measurements can be made. Unfortunately, optimal conditions are not used, including incomplete CPN activation. Furthermore, since the mechanism of CPN activity is not fully understood, nor is the effect of cobalt activation of CPN, the consequence of activation may depend on external factors, which could be altered in the different diseased states. Moreover, continuous measurement of hippuric acid release is not as sensitive as methods measuring total hippuric acid extracted from a reaction mixture, as in the Schweisfurth method. Finally, the conditions relating to blood collection were not outlined. These researchers have obtained blood samples from multiple physicians at multiple locations under inconsistent conditions. Many factors are known to affect the quality of blood drawn; for the related kininase ACE, the level of activity widely varies based on conditions such as level of physical activity and length of fast prior to veni-puncture (*360;374*). These issues may challenge the results presented by these researchers.

An assay developed by Koheil *et al.* (*375*) is similar to the Schweisfurth method, but with some modifications; namely the activation of serum samples with $CoCl_2$. Diluted, activated serum is incubated with hippuryl-L-lysine and the resulting hippuric acid is extracted and measured as in the Schweisfurth procedure. This method was used to determine the activity of CPN in patients with cystic fibrosis (*375*). Compared to control

patients, those with cystic fibrosis had significantly lower CPN activity; and the researchers found no significant differences in the results from male versus female patients. This is an interesting result, as the researchers hypothesize that deficient basic carboxypeptidase activity in cystic fibrosis patients could lead to an accumulation of positively charged peptides with ciliostatic activity, which exacerbate the disorder (*375;376*). This theory was based on previous studies which have demonstrated the destruction of ciliary dyskinesia, and improvement of patient condition, by treatment with pancreatic CPB (*376-378*).

Unfortunately, the control population used for this end-point assay was not composed of healthy people, but rather patients with variable disorders (including asthma, diabetes, familial dysplasia of lung, exocrine pancreatic insufficiency and neutropenia), miscellaneous ambulatory outpatients, and lab personnel. This unregulated control population may have an average CPN activity unlike that of healthy patients, and may therefore cause incorrect conclusions regarding the CPN activity in patients with cystic fibrosis.

Lieberman *et al.* (*379*) developed an assay similar to the Schweisfurth method., but with only the substrate hippuryl-L-arginine. It also follows different assay conditions with respect to serum dilution and substrate concentration. These researchers examined the activity of CPN in patients with lung disease and cystic fibrosis (*379*). In direct opposition of Koheil *et al.* (*375*), these researchers found CPN activity in patients with cystic fibrosis to be unchanged from healthy control patients. However, when separated by gender, they found CPN activity to be increased in female patients with cystic fibrosis, but unchanged in males with cystic fibrosis. Additionally, they found elevated CPN activity in patients with various lung diseases (in agreement with Schweisfurth *et al.* (*368*)), however this could be due to the activity of CPM, as mentioned previously.

The method of Lieberman shares the same limitations as that of Koheil, as well as having additional problems: noticeably suboptimal conditions were used, including a large amount of undiluted serum, and an extremely low concentration of substrate. Furthermore, only a very small number of patients were analyzed.

Plummer *et al.* developed a simple spectrophotometric assay using the substrates benzoyl-Ala-Arg and FA-Ala-Lys for the measurement of CPN activity in plasma (*205*). Plasma samples are activated with $CoCl_2$ and then added to the substrate solution. The reaction is followed continuously by the change in absorbance at 254 nm (for benzoyl-Ala-Lys) or 336 nm (for FA-Ala-Lys). This method was used by Chercuitte *et al.* to examine the activity of CPN in blood and synovial fluid of patients with various forms of arthritis (*380*). They found that the activity of CPN was increased in patients with rheumatoid and psoriatic arthritis, but not in those with osteoarthritis. They also found the activity in synovial fluid to be consistently lower than that in the matched blood samples. However, the values largely overlapped those of the healthy control patients in all cases; therefore deviance from normal CPN activity cannot be concluded.

This continuous assay by Plummer, like all other assays using cobalt-activated CPN, may have altered CPN activity in a manner which cannot be predicted or interpreted. Also, these researchers demonstrate a lack of consistency of blood collection procedure, which could alter the activity of CPN, as mentioned previously. Additionally, this assay uses plasma rather than serum samples, which may have small unknown effects on the activities measured, which limits comparability.

A novel assay developed by Corbin *et al.* (*249*) uses a non-synthetic substrate, salmine, for CPN quantification. Salmine is a component of the array of fish sperm proteins collectively known as protamine. Like all protamines, the final four carboxy-terminal residues of salmine are arginine. For this assay, diluted serum samples are activated with $CoCl_2$ and added to salmine. The reaction is stopped with trichloroacetic acid, centrifuged, and ninhydrin reagent is added to the supernatant. The absorbance of the ninhydrin imino derivative (Ruhemann's purple) is read at 570 nm. Corbin *et al.* determined CPN activity to be decreased in patients in dextran shock and those with dengue fever (*249*). They also examined the activity of CPN in patients with cystic fibrosis; they found the activity to be unchanged compared to control patients, which is in direct contradiction of Koheil *et al.* (*375*), who found CPN activity to be decreased in patients with cystic fibrosis and Lieberman *et al.* (*379*), who found CPN activity to be increased in female patients with cystic fibrosis.

This unique end-point assay, like all other assays, has several pros and cons. CPN is not fully activated under the conditions used, and there is likely some residual TAFIa activity due to the room temperature activation step. Additionally, due to the unique choice of salmine as a substrate, other problems arise. Firstly, each salmine molecule has four carboxy-terminal arginine residues, however the relative rates of hydrolysis of each residue are unknown (particularly the fourth arginine from the end, which would have a different neighbouring residue on the amino terminal side; the identity of the penultimate residue is known to affect the affinity and reaction rate of CPN). The apparent activity in samples with extremely high activity may be lower than actuality if the fourth arginine residue from the terminus has a neighbouring residue which decreases the rate of cleavage. Furthermore, the method of quantification of the hydrolysis reaction is through measurement of free arginine in the solution. However arginine is present in plasma at a concentration of 20-140 μM, and may change under different pathological conditions; therefore, the apparent CPN activity measured may be more or less than the true value. Finally, by their own admission "commercial protamine preparations obtained from salmon sperm contain several nearly identical polypeptides", therefore salmine is not as consistent as synthetic substrates, and the lot to lot variation may be large.

Although many interesting and creative methods have been developed to measure the concentration of CPN in patient samples, they all suffer many flaws, the most important of which is that they measure CPN specific activity without measuring CPN concentration. The activity of CPN is related not only to its concentration, but also its structure (for example the degree of plasmic activation), the presence of inhibitors, and the presence of endogenous substrates. All these factors must be considered when calculating the true concentration of CPN. Unfortunately, many of these aspects are not yet understood in the context of CPN activity. Further complicating matters is the variable presence of TAFIa, the lack of regulation regarding blood collection, suboptimal conditions and poor choices of substrates. Overall, these issues make the current knowledge of CPN activity in diseased and healthy states uncertain.

1.1.4.4.2. CPN deficiency

Of significant clinical interest is the discovery of several patients with CPN deficiencies. The first such patient was a 65 year old man who retained only 21% of normal CPN activity (*255;355;356;358*). He had an eleven year history of severe angioedema, with

approximately 40 episodes per year, none of which were related to diet, environment or emotional stress (*356*). During his attacks there was no elevation of serotonin or kinin activity, but his plasma histamine concentration increased, and there was a marked decrease in the inactivation of C3a and kallidin. All other test results were negative or normal. The researchers took 10 blood samples over a two-year period, all of which demonstrated reduced CPN activity (*356*). His CPN clearance was found to be unchanged from that of healthy volunteers, concluding that he has a normal fractional catabolic rate (*358*). His serum did not inhibit the activity of CPN in normal serum (towards chromogenic substrates). This indicates the absence of a unique CPN inhibitor in his blood, therefore a reduced rate of CPN synthesis was concluded to be the cause of his deficiency (*356;358*).

Such an abnormality would be consistent with an allelic gene defect., and his family was therefore examined: 14 of his relatives also presented with CPN activity lower than normal; however, only one of his siblings (a sister) had similarly low CPN activity. She had suffered intermittent angioedema during a period of 1 to 2 years, but had suffered no recent episodes for over 35 years (*356*).

Genetic sequencing determined that this man has a frameshift mutation in exon 1 of the catalytic subunit (a guanine insertion at position 385) which produces a truncated protein of only 164 residues (compared to the 458 residues of wild type CPN1) and which lacks a complete active site (*255;296;355;356;358*). Additionally, his catalytic subunit gene has a missense mutation (resulting in Gly178Asp) in exon 3. This mutation may result in a decreased activity (explaining his 21% residual CPN activity, as opposed to the predicted 50%) as it occurs is a highly conserved site across many species and many related human carboxypeptidases (*255;296;355;356;358*). Based on these results, it was hypothesized that the abnormality is transmitted in an autosomal recessive manner. All the children of this man and of his sister (assuming they are homozygous recessive) would be heterozygotes (assuming their respective spouses are homozygous dominant). This would be expected to produce intermediate CPN activity in these children, and this is indeed the case (*356*).

Recently, another woman with decreased CPN activity was identified after she developed angioedema following initiation of ACE inhibitor therapy. Despite having very low CPN

activity, her symptoms disappeared after stopping ACE inhibitor therapy and beginning treatment with antihistamines (*255;381*). This patient has a major CPN deficiency, with only 3% residual CPN activity. The deficiency is not due to the presence of an unknown CPN inhibitor, as demonstrated by lack of inhibition when spiked into normal plasma (*381*). As with the previously described patient, all other laboratory tests came back negative or normal. This patient represents the first documented case of a nearly complete CPN deficiency, and surprisingly, she is without overt phenotype.

1.1.4.5. The structure of CPN

Through the work of several research laboratories, CPN was purified to homogeneity (*188;195;202;209;210*). Early characterization of the enzyme revealed its multi-subunit structure, a unique attribute compared to all other human carboxypeptidases (*188;189;203*). CPN was determined to be a 280 kDa heterotetramer of two 48-55 kDa subunits and two 83 kDa subunits (*188;192;195;201-204*). The smaller subunit was identified as being catalytic, based on labelling with *N*-bromoacetyl-D-[5-^{14}C]arginine, a substrate mimic, which bound almost exclusively to the 48-55 kDa subunit (*201*). The molecular mass determinations were based upon an amalgamated average of ultracentrifugation and polyacrylamide gel electrophoresis (PAGE) analysis.

The molecular mass, as determined by native PAGE analysis (using 6 to 10% gels and calculating versus the retention value (R_M) in comparison to proteins of known molecular mass), is 290 kDa (*192*). The molecular mass, as determined by sedimentation ultracentrifugation (using interference as the method of detection), ranges from 300-325 kDa (*188;192*). CPN contains a significant amount of carbohydrate (approximately 17% by mass), including glucosamine, mannose, galactose, fucose, and sialic acid. The types and ratios of carbohydrate present are consistent with that of other plasma proteins, and it is likely asparaginyl-linked to the CPN2 subunit (*188;205*). In the absence of carbohydrate, CPN is predicted to have a molecular mass of 216.6 kDa, as determined by sequence analysis for two CPN1 and two CPN2 subunits (*198*).

Isoelectric focusing (of partially purified CPN from human serum) found two distinct forms; one with an isoelectric point (pI) of 3.8 and the other with a pI of 4.3. The two forms are due to differing sialic acid content, and when CPN was desialated prior to focusing, only a single homogenous peak, with a pI of 5.2, was found (*382*). Although

only the regulatory CPN2 subunit carries carbohydrate, removal of sialic acid reduced the activity of CPN, especially during refocusing. This strengthens the prediction that the carbohydrate is important for the stabilization of CPN and maintenance in the circulation (*382-384*). Multimeric CPN is stable at 37°C for at least 2 hours, while the isolated catalytic subunit loses 75% of its activity within the same timeframe. Additionally, CPN proteolyzed by plasmin, chymotrypsin, urinary kallikrein, or plasma kallikrein (at room temperature, for 6-24 hours) rapidly lost activity when incubated at 37°C (*201*). Interestingly, the isolated catalytic subunit, when incubated with a 5-fold molar excess of the regulatory subunit, (versus bovine serum albumin as a control), lost only 37% of its activity when incubated at 37°C for 2 hours, suggesting a crucial role of CPN2 in stabilizing CPN1 at physiological temperatures (*201*).

The subunits of CPN are held together through ionic forces, as guanidine hydrochloride and other ionic detergents can dissociate the subunits, while non-ionic detergents are ineffective (*201;204*). Furthermore, no inter-subunit disulfides are present, as reduction is not necessary for the dissociation (*385*). The isolated subunits can be recombined (with a relatively poor success rate of 30%) to form a multimer which remains a stable throughout gel filtration (*198*). Re-assembly of the multimer may be facilitated by hydrophobic interactions. The overall amino acid composition of CPN shows elevated aromatic and hydrophobic residues, 49% and 32% higher than average, respectively (*188*). Furthermore, the polar to apolar ratio is 1.18, which is highly hydrophobic, and could therefore favour the aggregation of CPN (*188*).

The overall shape and inter-chain interactions of CPN are not yet known; however, some experimental results provide modest clues to the structure. Antibodies generated toward the intact, multimeric CPN also recognize each isolated subunit. Additionally, antibodies generated toward either isolated subunit recognize multimeric CPN (*201*). This suggests that at least some portion of each subunit is exposed to the external environment. Unfortunately, these researchers did not provide information regarding the binding affinities of the antibodies produced. Proteolytic degradation at the carboxy-terminus of CPN1 can occur during prolonged thawing of incompletely purified material, and this is often correlated with dissociation of CPN, therefore the carboxy-terminus of CPN1 may be involved in complex formation (*242*). Based on x-ray crystallographic data on the isolated recombinant CPN1 subunit (see below), Keil *et al.*, predict that the structure

contains side by side CPN2 subunits, associated by polar and electrostatic interactions, with CPN1 subunits bound to the outer edge of each CPN2 subunit, associated by hydrophobic interactions (*189*).

A good understanding of the subunit interactions of CPN becomes even more important when considering the effects of proteases on CPN structure and activity. Plasmin is predicted to hydrolyze CPN *in vivo*; therefore, *in vitro* experiments were conducted to examine the effects of plasmin-mediated CPN cleavage. When equimolar plasmin (or trypsin) was incubated with CPN at 37°C, the activity toward the synthetic CPN substrate dansyl-Ala-Arg increased to a maximum of 150%, following 3 hours of incubation (*198*). Based on immunoblot analysis, hydrolysis of both the regulatory and catalytic subunits begins as early as 5 min after addition of plasmin. The regulatory 83 kDa subunit is cleaved to form products of 72 kDa and 13 kDa. Both of these products, like intact CPN2, contain carbohydrate. The 55 kDa catalytic subunit is cleaved into a 48 kDa product, and then further hydrolyzed to the non-covalently linked products of 27 kDa (originating from the amino-terminus) and 21 kDa (originating from the carboxy-terminus). A comparison of immunoblot and activity analysis demonstrated that the increase in CPN activity (when observed) is associated with the cleavage of the 55 kDa subunit to the 21 kDa and 27 kDa products (*166;188;189;198;201;385*). CPN isolated from plasma contains equimolar concentrations of the 55 kDa CPN1 and the 48 kDa CPN1 and these are thought to originate due to constitutive proteolysis in the circulation, and therefore co-exist in tetrameric CPN. The 48 kDa product has a similar substrate specificity as the full-length CPN1, but may in fact be slightly more active toward certain substrates (*166;188;198;201*).

The human gene for CPN1 is located on chromosome 10q24 and contains 9 exons (*191;296;386*). Interestingly, the related carboxypeptidases family members all reside on different chromosomes; *cpu* on chromosome 13, *cpa* on chromosome 7, *cpd* on chromosome 17, *cph* on chromosome 4, and *cpm* on chromosome 12 (*191*). *cpn1* is 1.7 kbp in length, with 5' and 3' untranslated regions of 213 and 115 bp, respectively (*242*). *cpn1* lacks a TATA, CAAT or G/C-rich initiation site, and also lacks a canonical polyadenylation signal (*191;242*). Mouse *cpn1* has been found to contain liver-specific transcription factor and repressor binding sites (*191*). The translated CPN1 is 458 amino acids in length, including a 20 residue signal sequence peptide (*204;206;242;387*). The

core CPN1 protein contains a 319 residue long amino-terminal catalytic domain and a 79 residue long transthyretin-like (TT) domain (*199*). The calculated molecular mass of CPN1, in the absence of the catalytic zinc ion, is 50 039 Da (*242*). CPN1 is quite hydrophobic, which can cause problems during isolation of the subunit, as it has the tendency to stick to column matrices via hydrophobic interactions (*201;204*).

CPN1 retains a high degree of amino acid identity with related basic carboxypeptidases, and even its more distantly related family members, such as CPA, have conserved residues important for glycosylation, zinc binding, substrate binding and catalysis (*200*). The zinc ligating residues in CPN are His^{66}, Glu^{74}, and His^{196} (*197;242;388*), the substrate binding residues are Asn^{140}, Arg^{141}, Asn^{142}, and Tyr^{240} (*197;388;389*), and the active site residues are Tyr^{240}, Glu^{288}, and Leu^{289} (Figure 1.17 and Figure 1.18) (*197;242;388*). All five cysteine residues of CPN1 are in the same position as in CPE, and the following intra-chain disulfides exist: Cys^{22}-Cys^{84}, and Cys^{251}-Cys^{291} (*189;242*).

The catalytic mechanism of CPN has been predicted based on multiple x-ray crystal structures of related carboxypeptidases, molecular modelling approaches, and a single x-ray crystal structure of an isolated recombinant truncated two-chain CPN1 subunit (Figure 1.17). CPN hydrolysis of a substrate with a carboxy-terminal arginine (such as bradykinin), would likely proceed through the following mechanism (Figure 1.19). The guanidyl group of the P_1' arginine would form a forked hydrogen bond to the carboxylate of Asp^{207} and the carbonyl group of Tyr^{266}. Additionally, the P_1' carboxylate group and amide nitrogen would have polar interactions with the guanidyl groups of Arg^{131} and Arg^{141} and the hydroxyl group of Tyr^{266}. The P_1 side chain would extend into a large hydrophobic groove (which explains the preference for medium to large and bulky sized P_1 residues). Additional enzyme-substrate interactions would come from hydrogen bonds between the amido nitrogen of P_1 and the carbonyl group of P_2 to the hydroxyl group of Tyr^{266} and ε-ammonium group of Lys^{293}.

The catalytic water molecule in the active site should demonstrate a low pK value due to zinc binding and at physiological pH would be easily deprotonated. The carboxylate of Glu^{288} would abstract the H^+ from the catalytic water, forming a nucleophilic hydroxyl, which could attack the carbonyl of the scissile peptide bond creating a tetrahedral intermediate. The attack would be facilitated by polarization of the scissile bond through

interactions with the zinc ion and the Arg^{131} guanidinyl group. Subsequently, the proton abstracted by Glu^{288} would be transferred to the leaving group P_1' nitrogen, and following breakdown of the tetrahedral intermediate, the carboxy-terminal arginine would be released. A similar mechanism for hydrolysis of substrates with carboxy-terminal lysine can be predicted, but with the P_1' lysine side chain approaching Asp^{207} at an improved angle, which explains the preference of CPN for substrates with carboxy-terminal lysine over arginine residues (*189*). This model can be compared to that of related carboxypeptidases, which explains the differing substrate preferences of the enzymes (*195;197;203;216;388;390-392*). Interestingly, a hill plot (to determine the number of active sites per enzyme molecule) exhibits a slope of 1.02 for substrate benzoyl-Gly-Lys and 1.04 for benzoyl-Gly-Arg, indicating that perhaps only a single catalytic site is active at any one time (*220*).

Human CPN1 is topologically quite similar to human CPM (Figure 1.20) (*189*). It consists of a catalytic domain, extending from residues Val^1 to His^{319}, and a TT domain, comprising residues Gln^{320} to Ala^{438}. The catalytic domain is characteristic of all carboxypeptidases, represented by an α/β hydrolase fold, which has an 8 stranded β-sheet of mixed parallel-antiparallel orientation, and flanked by 9 α-helices running approximately antiparallel to the β-sheets (*189;393*). The core of the catalytic domain is mainly hydrophobic with the exception of an ion pair (Lys^{60}-Glu^{284}), and two salt bridges (His^6-Glu^{74}, and Arg^{27}-Glu^{44}-Arg^{102}) (*189*). The loop region containing the protease sensitive Arg^{218}-Arg^{219} peptide bond is not well defined in the crystal structure, but is predicted to extend towards the active site groove. This could explain the enhanced activity as a result of its cleavage, due to a less constrained approach for the substrate into the active site (*189*). The TT domain is unique to the CPN/E subfamily members, and consists of a seven stranded mixed and antiparallel β-barrel (*189*). The function of the TT domain is not yet known.

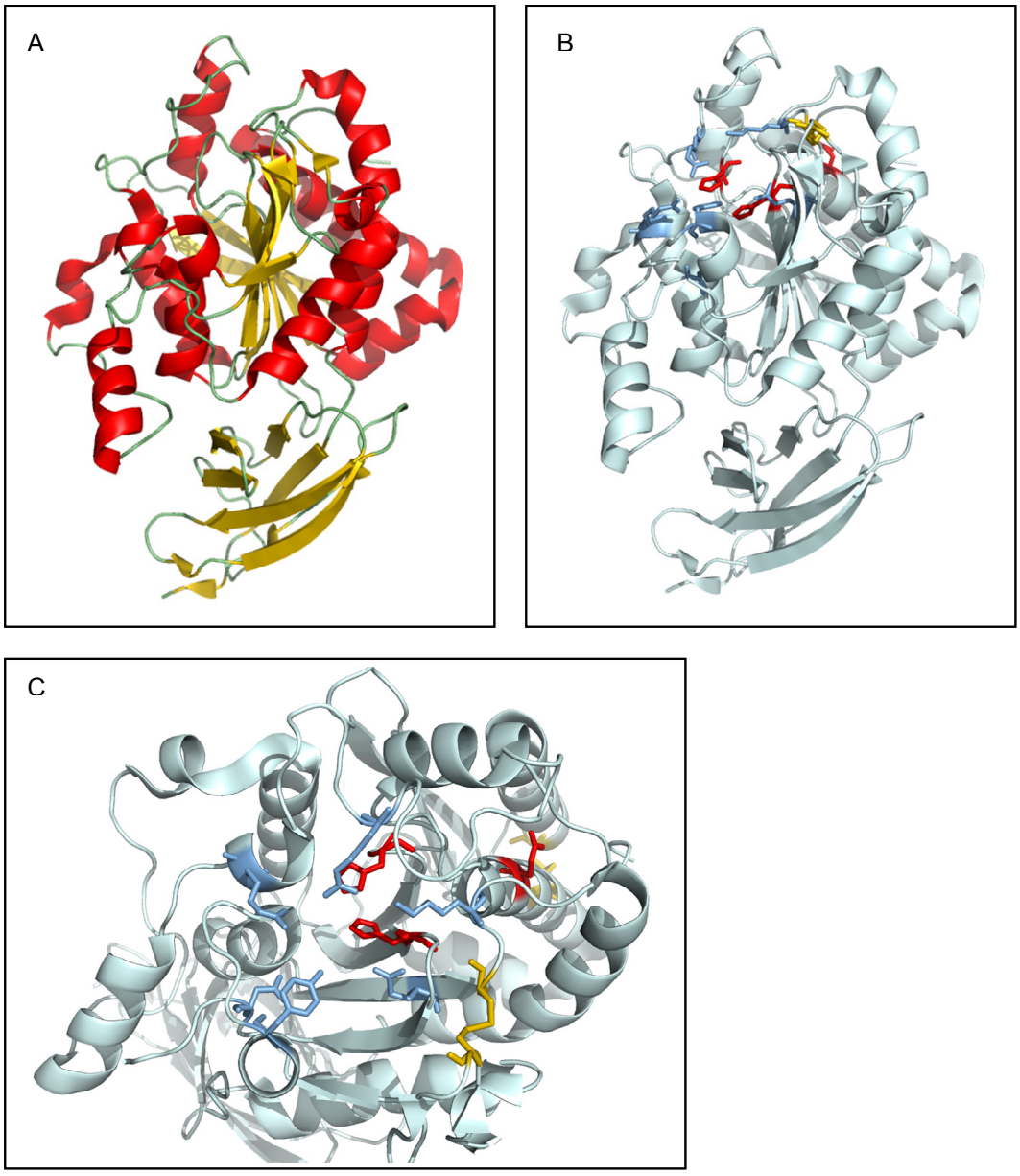


Figure 1.17: The crystal structure of CPN1. Panel A: the structure of the catalytic subunit of CPN. α-helices are in red, β-sheets are in yellow, and coils are in green. The catalytic domain α/β hydrolase fold is at the top, and the TT-domain β-barrel is at the bottom. The active site zinc ion was not retained in the molecule during crystallization and is therefore absent. Panel B: the general structure of CPN1 is coloured in light grey, with disulfides in yellow, zinc-coordinating residues in red, and residues implicated in catalysis and substrate specificity are in blue. Panel C: an expanded view of the active site groove. The general structure of CPN1 is coloured in light grey, with zinc-coordinating residues in red, and residues implicated in catalysis and substrate specificity are in blue. This figure was generated with PyMOL software. The CPN1 structure was obtained from PDB 2NSM.

A

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CPN1        VTFRHHRYDDLVRTLYKVQNECPGITRVYSIGRSVEGRHLYVLEFSDHPGIHEPLEPEVKYVGNMHGNEALGRELMLQLS  80
CPE/H       ISFEYHRYPELREALVSVWLQCTAISRIYTVGRSFEGRELLVIELSDNPGVHEPGEPEFKYIGNMHGNEAVGRELLIFLA 128
CPM         LDFNYHRQEGMEAFLKTVAQNYSSVTHLHSIGKSVKGRNLWVLVVGRFPKEHRIGIPEFKYVANMHGDETVGRELLLHLI  97
CPD         KDFHHHHFPDMEIFLRRFANEYPNITRLYSLGKSVESRELYVMEISDNPGVHEPGEPEFKYIGNMHGNEVVGRELLLNLI 168
CPB         EHFEGEKVFRVNVEDENHINIIRELASTT--QIDFWKPDSVTQIKPHSTVDFRVKAEDTVTVENVLKQNELQYKVLISNL  97
mcCPA       VRFDREKVFRVKPQDEKQADIIKDLAKTN--ELDFWYPGATHHVAANMMVDFRVSEKESQAIQSALDQNKMHYEILIHDL  96
CPA1        EDFVGHQVLRISVADEAQVQKVKELEDLEHLQLDFWRG----PAHPGSPIDVRVPFPSIQAVKIFLESHGISYETMIEDV  93
CPA2        ETFVGDQVLEIVPSNEEQIKNLLQLEAQEHLQLDFWKS----PTTPGETAHVRVPFVNVQAVKVFLESQGIAYSIMIEDV  93
TAFI        FAFQSGQVLAALPRTSRQVQVLQNLTTTY--EIVLWQPVTADLIVKKKQVHFFVNASDVDNVKAHLNVSGIPCSVLLADV  98
                  *   :                 :        .                      .   :       :  . ::

CPN1        EFLCEEFRNRNQRIVQLIQDTRIHILPSMNPDGYEVAAAQGPNKPGYLVGRNNANGVDLNRNFPDLNTYIYYNEKYGGPN 160
CPE/H       QYLCNEYQKGNETIVNLIHSTRIHIMPSLNPDGFEKAASQPGELKDWFVGRSNAQGIDLNRNFPDLDRIVYVNEKEGGPN 208
CPM         DYLVTSDGKDPE-ITNLINSTRIHIMPSMNPDGFEAVKKPDC---YYSIGRENYNQYDLNRNFPDAFEYNNVSRQ----- 168
CPD         EYLCKNFGTDPE-VTDLVHNTRIHLMPSMNPDGYEKSQEGDS---ISVIGRNNSNNFDLNRNFPDQFVQITDPTQ----- 239
CPB         RNVVEAQFD------SRVRATG-HSYEKYNK-----------------WETIEAWTQQVATENPALISRSVIGTTFEG-- 151
mcCPA       QEEIEKQFD------VKEDIPGRHSYAKYNN-----------------WEKIVAWTEKMMDKYPEMVSRIKIGSTVED-- 150
CPA1        QSLLDEEQEQMFAFRSRARSTDTFNYATYHT-----------------LEEIYDFLDLLVAENPHLVSKIQIGNTYEG-- 154
CPA2        QVLLDKENEEMLFNRRRERSGN-FNFGAYHT-----------------LEEISQEMDNLVAEHPGLVSKVNIGSSFEN-- 153
TAFI        EDLIQQQISN----DTVSPRASASYYEQYHS-----------------LNEIYSWIEFITERHPDMLTKIHIGSSFEK-- 155
                                        :                       :  . *

CPN1        HHLPLPDN----WKSQVEPETRAVIRWMHSFNFVLSANLHGGAVVANYPYDKSFEHRVRGVRRTASTPTPDDKLFQKLAK 236
CPE/H       NHLLKNMKKIVDQNTKLAPETKAVIHWIMDIPFVLSANLHGGDLVANYPYD---ETRSGSAHEYSSS--PDDAIFQSLAR 283
CPM         ------------------PETVAVMKWLKTETFVLSANLHGGALVASYPFD---NGVQATGALYSRSLTPDDDVFQYLAH 227
CPD         ------------------PETIAVMSWMKSYPFVLSANLHGGSLVVNYPFD---DDEQG---LATYSKSPDDAVFQQIAL 295
CPB         --------------RAIYLLKVG-KAGQNKPAIFMDCGFHAREWISPAFCQWFVREAVRTYGREIQVTELLDKLDFYVLP 216
mcCPA       --------------NPLYVLKIG-EKNERRKAIFMDCGIHAREWVSPAFCQWFVYQATKTYGRNKIMTKLLDRMNFYILP 216
CPA1        --------------RPIYVLKFS-TGGSKRPAIWIDTGIHSREWVTQASGVWFAKKITQDYGQDAAFTAILDTLDIFLEI 219
CPA2        --------------RPMNVLKFS-TGGDK-PAIWLDAGIHAREWVTQATALWTANKIVSDYGKDPSITSILDALDIFLLP 217
TAFI        --------------YPLYVLKVSGKEQAAKNAIWIDCGIHAREWISPAFCLWFIGHITQFYGIIGQYTNLLRLVDFYVMP 221
                           . .        : :. .:*.   :                                 :   :
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CPN1        VYSYAHGWMFQGWN--CG-----DYFPDGITNGASWYSLSKGMQDFNYLHTNCFEITLELSCDKFPPEEELQREWLGNRE 309
CPE/H       AYSSFNPAMSDPNRPPCRKNDDDSSFVDGTTNGGAWYSVPGGMQDFNYLSSNCFEITVELSCEKFPPEETLKTYWEDNKN 363
CPM         TYASRNPNMKKGDE--CKNKMN---FPNGVTNGYSWYPLQGGMQDYNYIWAQCFEITLELSCCKYPREEKLPSFWNNNKA 302
CPD         SYSKENSQMFQGRP--CKNMYPNEYFPHGITNGASWYNVPGGMQDWNYLQTNCFEVTIELGCVKYPLEKELPNFWEQNRR 373
CPB         VLNIDGYIYTWTKSRFWRKTRSTHTGSSCIGTDPNRNFDAG-WCEIGASRNPCDETYCGPAAESEKETKALADFIRNKLS 295
mcCPA       VFNVDGYIWSWTKNRMWRKNRSKNQNSKCIGTDLNRNFNAS-WNSIPNTNDPCADNYRGSAPESEKETKAVTNFIRSHLN 295
CPA1        VTNPDGFAFTHSTNRMWRKTRSHTAGSLCIGVDPNRNWDAG-FGLSGASSNPCSETYHGKFANSEVEVKSIVDFVKDHG- 297
CPA2        VTNPDGYVFSQTKNRMWRKTRSKVSGSLCVGVDPNRNWDAG-FGGPGASSNPCSDSYHGPSANSEVEVKSIVDFIKSHG- 295
TAFI        VVNVDGYDYSWKKNRMWRKNRSFYANNHCIGTDLNRNFASKHWCEEGASSSSCSETYCGLYPESEPEVKAVASFLRRNIN 301
                                          .                 * :       .    : :     :

CPN1        ALIQFLEQVHQGIKGMVLDEN-YNNLANAVISVSGINH--DVTSGDHGDYFRLLLPGIYTVSATAPGYDPETVTVTVG-- 384
CPE/H       SLISYLEQIHRGVKGFVRDLQ-GNPIANATISVEGIDH--DVTSAKDGDYWRLLIPGNYKLTASAPGYLAITKKVAVP-- 438
CPM         SLIEYIKQVHLGVKGQVFDQN-GNPLPNVIVEVQDRKHICPYRTNKYGEYYLLLLPGSYIINVTVPGHDPHITKVIIP-- 379
CPD         SLIQFMKQVHQGVRGFVLDATDGRGILNATISVAEINH--PVTTYKTGDYWRLLVPGTYKITASARGYNPVTKNVTVKSE 451
CPB         SIKAYLTIHSYSQMMIYPYSYAYKLGENNAELNALAKATVKELASLHG-TKYTYGPGATTIYPAAGGSDDWAYDQGIR-- 372
mcCPA       EIKVYITFHSYSQMLLFPYGYTSKLPPNHEDLAKVAKIGTDVLSTRYE-TRYIYGPIESTIYPISGSSLDWAYDLGIK-- 372
CPA1        NIKAFISIHSYSQLLMYPYGYKTEPVPDQDELDQLSKAAVTALASLYG-TKFNYGSIIKAIYQASGSTIDWTYSQGIK-- 374
CPA2        KVKAFITLHSYSQLLMFPYGYKCTKLDDFDELSEVAQKAAQSLRSLHG-TKYKVGPICSVIYQASGGSIDWSYDYGIK-- 372
TAFI        QIKAYISMHSYSQHIVFPYSYTRSKSKDHEELSLVASEAVRAIEKTSKNTRYTHGHGSETLYLAPGGGDDWIYDLGIK-- 379
             :  ::     .              :       .                       :     .        :
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B

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CPM         LDFNYHRQEGMEAFLKTVAQNYSSVTHLHSIGKSVKGRNLWVLVVGRFPKEHRIGIPEFKYVANM H GDETVGR E LLLHLI 97
CPD         KDFHHHHFPDMEIFLRRFANEYPNITRLYSLGKSVESRELYVMEISDNPGVHEPGEPEFKYIGNM H GNEVVGR E LLLNLI 168
CPN1        VTFRHHRYDDLVRTLYKVQNECPGITRVYSIGRSVEGRHLYVLEFSDHPGIHEPLEPEVKYVGNM H GNEALGR E LMLQLS 80
CPE/H       ISFEYHRYPELREALVSVWLQCTAISRIYTVGRSFEGRELLVIELSDNPGVHEPGEPEFKYIGNM H GNEAVGR E LLIFLA 128
              *.:*:   :   *  .  : . :::::::* :*.:.*.* *: ..  *  *.   **.**:.* * **:*.:* * **:: *

CPM         DYLVTSDGKDPE-ITNLINSTRIHIMPSMNPDGFEAVKKPDC---YYSIG R ENYNQYDL NRN FPDAFEYNNVSRQ----- 168
CPD         EYLCKNFGTDPE-VTDLVHNTRIHLMPSMNPDGYEKSQEGDS---ISVIG R NNSNNFDL NRN FPDQFVQITDPTQ----- 239
CPN1        EFLCEEFRNRNQRIVQLIQDTRIHILPSMNPDGYEVAAAQGPNKPGYLVG R NNANGVDL NRN FPDLNTYIYYNEKYGGPN 160
CPE/H       QYLCNEYQKGNETIVNLIHSTRIHIMPSLNPDGFEKAASQPGELKDWFVG R SNAQGIDL NRN FPDLDRIVYVNEKEGGPN 208
            ::*  .  .  : :.:*::.****::**:****:*            :* * .* :  ** *** ***        :

CPM         ------------------PETVAVMKWLKTETFVLSANL H GGALVASYPF D ---NGVQATGALYSRSLTPDDDVFQYLAH 227
CPD         ------------------PETIAVMSWMKSYPFVLSANL H GGSLVVNYPF D ---DDEQG---LATYSKSPDDAVFQQIAL 295
CPN1        HHLPLPDN----WKSQVEPETRAVIRWMHSFNFVLSANL H GGAVVANYPY D KSFEHRVRGVRRTASTPTPDDKLFQKLAK 236
CPE/H       NHLLKNMKKIVDQNTKLAPETKAVIHWIMDIPFVLSANL H GGDLVANYPY D ---ETRSGSAHEYSSS--PDDAIFQSLAR 283
                         *** **: *:    ******* * ** :*..**: *    :         : :  *** :** :*

CPM         TYASRNPNMKKGDE--CKNKMN---FPNGVTNGYSW Y PLQGGMQDYNYIWAQCFEITL EL SCC K YPREEKLPSFWNNNKA 302
CPD         SYSKENSQMFQGRP--CKNMYPNEYFPHGITNGASW Y NVPGGMQDWNYLQTNCFEVTI EL GCV K YPLEKELPNFWEQNRR 373
CPN1        VYSYAHGWMFQGWN--CG-----DYFPDGITNGASW Y SLSKGMQDFNYLHTNCFEITL EL SCD K FPPEEELQREWLGNRE 309
CPE/H       AYSSFNPAMSDPNRPPCRKNDDDSSFVDGTTNGGAW Y SVPGGMQDFNYLSSNCFEITV EL SCE K FPPEETLKTYWEDNKN 363
             *:  :  * .     *        * .* *** :* *  :  ****:**: ::***:*: ** .*  * :* *: *   *  *:

CPM         SLIEYIKQVHLGVKGQVFDQN-GNPLPNVIVEVQDRKHICPYRTNKYGEYYLLLLPGSYIINVTVPGHDPHITKVIIP-- 379
CPD         SLIQFMKQVHQGVRGFVLDATDGRGILNATISVAEINH--PVTTYKTGDYWRLLVPGTYKITASARGYNPVTKNVTVKSE 451
CPN1        ALIQFLEQVHQGIKGMVLDEN-YNNLANAVISVSGINH--DVTSGDHGDYFRLLLPGIYTVSATAPGYDPETVTVTVG-- 384
CPE/H       SLISYLEQIHRGVKGFVRDLQ-GNPIANATISVEGIDH--DVTSAKDGDYWRLLIPGNYKLTASAPGYLAITKKVAVP-- 438
            :**.:::*:* *::* * *    . : *. :.*   .*     : . *:*: **:** * :..:. *: .  .* :
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Figure 1.18: Alignment of the primary sequence of CPN1 with related carboxypeptidases of the MC clan. Panel A: the alignment includes the sequence of human CPN1, CPE/H, CPM, CPD, CPB, mcCPA, CPA1, CPA2, and TAFI. Panel B: the alignment of CPN/E family carboxypeptidases only. Residues that are identical across all species are indicated with an asterisk (*), conservative changes are indicated with a colon (:), and semi-conservative changes are indicated with a full-stop (.). Important residues for catalysis (green), zinc coordination (blue) and substrate binding (red) are outlined in panel B. Signal sequences are not included in the alignments. This figure was generated with the CLUSTALW sequence alignment program.

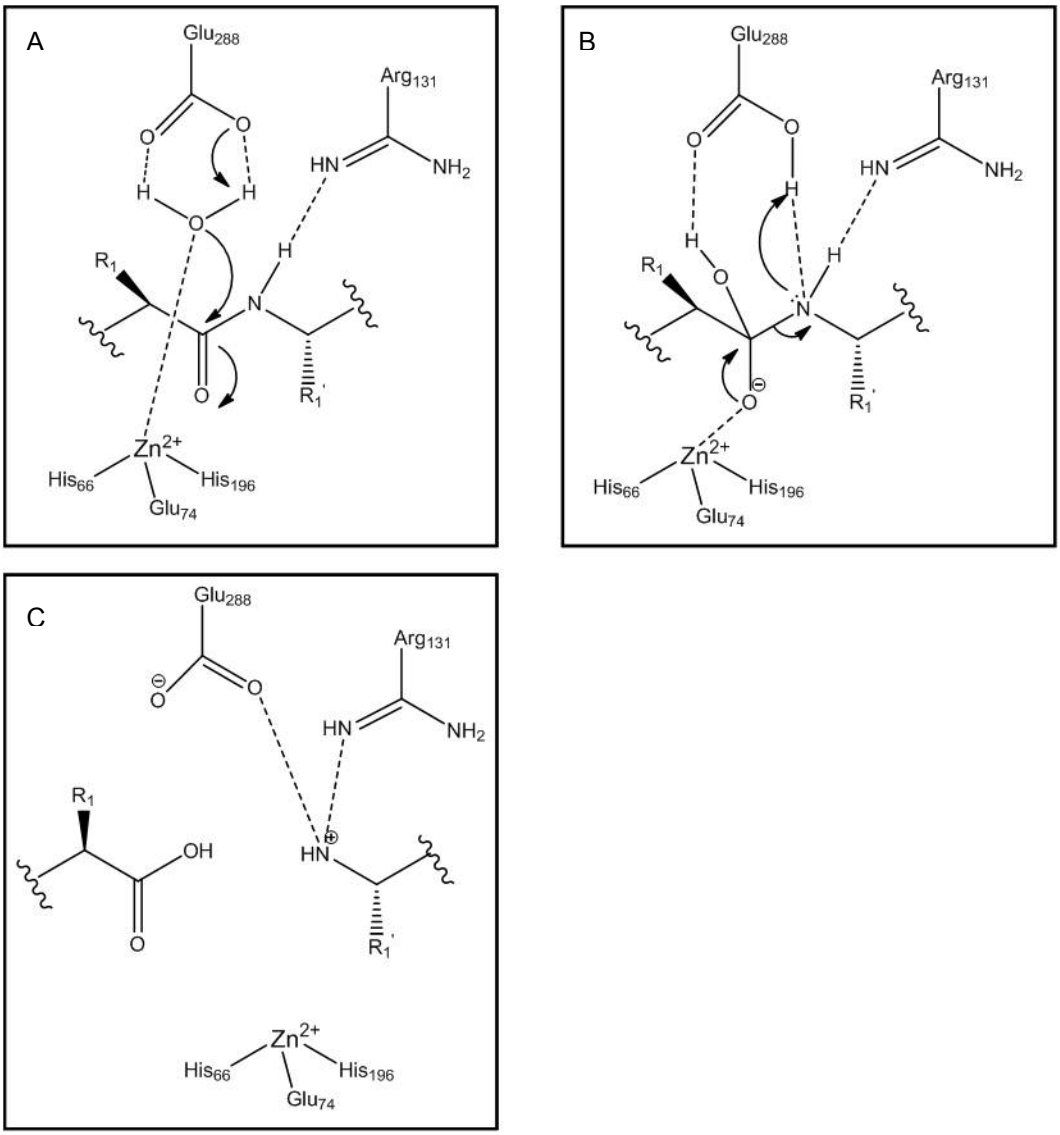

Figure 1.19: The predicted reaction mechanism of CPN. Glu^{288} abstracts H^+ from the catalytic water and the nucleophilic hydroxyl attacks the scissile bond (panel A). This bond is polarized through interactions with the catalytic zinc ion. The H^+ abstracted by Glu^{288} is transferred to the leaving group nitrogen (panel B), and the carboxy-terminal arginine residue is released following breakdown of the tetrahedral intermediate (panel C).

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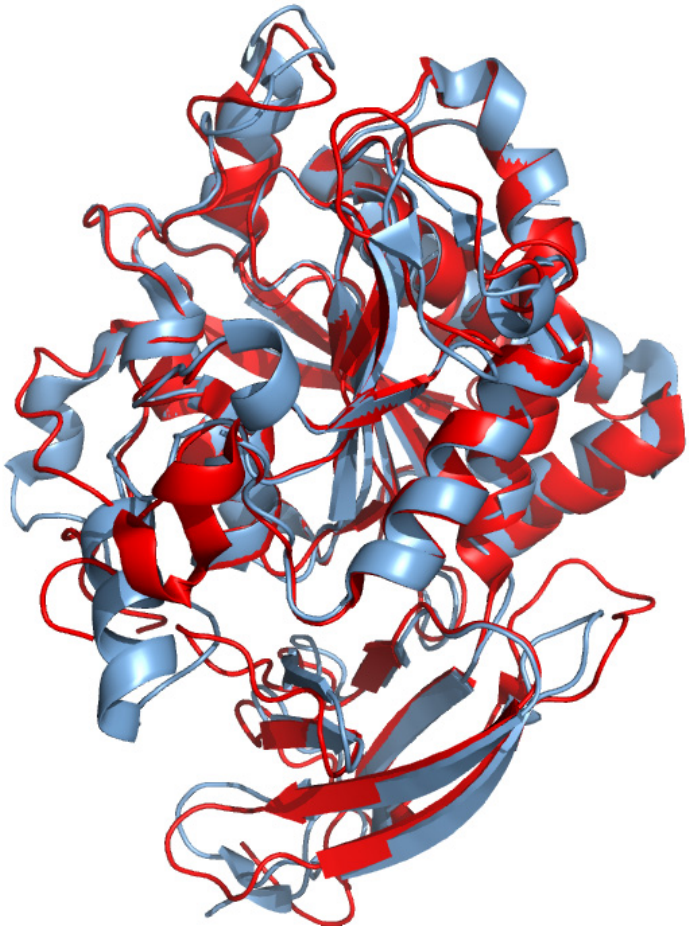


Figure 1.20: A comparison of the crystal structures of CPN1 and CPM. CPN is coloured in blue and CPM in red. This figure was generated with PyMOL software. The CPN1 structure was obtained from PDB 2NSM and the CPM structure was obtained from PDB 1UWY.

There are several pairs of basic residues at the carboxy-terminal end of CPN1, including Lys^{396}-Arg^{397}, Arg^{406}-Arg^{407}, Arg^{411}-Arg^{412}, Arg^{424}-Lys^{425}, and Lys^{425}-Lys^{426}, which are potential cleavage sites for plasmin and other serine proteases of tryptic specificity, and could be responsible for the formation of the 48 kDa form of CPN1 (*198;204;206;242;387*). The responsible cleavage site is known to be in the carboxy-terminus because sequencing indicates that the amino-terminal sequence for the 48 kDa form is identical to that of the 55 kDa form (*198*). However, the exact location of cleavage is still open to debate. One additional dibasic site is the Arg^{218}-Arg^{219} peptide bond, cleavage of which results in the two-chain form of CPN1 (*201;242*). Both chains are necessary for catalytic activity, as the amino terminal fragment has all three zinc chelating residues and several substrate binding residues, while the carboxy-terminal fragment has the critical Glu^{288} and additional substrate binding residues (*198*).

Although the two-chain form of CPN1 seems to have enhanced activity toward some substrates, intact CPN1 has consistent activity, whether associated with the regulatory subunits or not. For example, hydrolysis of bradykinin proceeds at a rate of 0.1 mM min^{-1} μmol^{-1} for CPN and of 0.11 mM min^{-1} μmol^{-1} for the isolated CPN1 subunit. Similarly, benzoyl-Ala-Lys is hydrolyzed at rates of 9 mM min^{-1} μmol^{-1} and 7.5 mM min^{-1} μmol^{-1} for CPN and CPN1, respectively. Inhibitory constants are also similar, as GEMSA inhibits the reaction with benzoyl-Ala-Lys with an IC_{50} of 25 μM and 34 μM, for CPN and CPN1 respectively (*201*). Activation by cobalt is similar for CPN and CPN1, with an increase in catalytic activity of 2- to 6-fold at neutral pH, depending on the substrate. There have been some reports, however, stating that the full tetrameric CPN demonstrates slightly altered reaction kinetics compared to the isolated catalytic subunit (*198;201;203;207*), and any increase in catalytic activity from CPN to CPN1 is mainly due to an increase in the catalytic rate constant (k_{cat}) (*187;209;211;212*).

The regulatory subunits are thus termed due to their proposed role in stabilizing CPN1 by preventing its removal from the bloodstream by glomerular filtration, and by preventing degradation of enzymatic activity at 37ºC (*201*). The gene for CPN2, located on chromosome 3q29, is a single exon, 2870 bp in length and contains a long 3' untranslated region. Due to multiple potential polyadenylation signals, multiple mRNA forms of CPN2 may exist (*204*). *cpn2* codes for a 545 residue long polypeptide with no sequence relation to any known carboxypeptidase, and is divided into a 52-residue cysteine-rich amino terminal region, a 312-residue leucine rich repeat (LRR) region and a 145-residue cysteine rich carboxy-terminal region (*198;199;204*). The 83 kDa CPN2 is 28% carbohydrate, by mass, and the calculated molecular mass for the protein alone is 58 762 Da, which closely approximates the 61 kDa determined for the chemically deglycosylated subunit (*188;192;193;195;198;201;204;206;242;387;388*). There are seven potential asparaginyl-linked glycosylation sites in CPN2, five of which fall within the bounds of the LRR region (*204*). The heavy glycosylation, and particularly the sialic acid residues, protect multimeric CPN from uptake by liver lectins and clearance from the circulation (*204*).

Twenty percent of the amino acids in CPN2 are leucines, and 54% of CPN2 is contained within the rigid LRR region (*198;199;203-206;242;387*). The core of the CPN2 molecule consists of 12 leucine rich repeats, each of 24 amino acids in length (*204;206;242;387*).

Each repeat approximately follows the sequence: P-X-X-α-F-X-X-L-X-X-L-X-X-L-X-L-X-X-N-X-L-X-X-L (where P is proline, F is phenylalanine, L is leucine, N is asparagine, X is any amino acid and α is any aliphatic amino acid) (*204*). All LRR proteins that have been crystallized to date have a very similar horseshoe-like structure. Generally a curved β-sheet lines the inner concave surface with α-helices and β-turns along the outer circumference (*198*). Over 2 000 proteins from a wide range of species contain LRRs; these generally mediate binding interactions and supply rigid structural domains for the protein (*199;394-396*).

Analysis with the Kyte and Doolittle hydropathy plot (using a window of 24 amino acids) reveals that the amino three quarters of CPN2 is strongly hydrophobic, with maximum hydrophobicity in the central region Gln^{235}-Met^{276}, and the carboxy-terminal region is more hydrophilic (*188;203;204*). The carboxy-terminal portion of CPN2 is predicted to be the most flexible, and likely exposed to the exterior environment due to its high level of antigenicity (*198;199;204*). The LRR is predicted to mediate CPN1-CPN2 association, while the positively charged carboxy-terminus of each CPN2 molecule is thought to interact with the negatively charged amino-terminus of the other CPN2 molecule and therefore mediate tetramer formation (*198;204;206;397*).

CPN2, like CPN1, is also susceptible to proteolytic cleavage by plasma serine proteases. Cleavage releases a 13 kDa (88-residue) carboxy-terminal docking peptide from the remaining 72 kDa regulatory subunit (*166;188;198;201*). Cleavage by plasmin has been suggested to occur at the Arg^{457}-Ser^{458} peptide bond, which resides in the flexible, hydrophilic region of CPN2 (*198*). The released 13 kDa peptide is glycosylated and therefore acts as a larger peptide than would be expected based on amino acid composition (7304 Da) (*198*).

1.1.5. The apparent absence of a role for CPN in fibrinolysis

Circulating basic carboxypeptidases may play an important role in antifibrinolysis. Removal of the carboxy-terminal lysine residues of partially degraded fibrin attenuates plasminogen activation thereby slowing fibrinolysis (*77;180;182*). There are two known basic plasma carboxypeptidases, TAFIa and CPN. Despite the presence of both enzymes in the circulation and their similar substrate specificity (including sharing substrates such

as bradykinin, C3a, and C5a), the roles of these enzymes appear to differ greatly; particularly with respect to their antifibrinolytic activities.

The role of TAFIa in fibrinolysis is well documented, while the role of CPN in fibrinolysis has been dismissed. When purified TAFIa is added to whole blood clots bathed in plasma, clot lysis is significantly attenuated. If PTCI, an inhibitor of TAFIa, is also added, the effect of TAFIa is overcome. Furthermore, plasma depleted of TAFI displays accelerated clot lysis compared to untreated plasma. In contrast, when purified CPN is added to whole blood clots bathed in plasma, clot lysis is only very minimally attenuated; and after 1.5 hours of incubation, the extent of clot lysis is identical in the presence or absence of purified exogenous CPN (*90*). Similarly, addition of a specific CPN inhibitor to clots formed from (TAFI-depleted) plasma did not potentiate fibrinolysis (*165;166*).

This disparity can be explained by the degree of lysine removal from the fibrin clot by TAFIa versus CPN. Partially plasmin-digested fibrin degradation products arising from whole blood clots display high concentrations of specific plasminogen binding sites. When these fibrin degradation products are incubated with purified TAFIa (at a concentration representing full activation of all the TAFI in the circulation), the specific binding of plasminogen is reduced by greater than 90%. In contrast, when these products are incubated with the plasma concentration of purified CPN for the same period of time, fewer than 50% of the specific plasminogen binding sites were removed (*90*).

The antifibrinolytic activity of TAFIa occurs through a tPA-dependent threshold mechanism. Due to the spontaneous decay of TAFIa, the concentration of the active enzyme varies over time: TAFIa is spontaneously inactivated, while at the same time, during ongoing fibrinolysis, further TAFI is activated to TAFIa by plasmin (*90*). However, if the rate of decay of TAFIa overwhelms the rate of activation of TAFI, the antifibrinolytic activity can drop below the threshold value (the value above which fibrinolysis is kept in check, and below which clot lysis accelerates) (*182;183*). CPN, on the other hand, appears to have very little effect on fibrinolysis.

The role of CPN in attenuation of plasminogen activation appears to be related not to fibrin-dependent activation, but rather cell-surface activation (*90;198;398*). Plasma

membrane proteins of certain cell types have carboxy-terminal lysine residues that act as receptors for plasminogen binding. Freshly isolated leukocytes express 100-fold fewer plasminogen binding sites compared to cultured leukocytes. This is predicted to be due to constitutive removal in the circulation of the carboxy-terminal lysine residues of these receptors by CPN (*399-401*). Furthermore, cells incubated in plasma exhibit significantly reduced specific plasminogen binding, and this effect can be overcome by addition of the CPN inhibitor GEMSA. Similarly, cells incubated with purified CPN also displayed reduced plasminogen binding, and again, the effect can be overcome by GEMSA (*90*). It appears that the role of CPN in decreasing plasminogen binding and attenuating plasminogen activation is likely related to decreasing extracellular matrix degradation and cellular migration rather than attenuating fibrinolysis (*90;198;398*).

On the other hand, it is possible that the the plasmin-cleaved two-chain form of CPN may play a more significant role in fibrinolysis than the native single-chain CPN. When TAFIa is added to clots formed from TAFI-depleted plasma, a significant attenuation in clot lysis is exhibited, while addition of CPN only results in an appreciable attenuation of clot lysis when CPN is added at a concentration 6-fold or greater than that found in plasma. However, when plasmin-cleaved CPN (CPNc) is added, a significant attenuation of clot lysis is noted (*166*). The antifibrinolytic effect of CPNc is dependent upon the concentration of tPA, and the stable activity appears to follow a threshold mechanism, similar to TAFIa (*165;166;181;402*). GEMSA abrogates the antifibrinolytic effects of CPNc. Curiously, the (minimally 8-fold) increase in antifibrinolytic activity of CPNc compared to CPN is not reflected by an increase in activity toward small chromogenic substrates; CPNc is only 50% more active toward dansyl-Ala-Arg and only 7% more active toward FA-Ala-Lys (*166*).

The role of plasmin in activating CPN during fibrinolysis is not clear. CPN is activated by plasmin to CPNc *in situ* during clot lysis; however, this activated CPNc does not nearly produce the magnitude of antifibrinolytic effects expected based on the results with pre-cleaved CPNc. It has been proposed that plasmin cannot activate CPN to locally antifibrinolytic concentrations (*166*). CPN is also known to be cleaved by other proteases, however the effect of these cleavages on fibrinolysis is not yet known (*188;198;201;206*). It is possible that activation of CPN mirrors that of TAFI; TAFI can be activated by plasmin, but this is not its primary physiological activator. Similarly,

CPN may also be activated by an alternate serine protease of tryptic specificity, and plasmin may merely act as a surrogate for this enzyme during instances of increased plasmin generation and fibrinolysis. Therefore the physiological relevance of plasmin-cleaved CPNc in attenuating plasminogen activation is unknown, but it has been suggested that CPN is responsible for a low-level long-term antifibrinolytic activity, and that TAFIa is the important carboxypeptidase during instances of major fibrinolysis (*166*).

1.1.6. The apparent lack of regulation of CPN

CPN has been implicated in the modulation of activity of multiple systems due to its varied substrate specificity (*184-188*). Numerous substrates of CPN have been identified, including: bradykinin (*218;219*), kallidin (*218;219*), the complement derived anaphylatoxins (*192;247;248*), hexapeptide enkephalins (*187*), fibrinopeptides and fibrin degradation products (*166;181;184*), protamine (*249*), creatine kinase MM- and MB-isoenzymes (*186;250;251*), stromal cell derived factor-1 α (*185*), AIIt receptor (*90;252*), the α-chain of hemoglobin (*253*), and most recently chemerin (*254*). These proteins and peptides are involved in a wide range of (patho)physiological pathways, which suggests a role for CPN in the modulation of many varied processes, such as inflammation, fibrinolysis, cell homing, and pain regulation.

No regulatory mechanisms governing the activity of CPN have been discovered; however it seems unlikely that an unregulated, constitutively active enzyme plays a role in such a diverse set of processes. Other plasma enzymes appear to also be major regulators of many of these substrates *in vivo*. For example, although bradykinin is a substrate of CPN, the major inactivation pathway of this inflammatory mediator is thought to be through degradation by ACE (*279*). Similarly, TAFIa inactivates the anaphylatoxin C5a much more rapidly than CPN in both purified systems and in plasma or serum samples (*297*). TAFIa is also the more potent carboxypeptidase against fibrin and fibrin degradation products in a purified system, and appears to be the only relevant carboxypeptidase when measured in plasma samples (*166*).

However, regulation by redundancy is not likely the mechanism, given the energy expended to create such a complex molecule as CPN; evolution would likely preclude the presence of CPN in the circulation. There is significant sequence similarity between CPN

of varied species, which indicates an important role for the enzyme, one that evolution has deemed important to maintain in the species. Additionally, the absence of patients who completely lack the enzyme suggests an important physiological role.

The activity of CPN measured in individuals around the globe with healthy and unhealthy dispositions is comparable; and its activity is considered to be constitutive. When mice are challenged with lipopolysaccharide (to induce complement activation), the concentration of CPN mRNA is unchanged (*296;359*). Similarly, the activity of CPN in patients with various diseases is relatively unchanged with the exception of those with hepatic dysfunction (because CPN is synthesized in the liver) or in metastatic disorders. How could an enzyme with such important and varied roles be allowed to function in an unchecked manner? Although no regulatory mechanisms have been discovered for CPN, it is likely that the complexities of studying CPN have precluded their discovery.

1.2. Hypotheses and aims

CPN plays a major role in the regulation of fibrinolysis, and its own activity can be regulated through structural changes. CPN regulation comprises proteolytic activation by circulating plasmin and inhibition by a proteinacious plasma inhibitor derived from the CPN2 subunit. CPN is a trimeric molecule consisting of a single catalytic and two regulatory subunits.

The aim of my thesis project is four-fold:

1. To demonstrate the effective and physiologically relevant cleavage of basic carboxy-terminal residues from fibrin degradation products by CPN
2. To determine the relationship of fibrin cleavage by CPN and antifibrinolysis
3. To determine the stoichiometry of subunits in multimeric CPN
4. To demonstrate that CPN regulation is possible through positive control mechanisms (plasmic cleavage) and negative control mechanisms (CPN inhibitor)

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. General reagents

ε-amino-caproic acid (εACA), benzamidine, epichlorohydrin, arginine, lysine, lysine oxidase, horseradish peroxidase (HRP), and SigmaFast *o*-Phenylenediamine dihydrochloride tablets were from Sigma-Aldrich (St. Louis, MO, USA). Amplex red was from Invitrogen (Burlington, ON, Canada). Q-Sepharose, Sepharose 6B, DEAE and Sephacryl S-200 column resins were from Sigma-Aldrich. Benzamidine-Sepharose column resin, and pre-packed Superose-6 and Superose-12 gel filtration columns were from GE-Pharmacia (Uppsala, Sweden). CNBr-activated Sepharose 4B was from GE Healthcare (Piscataway, NJ, USA). Chemiluminescence reagent (Western Lightning) was from PerkinElmer (Waltham, MA, USA). Nitrocellulose filters for blotting were from BioRad (Hercules, CA, USA). Amicon Spin concentrators were from Millipore (Billerica, MA, USA). DNA markers were from New England Biolabs (Pickering, ON, CA) and protein molecular mass markers were from BioRad. The antibiotics ampicillin and kanamycin were purchased from Sigma-Aldrich. SYBR Safe DNA gel stain was from Invitrogen. Clear and black polystyrene microwell plates were Corning brand from Sigma-Aldrich. All other chemical reagents were enzyme-grade.

2.1.2. Inhibitors and substrates

2-guanidinoethylmercaptosuccinic acid (GEMSA), DL-mercaptomethyl-3-guanidino-ethylthiopropanoic acid (MERGETPA), D-valylphenylalanyllysyl chloromethylketone (VFKCK) and D-phenylalanylprolylarginyl chloromethylketone (PPACK) were purchased from Calbiochem (San Diego, CA, USA). Potato tuber carboxypeptidase inhibitor (PTCI) was from Sigma-Aldrich. 3-(2-furyl)acryloyl-Ala-Lys-OH (FA-Ala-Lys) was purchased from Bachem Bioscience (King of Prussia, PA, USA) and H-D-Valyl-L-Leucyl-L-Lysine-p-Nitroaniline dihydrochloride (S-2251) was from Diapharma (West Chester, OH, USA).

2.1.3. Purchased and gifted proteins

Human fibrinogen was purchased from Enzyme Research Laboratories (San Francisco, CA, USA), Sigma-Aldrich, American Diagnostica (Stamford, CT, USA), and

Calbiochem. Human CPB was from Roche (Basel, Switzerland), and human TAFI was from Affinity Biologicals (Ancaster, ON, Canada). Solulin (a soluble recombinant variant of human thrombomodulin) was from Paion (Aachen, Germany). Thrombin and normal human plasma were from Enzyme Research Laboratories and human tPA was a gift from Dr. G. Vehar (Genentech, Richmond, CA, USA). Sheep polyclonal anti(human)-CPN (antiCPN) was provided as serum by Affinity Biologicals using CPN supplied by the Bajzar Lab. The serum was affinity purified on a CPN-Sepharose column. Sheep polyclonal anti(human)-CPN1 (antiCPN1) was produced and purified similarly, using purified CPN1. CPN1 was purified by separating CPN into individual subunits by treatment with 6 M guanidinium hydrochloride and the CPN1 subunit was isolated by gel filtration on Superose-6 column. Donkey antiSheep IgG conjugated to horseradish peroxidase (DAS-HRP) was also from Affinity Biologicals.

2.1.4. DNA cloning and cell culture materials

The DH5-α strain of *E. coli* was purchased from Invitrogen. All DNA cloning enzymes, including restriction endonucleases (*Eco*RI, *Bam*HI, *Not*I, and *Hind*III), polymerases (*Taq*, and *Pfu*), ligases (T_4 ligase), and various cloning enzymes (CIP, and *Dpn*1) as well as their respective buffers were from New England Biolabs. DNA primers were purchased from Integrated DNA Technologies, IDT (Coralville, IA, USA). All sequencing was performed by the Department of Biochemistry DNA Core Services Lab at the University of Alberta (Edmonton, AB, CA). The cloning vectors pEGFP-N1 and pEGFP-C1 were from Clontech Laboratories (Mountain View, CA, USA), and the vectors pCMV-Sport6, pcDNA5/TO, and pcDNA3.1+ were from Invitrogen. Kits were purchased for PCR clean-up (QIAquick PCR purification kit), gel extraction (QIAquick gel extraction kit) and plasmid isolation (QIAprep spin miniprep kit) from Qiagen (Mississauga, ON, CA). The BHK-21 cell line was from the American type culture collection, ATCC (Manassas, VA, USA). The T-REx 293 cell line was purchased from Invitrogen. Most cell culture media and supplements; including Minimum essential media (MEM), Dulbecco's modified Eagle medium (DMEM), Opti-MEM, fetal bovine serum (FBS), trypsin, sodium pyruvate, non-essential amino acids, L-glutamine, insulin-transferrin-selenium (ITS+), Geneticin, Blasticidin S-HCl, Hygromycin-B and penicillin-streptomycin; were from Gibco (Burlington, ON, Canada). The antibiotic doxycycline hydrochloride hemiethanolate hemihydrate was from Sigma-Aldrich. The transfection reagent Lipofectamine was purchased from Invitrogen.

2.1.5. Synthesis of arginine-Sepharose and lysine-Sepharose

One hundred millilitres of Sepharose 6B was activated with epichlorohydrin (10% v/v final) in 0.4 M NaOH over 2 hours at 60°C, with vigorous mixing. Following removal of epichlorohydrin (by washing with water), arginine was added (10 % w/v final) to the resin in 0.01 M NaOH at a final volume of 150 mL. Coupling occurred over 2 hours at 60°C with gentle shaking, following which the resin was allowed to cool to room temperature, was well washed and stored in Hepes buffer (0.02 M HEPES, pH 7.4 – HB) with 0.02% sodium azide.

2.1.6. Purification of CPN from human plasma

CPN was purified from citrated fresh frozen normal human plasma (NHP) according to Walker *et al.*, (*166*) with modifications. Briefly, material precipitating from 1 L of NHP between 30% and 50% ammonium sulfate was resuspended in Hepes-buffered saline (0.02 M HEPES / 0.15 M NaCl, pH 7.4 – HBS) and dialyzed extensively versus the same. The material was loaded onto a 500 mL Q-Sepharose anion exchange column equilibrated in HBS and developed with a 0.15–0.50 M NaCl gradient in HB. CPN-containing fractions were identified by chromogenic FA-Ala-Lys assay (section 2.3.1.), pooled, diluted in HB to 0.05 M NaCl and passed over a 50 mL arginine-Sepharose column (section 2.1.5.) equilibrated in HB containing 0.05 M NaCl. The column was washed with HB containing 0.2 M NaCl and developed with a 0.2–0.5 M NaCl gradient in HB. Pooled fractions were concentrated using a 10 mL Q-Sepharose column, followed by further concentration with 60% ammonium sulfate. The pelleted protein was resuspended in HBS and loaded on a 24 mL Superose-12 gel filtration column equilibrated in HBS with 0.001% Tween80 (HBSmT). Following elution with HBSmT, the CPN-containing peak was concentrated by Amicon Spin concentrator with a molecular mass cut-off of 30 000 Da. The protein was identified by immunoblotting and the purity was verified by SDS-PAGE (section 2.3.4.). The concentration was determined by A_{280} using a $\varepsilon_{1\%}$ of 11.3 and a molecular mass of 280 000 Da, and activity was compared to previous preparations by FA-Ala-Lys assay. CPN preparations were aliquoted and stored at -80°C.

2.1.7. Purification of recombinant CPN2 from cell culture

Three hundred millilitres of pooled conditioned media from CPN2 transformed T-REx 293 cells was centrifuged at 4 000 g for 20 min at room temperature to remove

precipitate. The supernatant was diluted 1:3 with HB with 0.001% Tween20 and was loaded onto a 5 mL Q-Sepharose column equilibrated with HB. The column was washed with HB and rCPN2 was eluted in HB with 0.5 M NaCl. rCPN2-containing fractions were determined by A_{280} and dot blot using Sheep antiCPN. Dot blots were performed as follows: 2 µL of each fraction was dotted on nitrocellulose paper, dried, blocked for one hour in 5% milk in Tris-buffered saline with Tween (20 mM Tris / 0.15 M NaCl / 0.01% Tween20 – TTBS), incubated for 1 hour with Sheep antiCPN diluted 1/40 000 in 5% milk in TTBS, washed extensively with TTBS and TBS, developed with Western Lightning (according to manufacturer's protocol) and visualized on Kodak ImageStation. rCPN2-containing fractions were pooled and diluted 1:3.33 with HB and loaded onto a 1 mL antiCPN-Sepharose column equilibrated with HBS. This column was washed with HBS and rCPN2 was eluted with 0.2 M glycine pH 2.5, 5 into 1 M Tris pH 8.0 to neutralize the acid. rCPN2-containing fractions were pooled and diluted 1:3 with HB plus 0.001% Tween20 and loaded onto a 1 mL Q-Sepharose column equilibrated in HB. The column was washed with HB and rCPN2 was eluted in HB containing 0.5 M NaCl. rCPN2-containing fractions were pooled and concentrated at 2 000 g with an Amicon spin concentrator with a molecular mass cut-off of 30 000 Da, followed by buffer exchange with HB to lower the concentration of NaCl to 50 mM. Protein concentration was verified by A_{280} using a using a $\varepsilon_{1\%}$ of 14.8 and a molecular mass of 83 000 Da and by antiCPN ELISA (section 2.3.2.). Purity was determined by 5-15% SDS-PAGE followed by Coomassie blue staining and immunoblotting. rCPN2 preparations were aliquoted and stored at -20°C.

2.1.8. Purification of plasminogen

Plasminogen was isolated using affinity chromatography on lysine-Sepharose, according to Deutsch *et al.*, (*403*) with modifications. Four hundred millilitres of TAFI-depleted citrated plasma were passed over a 50 mL lysine-Sepharose column equilibrated in HBS, prepared similar to Deutsch *et al*., and plasminogen was eluted with HBS containing 20 mM εACA. The protein-containing peak was determined by A_{280}, pooled, diluted 1:3 with HB and passed over a 10 mL Q-Sepharose column equilibrated in HB. Plasminogen was eluted with HB containing 0.5 M NaCl. The protein-containing peak was pooled and concentrated by Amicon Spin concentrator with a molecular mass cut-off of 30 000 Da. Concentration was determined by A_{280} using a $\varepsilon_{1\%}$ of 16.1 and a molecular mass of 92 000 Da and the homogeneity was verified by SDS-PAGE. Purified plasminogen was

compared to previous preparations by activation with tPA and cleavage analysis of the plasmin substrate H-D-Valyl-L-Leucyl-L-Lysine-p-Nitroaniline dihydrochloride (S-2251) over time at 37ºC in a Versamax plate reader. Plasminogen preparations were aliquoted and stored in 50% (v/v) glycerol at -20°C.

2.1.9. Purification of plasmin

Plasmin was prepared by uPA hydrolysis, according to Walker *et al.*, and Bajzar *et al.* (*5;163*) with modifications. Plasminogen was dissolved in Tris-buffered saline (0.05 M Tris / 0.15 M NaCl, pH 8.0 – TBS) to a final concentration of 1 mg/mL. This solution was made 25% (v/v) in glycerol, εACA was added to a final concentration of 50 mM and 50 U/mL of uPA was added to begin activation. This solution was incubated at 37°C for 2 hours, followed by addition of another 50 U/mL of uPA and incubation for a further 2 hours at 37°C. Following the second incubation, the solution was loaded on a 5-10 mL benzamidine-Sepharose column, pre-equilibrated in TBS. Following a TBS wash, the plasmin was eluted with 20 mM benzamidine in TBS. Plasmin-containing fractions were identified based on activity against the plasmin substrate S-2251. Benzamidine and urokinase were removed using a 10 mL lysine-Sepharose column, and plasmin was eluted with 20 mM εACA. Plasmin-containing fractions were concentrated using a 1 mL Q-Sepharose column pre-equilibrated in TBS, and eluted with 0.5 M NaCl in TBS. The protein fractions (as determined by A_{280}) were pooled, made to 50% (v/v) glycerol, and stored at -20°C

2.1.10. Purification of (DD)E

The terminal fibrin degradation product (DD)E was prepared by plasmin-mediated lysis of a cross-linked fibrin clot, similar to Stewart *et al.*, (*72*) with important modifications. Fibrinogen was rendered CPN-deficient by passage over a polyclonal antiCPN-Sepharose column. Similarly, CPN-deficient fibrinogen was rendered TAFI-deficient by passage over a polyclonal antiTAFI-Sepharose column. Elimination of contaminating CPN and TAFIa activity was verified by lack of demonstrable FA-Ala-Lys hydrolysis over background. Finally, fibrinogen was rendered α_2-antiplasmin deficient by passage over an antiα_2-antiplasmin column. Flow through was reapplied until there was no difference in the lysis time during clot lysis assay (section 2.3.5.) Twenty milligrams of CPN-, TAFI-, and α_2-antiplasmin-deficient fibrinogen were incubated for up to 3 hours at room temperature with (final concentrations) 64 nM thrombin, 400 nM plasminogen, and

43 nM tPA in the presence of 10 mM calcium chloride and 0.5 M sodium chloride in HB. Following incubation, D-phenylalanylprolylarginyl chloromethylketone (PPACK; 1 μM final concentration) and D-valylphenylalanyllysyl chloromethylketone (VFKCK; 5 μM final concentration) were added to inhibit the tPA and thrombin, and εACA was added to a final concentration of 1 mM (to compete with binding of tPA, plasminogen and plasmin to the fibrin degradation products and release these fibrinolytic components from the final purified products). The solution was separated on a Sephacryl S-200 gel filtration column equilibrated in HB with 0.5 M sodium chloride, (pre-run with HB 0.5 M NaCl / 1 mM εACA), at a speed of 0.5 mL/min. Protein-containing fractions (as determined by A_{280}) were analyzed by SDS-PAGE and only fractions of pure (DD)E with no other fibrin degradation products were chosen and pooled. The pooled concentration was determined by A_{280} using a $\varepsilon_{1\%}$ of 18.4 (section 2.3.8.) and a molecular mass of 250 000 Da. The integrity and cofactor activity were measured by lysine release assay (section 3.2.2.) and plasminogen activation assay (section 2.3.6.) respectively.

2.1.11. Purification of α_2-antiplasmin

Human α_2-antiplasmin was isolated using affinity chromatography on kringle-Sepharose, according to Wiman (*404*), with modifications. One litre of TAFI- and plasminogen-depleted citrated plasma was passed (30 mL at a time) over a 10 mL kringle-Sepharose column, equilibrated in HBS. The column was washed with HBS, and α_2-antiplasmin was eluted with HBS containing 20 mM εACA. Protein-containing peaks were determined by A_{280} and pooled. α_2-antiplasmin was concentrated using a 5 mL Q-Sepharose column equilibrated in HB. This column was washed with HB and α_2-antiplasmin was eluted with a gradient of NaCl from 0-0.5 M in HB. Fractions were pooled based on A_{280} and inhibitory activity in a plasmin / S-2251 assay. α_2-antiplasmin was then concentrated with 75% ammonium sulfate, resuspended in HBS and run over 50 mL Sephacryl S-200 column, followed by a final concentration step using an Amicon Spin concentrator with a molecular mass cut-off of 30 000 Da. Purity was verified by SDS-PAGE, concentration was determined by A_{280} using a $\varepsilon_{1\%}$ of 13.0 and a molecular mass of 70 000 Da, and activity was demonstrated by inhibition of plasmin towards S-2251 (section 2.1.9.).

2.2 Equipment and software

The Spectramax and Versamax absorbance spectrophotometers, the Spectramax Gemini fluorescence spectrophotometer and their common software (Softmax) were purchased

from Molecular Devices (Sunnyvale, CA, USA). The Kodak ImageStation was from Carestream Molecular Imaging (Rochester, NY, USA). The BioLogic LP chromatography system and its software (BioLogic LP Data Viewer) were from Bio-Rad and the ÄKTAexplorer chromatography system and its software (Unicorn) were from GE-Pharmacia. All components of the multi-angle laser light scattering (MALLS) system (miniDawn TREOS, WyattQUELS, Optilab rEX) and the software (ASTRA V) were purchased from Wyatt (Santa Barbara, CA, USA). The cell culture cabinet was a Purifier Logic Class II from Labconco (Kansas City, MO, USA) and the incubator from Thermo Scientific (Waltham, MA, USA). The centrifuges used were purchased from Beckman (Brea, CA, USA), and the PCR machine was from Biometra (Goettingen, Germany). All statistical analysis and graphs were produced using GraphPad Prism 4 by the GraphPad Software Company (La Jolla, CA, USA). All molecular diagrams were created using software by PyMOL from DeLano Scientific LLC (Palo Alto, CA, USA) or ICM-Browser from MolSoft (La Jolla, CA, USA).

2.3. Methods

2.3.1. Furylacryloyl-Ala-Lys (FA-Ala-Lys) chromogenic carboxypeptidase assay

Samples of purification fractions, purified carboxypeptidase or normal human plasma (NHP), diluted as required in HBS with 0.01% Tween80 (HBST), were added (5-75 µL) to a clear microwell plate. Any inhibitors, also diluted in HBST, were added to the appropriate wells. FA-Ala-Lys, diluted in HBST to a final assay concentration of 1 mM, was then added in quick succession to the appropriate wells to make a final assay volume of 100 µL. Plates were immediately mixed and the rate of FA-Ala-Lys hydrolysis was measured by the decrease in absorbance at 340 nm (or 346 nm for determinations of kinetic constants) as a function of time at 37ºC in a Versamax plate reader, with an interval time of 10 seconds. The range of CPN for standard curves varied by application, as described below..

For experiments examining the inhibition of CPN by NHP or NHP depleted of CPN (NHPdCPN) (section 3.5.2. and section 3.5.3.), the samples of CPN were titrated from 40-0.31 nM and NHP was titrated from 100% to 0.78% in HBST. These were added to an equal volume of 2 mM FA-Ala-Lys, in the presence or absence of rCPN2 (80-0.625 nM, final concentration).

For kinetic determinations (section 3.2.2.), CPN, plasmin-cleaved CPNc, CPB and TAFIa activities were compared. CPN was activated with plasmin (as described in section 2.3.9.), and TAFI was activated with thrombin / solulin. One micromolar TAFI was activated with 30 nM thrombin and 50 nM solulin in HBS containing 5 mM $CaCl_2$ for 15 minutes at room temperature. The reaction was quenched by the addition of 75 μM PPACK. All enzymes were compared in parallel assays at room temperature or at 37°C. Fifty microlitres of 10 nM carboxypeptidase was added to a microwell plate and the reaction was initiated by addition of 50 μL of FA-Ala-Lys at a concentration range of 10 mM to 156.25 μM. The slope of the initial rate of reaction was determined as a function of the ΔA for FA-Ala-Lys, corrected for the residual contribution of FA-Ala to the signal at A_{346}, at each concentration.

2.3.2. CPN-specific ELISA

Clear microwell plates were coated for one hour with polyclonal sheep antiCPN in carbonate buffer (15 mM sodium carbonate / 35 mM sodium bicarbonate, pH 9.6) and blocked with 1% bovine serum albumin (BSA) in HBS for one hour. Samples, diluted in 1% BSA in HBST, were incubated for one hour. After washing (with 137 mM sodium chloride / 8.1 mM sodium phosphate / 1.5 mM potassium phosphate / 3 mM potassium chloride / 0.1% Tween20, pH 7.4 – PBST), HRP-conjugated antiCPN was added to wells. HRP-conjugated antiCPN was produced using Pierce EZ-Link Plus Activated Peroxidase Kit (according to manufacturer's protocol). The plate was developed with 100 μL SigmaFast *o*-Phenylenediamine dihydrochloride tablets and the reaction was terminated with 50 μL 0.2 N sulfuric acid. The absorbance at 490 nm was read on Versamax plate reader. During all incubation periods, the plate was kept at room temperature.

Samples of fibrinogen were examined to identify a contaminating carboxypeptidase (section 3.1.3.). For these experiments, fibrinogen was diluted to 1.7 μg/mL or 3.4 μg/mL in 1% BSA in HBST prior to incubation in the coated microwell plates. For experiments measuring the concentration of CPN in conditioned media of CPN1- or CPN2-transfected cells, or for quantification during rCPN2 purification (section 3.4.3.), the conditioned media or column fractions were serially diluted in 1% BSA in HBST in order to load 100% to 0.0000078% of sample in the 100 μL load (percentage of sample in the load depended on the nature of the sample). Standard curves of purified CPN ranged from 1 nM to 15.625 pM.

2.3.3. Immunodepletion of fibrinogen

CNBr-activated Sepharose 4B was prepared according to the manufacturer's instructions. Affinity-purified antiCPN was coupled to prepared resin in HBS. Fifty microlitres of antiCPN-Sepharose beads were added to 500 μL of 25 mg/ml fibrinogen (or 17.42 mg/mL of ERL fibrinogen). Mixtures were incubated for one hour with mixing at room temperature. The beads were pelleted and the supernatant removed. The beads were washed twice in HBS for ten minutes with mixing, followed by elution of bound protein with 0.2 M glycine pH 2.8, which was quickly neutralized by 1 M Tris base. Both supernatant and eluate were kept for analysis.

2.3.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Unless otherwise noted, all SDS-PAGE gels were 5-15% gradient separating gels, with a 5% stacking gel. Gels were developed using the method of Neville (*405*), in a Tris-Borate buffer (40 mM Tris / 40 mM boric acid / 0.1% SDS, pH 8.4 – TB), typically at 50-120 volts. Samples were diluted in loading buffer (1% SDS / 10% glycerol – with 2% β-mercaptoethanol when needed to reduce samples) and heated at 100°C for 5 minutes prior to loading. Gels were either stained in Coomassie blue (7.5% ethanol / 5% acetic acid / 20 μM Coomassie brilliant blue R-250) or transferred to nitrocellulose filters for immunoblotting. Transfers were run in Towbin's buffer (25 mM Tris / 192 mM glycine / 20% methanol, pH 8.3) at 70 volts for one hour. The blots were then blocked in 5% skim milk powder in phosphate buffered saline (8.1 mM Na_2HPO_4 / 1.5 mM KH_2PO_4 / 3 mM KCl / 137 mM NaCl, pH 7.4 – PBS). Antibodies used for detection were diluted in the same, and blots were washed with PBS. Blots were developed with Western Lightning according to manufacturer's protocols and exposed on a Kodak ImageStation.

Fibrinogen immunodepletion eluates analyzed for the presence of CPN and CPN degradation products (section 3.1.4.) using 10% SDS-PAGE gels. These blots were incubated with sheep antiCPN primary and HRP-conjugated Donkey antiSheep secondary antibodies. Samples of CPN and rCPN2 were examined throughout their purification procedures (section 3.4.3.) with these same primary and secondary antibodies.

2.3.5. Clot lysis assays

Plasma-free clot lysis assays were performed using purified components. In a clear microwell plate, equal volumes of a solution of fibrinogen / plasminogen / α_2-antiplasmin and a solution of calcium / thrombin / tPA were combined. Final assay concentrations were: 3 mg/mL fibrinogen, 9.2 μg/mL plasminogen, 0-200 nM α_2-antiplasmin, 4 mM $CaCl_2$, 12 nM thrombin, and a dilution series of 25-800 pM tPA; all dilutions were in HBSmT. Plates were sealed with acetate plate sealers and the turbidity at 405 nm was measured as a function of time at 37ºC on a Versamax plate reader, with an interval time of 2.5 minutes. The lysis time (the time at which turbidity equals one-half of full-scale) was determined individually for each clot.

2.3.6. Plasminogen activation assay

The effect of carboxypeptidases on the plasminogen activation cofactor activity of the terminal fibrin degradation product (DD)E was measured by stationary phase (for plasma-containing samples), solution phase (for uncomplicated samples), or two-step solution phase (for timecourse examinations) assays.

2.3.6.1. Stationary phase plasminogen activation assay

Clear microwell plates were coated with 75 μL of 0.5 μM (DD)E (diluted in HB containing 0.5 M NaCl) and incubated for 2 hours at room temperature. Plates were washed three times with 150 μL HBST, followed by incubation with 100 μL of plasma or carboxypeptidase samples (with inhibitor when appropriate) for 2 hours at room temperature. Following a second wash step with HBST, 100 μL of developing solution was added; this solution contained (final concentrations, diluted in HBST): 0.25 μM plasminogen, 2 nM α_2-antiplasmin, 0.2 nM tPA, 10 mM $CaCl_2$, and 0.25 mM S-2251. Plates were sealed with acetate plate sealers and plasminogen activation (as a function of plasmin cleavage of S-2251) was monitored at 405 nm as a function of time at 37ºC on a Versamax plate reader, with an interval time of 2.5 minutes.

This assay was used to measure the inhibitory capacity of plasma toward antifibrinolysis by CPN (section 3.5.2. and section 3.5.3.). Samples were a dilution series of NHP or NHPdCPN (5% - 0.0078%, in HBST) plus or minus 5 nM of purified CPN. For comparison purposes, a standard curve of purified CPN was included (5 nM - 39 pM final concentrations, in HBST), and a negative control of HBST alone. NHP was depleted of

CPN by running it over antiCPN-Sepharose column; CPN depletion was verified by FA-Ala-Lys activity assay and immunoblots.

2.3.6.2. Solution phase plasminogen activation assay

A dilution series of samples were prepared in HBSmT, plus or minus 5 nM (final assay concentration) of purified CPN. Also, a standard curve of purified CPN (5 nM - 39 pM final assay concentration, in HBSmT), and a negative control of HBSmT alone were prepared. These were added to a clear microwell plate in a final volume of 25 μL. To each well was added 50 μL of solution containing (final assay concentration) 0.25 μM plasminogen, 2 nM α_2-antiplasmin, 10 mM $CaCl_2$ and 0.25 mM S-2251. Plasminogen activation was initiated by addition of 25 μL of a dilution series of tPA (final assay concentration: 0.2 nM – 25 pM); plates were sealed with acetate plate sealers and plasminogen activation (as a function of plasmin cleavage of S-2251) was monitored at 405 nm as a function of time at 37ºC on a Versamax plate reader, with an interval time of 2.5 minutes. All solutions were prepared and then added to plate in quick succession.

The solution phase method was used to analyze the CPN inhibitory activity of rCPN2 (section 3.5.4.). Samples of a dilution series of rCPN2 (20 nM – 0.156 nM; final assay concentration) plus or minus 5 nM purified CPN were monitored for enhancement of plasminogen activation.

2.3.6.3. Two-step solution phase plasminogen activation assay

Carboxypeptidase samples were prepared in HBSmT to 2x the desired incubation concentration. (DD)E was prepared in HBSmT to 4x the desired final assay concentration. An aliquot of carboxypeptidase solution and an aliquot of (DD)E solution were added in equivalent volumes according to a reverse timecourse from 0-600 min. All samples were kept on ice until use, and the timecourse incubations were conducted at room temperature. At the end of the incubation time, the carboxypeptidase activity in all samples was inhibited with 200 μM (final concentration) GEMSA. Fifty microlitres of each incubation mixture was added to a clear microwell plate and then plasminogen activation was initiated by addition of 50 μL of developing solution (final assay concentrations: 0.25 μM plasminogen, 200 pM tPA, 2 nM α_2-antiplasmin, 10 mM $CaCl_2$ and 0.25 mM S-2251; all diluted in HBSmT). Plates were sealed with acetate plate sealers and plasminogen activation (as a function of plasmin cleavage of S-2251) was

monitored at 405 nm as a function of time at 37ºC on a Versamax plate reader, with an interval time of 2.5 minutes.

This two-step solution phase method was used to compare the antifibrinolytic capabilities of CPN, CPNc, CPB and TAFIa (section 3.3.2.). CPN was activated with plasmin as described in section 2.3.9., and TAFI was activated with thrombin/solulin. In the latter case, one micromolar TAFI was activated with 30 nM thrombin and 50 nM solulin in HBS containing 5 mM $CaCl_2$ for 15 minutes at room temperature. The thrombin in the reaction was inhibited by the addition of 100 nM hirudin (PPACK, the normally used thrombin inhibitor interferes with tPA and plasmin activity during the plasminogen activation portion of the experiment; hirudin is not typically used due to its cost). (DD)E was diluted to an assay concentration of 200 nM and the carboxypeptidases were diluted to an incubation reaction concentration of 5 nM.

2.3.7. Combined plasminogen activation and lysine detection assay

To directly compare the release of carboxy-terminal lysine residues from (DD)E samples to the degree of reduction in the rate of plasminogen activation, aliquots of the same original samples were required. Therefore a two-step solution phase plasminogen activation assay (section 2.3.6.3.) was combined with the lysine detection assay (section 3.2.2.). This combined assay was used to compare the antifibrinolytic activities of CPN, CPNc, CPB and TAFIa.

TAFI was activated with thrombin/solulin. One micromolar TAFI was activated with 30 nM thrombin and 50 nM solulin in HBS containing 5 mM $CaCl_2$ for 15 minutes at room temperature. The thrombin in the reaction was inhibited by the addition of 100 nM hirudin, and CPN was activated with plasmin (as described in section 2.3.9.). To account for the effects of the α_2-antiplasmin used to quench the plasmin during CPN activation, after dilution (in HBSmT) of all the carboxypeptidases to 10 nM, α_2-antiplasmin was added to CPN, CPB and TAFIa to a concentration of 1 nM (similar to the concentration of α_2-antiplasmin in diluted CPNc); an equivalent volume of HBSmT was added to CPNc. (DD)E was prepared in HBSmT to 5 μM. An aliquot of each carboxypeptidase solution and an aliquot of (DD)E solution were added in equivalent volumes according to a reverse timecourse from 0-600 min. All samples were kept on ice until use, and the timecourse incubations were conducted at room temperature. At the end of the incubation

time, samples were quenched with 200 μM (final concentration) GEMSA in a large volume, to dilute the concentration of (DD)E to 400 nM.

Fifty microlitres of each incubation mixture was added to a clear microwell plate and then plasminogen activation was initiated by addition of 50 μL of developing solution (final assay concentrations: 0.25 μM plasminogen, 200 pM tPA, 2 nM α_2-antiplasmin, 10 mM $CaCl_2$ and 0.25 mM S-2251; all diluted in HBSmT). Plates were sealed with acetate plate sealers and plasminogen activation (as a function of plasmin cleavage of S-2251) was monitored at 405 nm as a function of time at 37ºC on a Versamax plate reader, with an interval time of 2.5 minutes. Reaction solution was prepared immediately before use and added to sample in quick succession.

The concentration of lysine in the samples was measured concurrently in the lysine detection assay (section 3.2.2.). Sixty-four microlitres of each incubation mixture was added to a black microwell plate containing 32 μL of amplex red (50 μM final concentration) per well. The reaction was initiated with 32 μL HRP / lysine oxidase mixture (final concentrations of 1 U/mL HRP and 0.1 U/mL lysine oxidase). The concentration of free lysine was quantified as a function of resorufin production by increase in fluorescence at 37ºC on a Spectramax Gemini fluorescence spectrophotometer with an excitation wavelength of 560 nm, an emission wavelength of 590 nm and a cut-off of 570 nm.

This combined plasminogen activation and lysine detection assay was also used to determine the maximum number of lysine residues that can be released per molecule of (DD)E by carboxypeptidases (section 3.2.4.) and to show that the number of released lysine residues is proportional to the decrease in cofactor activity. Concentrations ranging from 5-250 nM CPN, CPNc or CPB and 0.2-2.5 μM (DD)E were used for these analyses. The incubation reactions were performed at room temperature and 37ºC for 0-300 minutes.

2.3.8. Extinction coefficient calculation for (DD)E

The extinction coefficient typically quoted for (DD)E, $\varepsilon_{1\%}$ of 16.0 at A_{280}, was proposed by Stewart *et al.* (*72*). This appears to have been calculated incorrectly from the known extinction coefficients of fragments DD and E, as experimentally determined by Olexa *et*

al. (*68*). Olexa *et al.* determined the A_{280} of a 1 mg/mL solution of isolated fragment DD is 2.0; and of isolated fragment E is 1.2. Stewart *et al.*, who appears to have treated these as molar extinction coefficients, took the average of these two values (since each mole of (DD)E contains one mole of fragment DD and one mole of fragment E), to obtain their quoted 1.6 value. However, since these are mass extinction coefficients, this relation does not hold true; to determine the extinction coefficient for (DD)E, the relative masses of fragments DD and E must be taken into account. Each fragment D is 100 kDa and fragment E is 50 kDa, therefore the actual extinction coefficient is 1.84. The true calculated extinction coefficient is important for the determination of the number of moles of lysine released from (DD)E by carboxypeptidases. A falsely low extinction coefficient results in falsely inflated concentrations of substrate and ultimately a falsely decreased ratio of lysine:(DD)E.

2.3.9. Activation of CPN by plasmin

CPN was cleaved by plasmin to form the active CPNc for several kinetic and functional analyses. The activation solution was composed of 500 nM purified CPN, 10 nM plasmin, 5 mM $CaCl_2$ in HBSmT. This solution was incubated at 37°C for 2 hours and then quenched with 50 nM α_2-antiplasmin. CPNc was kept on ice until use.

2.3.10. Kinetic measurements

Kinetic parameters were determined for carboxypeptidase cleavage of FA-Ala-Lys and (DD)E using the lysine detection assay (section 3.2.2.). A dilution series, in HBSmT, of FA-Ala-Lys (from 5 mM to 78.125 µM, final concentrations) was added as the substrate, and CPN, CPNc, CPB or TAFIa was added to a final concentration of 100 pM to initiate the reaction. Similarly, a dilution series in HBSmT of (DD)E (from 200 nM to 50 nM, final concentrations) was added as the substrate, and CPN, CPNc, CPB, or TAFIa was added to a final concentration of 20 nM to initiate the reaction and determine the kinetic parameters for (DD)E hydrolysis. Kinetic parameters were determined using non-linear regression by GraphPad Prism software. CPN was activated (as described in section 2.3.9.). TAFI was activated with thrombin / solulin. One micromolar TAFI was activated with 30 nM thrombin and 50 nM solulin in HBS containing 5 mM $CaCl_2$ for 15 minutes at room temperature. The reaction was quenched by the addition of 75 µM PPACK.

2.3.11. DNA cloning and mammalian cell culture

2.3.11.1 DNA manipulations

The *cpn2* gene was obtained from G.R. Lambkin (University of Alberta, Edmonton, CA: originally purchased from ATCC; IMAGE clone 5190234) within the multiple cloning sites of pCMV-Sport6 and pcDNA3.1+ vectors. The gene was present in its native form (termed CPN2s), as well as containing additional sequence for hexaHis-tag and c-Myc epitope tag (termed CPN2r).

2.3.11.1.1. GFP fusion systems

The *cpn2* gene was amplified by polymerase chain reaction (PCR) from the given vector with addition of 5' *Eco*RI restriction site and 3' *Bam*HI restriction site. The primer sequences are described in Table 2.1. PCR reactions were assembled on ice using 0.5 U *Taq* polymerase, 1x dilution of *Taq* polymerase buffer with $MgCl_2$, 250 ng forward primer, 250 ng reverse primer, 50-100 ng template, and 200 μM dNTPs. The PCR thermocycling conditions were as follows: 1 cycle for 2 min at 95°C of denaturation, 30 cycles of 20 s denaturating at 95°C, 20 s annealing at 55°C, and elongation at 72°C for 90 s, and 1 cycle for 3 min at 72°C of final extension. PCR products were purified by electrophoresis on 1% agarose gel, stained with SYBR Safe, and extracted with QIAquick gel extraction kit. Purified products were quantified by A_{280}/A_{320} and 2.5 μg were digested with 20 U each of *Eco*RI and *Bam*HI restriction enzymes, both of which were compatible with NEB EcoRI buffer (100 mM Tris-HCl / 50 mM NaCl / 10 mM $MgCl_2$ / 0.025% Triton X-100, pH 7.5), supplemented with 100 μg/mL BSA. The digestion mixture was incubated for 1 hour at 37°C, and purified by QIAquick PCR purification kit.

The cloning vectors pEGFP-N1 and pEGFP-C1 encode an optimized variant of green fluorescent protein (GFP) which is flanked by a Kozak consensus translation initiation site and is under the control of the immediate early promoter of CMV. Genes cloned into the multiple cloning site of pEGFP-N1 will be expressed as fusions to the amino-terminus of GFP, and genes cloned into the multiple cloning site of pEGFP-C1 will be expressed as fusions to the carboxy-terminus of GFP. These vectors also contain an SV40 polyadenylation signal, SV40 mammalian origin of replication, and a neomycin-resistance cassette allowing for selection with kanamycin and Geneticin.

Table 2.1: The primer sequences for CPN cloning.

Primer	*Sequence*
Forward: CPN2 -> pEGFP-C1	5'-TACTGAATTCTATGCTCCCTGGAGCCTGG-3'
Reverse: CPN2 -> pEGFP-C1	5'-TAATGGATCCCTAGGGCCCTGCTGCCC-3'
Forward: CPN2 -> pEGFP-N1	5'-ATATGAATTCCCGCCACCATGCTCCCTGGAGCCTGG-3'
Reverse: CPN2 -> pEGFP-N1	5'-TATAGGATCCCGTCTGGGCCCTGCTGCCCG-3'
Forward: CPN2 -> pcDNA5/TO	5'-ATATGAATTCCCGCCACCATGCTCCCTGGAGCCTGG-3'
Reverse: CPN2 -> pcDNA5/TO	5'-TAT AGC GGC CGC CTA GGG CCC TGC TGC CCG-3'

pEGFP-N1 and pEGFP-C1 were digested with *Eco*RI and *Bam*HI restriction enzymes (as described above) and treated with calf intestinal alkaline phosphatase (CIP) to remove the 5' phosphate groups. Eight micrograms of doubly-digested vector was incubated with 10 U CIP in NEB buffer 3 (100 mM Tris-HCl / 100 mM NaCl / 10 mM $MgCl_2$ / 1mM dithiothreitol (DTT), pH 7.9) for 1 hour at 37°C, and purified by QIAquick kit. Ninety femtomoles of gene insert was incubated with 30 fmol of vector and 400 U of T_4 ligase in NEB T_4 ligase buffer (50 mM Tris-HCl / 10 mM $MgCl_2$ / 1 mM ATP / 10 mM DTT, pH 7.5) for 10 min at room temperature.

DH5-α *E. coli* were made competent with $CaCl_2$ transformation media (10 mM HEPES / 15 mM $CaCl_2$ / 250 mM KCl / 55 mM $MnCl_2$, pH 6.7), and competent cells (50 µL) were transformed with ligation mixture (3 µL) by heat shock at 42°C for 45 s. Cells were then placed on ice and 200 µL of Luria-Bertani broth (10 g/L NaCl / 10 g/L tryptone / 5 g yeast extract, pH 7.0 – LB) was added prior to spreading cells on LB plates containing 20 µg/mL kanamycin. Following a 16 hour incubation at 37°C, isolated colonies were selected and grown in LB-kanamycin broth overnight at 37°C and 250 rpm and the plasmid DNA was isolated by QIAprep spin miniprep kit. The presence of gene inserts

was verified by plasmid digestion with *Eco*RI and *Bam*HI and 1% agarose gel electrophoresis. Proper insertion was further verified by DNA sequencing.

2.3.11.1.2. Inducible expression systems

cpn2 was amplified by PCR from the pEGFP-C1 CPN2 vector construct, with the addition of 5' *Eco*RI and 3' *Not*I restriction sites. The primer sequences are described in Table 2.1. PCR reactions were assembled on ice as described in section 2.3.11.1.1. and purified by electrophoresis on 1% agarose gel with extraction by QIAquick gel extraction kit. Purified products were quantified by A_{280}/A_{320} and 2.5 µg were digested with 20 U each of *Eco*RI and *Not*I restriction enzymes, both of which were compatible with NEB EcoRI buffer, supplemented with 100 µg/mL BSA. The digestion mixture was incubated for 1 hour at 37°C, and purified by QIAquick PCR purification kit.

The cloning vector pcDNA5/TO is part of the T-REx system (tetracycline-regulated expression system for mammalian cells). Genes cloned into the multiple cloning site of pcDNA5/TO are under the control of the immediate early promoter of CMV which is controlled by two tandem tetracycline operator sequences which are binding sites for Tet repressor homodimers. A bovine growth hormone polyadenylation signal follows the inserted genes. This vector also confers resistance to ampicillin and hygromycin. When pcDNA5/TO is transfected into the complementary T-REx system cells, such as T-REx 293 (which contains the pcDNA6/TR plasmid and constitutively expresses Tet repressor proteins) expression of the desired protein is repressed. Addition of tetracycline (or the related doxycycline) de-represses the promoter and allows protein expression.

pcDNA5/TO was digested with *Eco*RI and *Not*I (as described above), CIP-treated, and purified by QIAquick kit. Ligations and competent cell transformations were carried out as described in section 2.3.11.1.1. Isolated colonies were then grown in LB broth containing 50 µg/mL ampicillin overnight at 37°C and 250 rpm, and the plasmid DNA was isolated by QIAprep spin miniprep kit. The presence of gene inserts was verified by plasmid digestion with *Eco*RI and *Not*I and 1% agarose gel electrophoresis and proper insertion was verified by DNA sequencing.

Additionally, *cpn1-gfp*, *cpn2-gfp* and *gfp* were sub-cloned into the pcDNA5/TO vectors. The vectors pEGFP-N1, pEGFP-N1 CPN1 and pEFGP-N1 CPN2 were digested with

*Hind*III and *Not*I to isolate the desired gene fusions. Digestions were performed as described above, in NEB buffer 2 (10 mM Tris-HCl / 50 mM NaCl / 10 mM $MgCl_2$ / 1 mM DTT, pH 7.5). Products were separated by 1% agarose gel electrophoresis and were extracted and purified with Qiagen kits. pcDNA5/TO was digested similarly with *Hind*III and *Not*I, CIP-treated, and purified by QIAquick kit. The inserts were ligated into the vectors, competent cells were transformed, and isolated colonies were analyzed all as described above and verified by DNA sequencing.

2.3.11.2 Mammalian cell culture

BHK-21 were selected for transfection with pEGFP-derived vectors. These cells are derived from Syrian golden hamster kidney fibroblasts. BHK-21 cells were grown in MEM supplemented with 10% FBS, 100 U penicillin, 100 µg streptomycin, 0.1 mM glycine, 0.1 mM L-alanine, 0.1 mM L-asparagine, 0.1 mM L-aspartic acid, 0.1 mM L-glutamic acid, 0.1 mM L-proline, 0.1 mM L-serine, and 1 mM sodium pyruvate. Cells transfected with pEGFP vectors were selected with 50 µg/mL geneticin.

T-REx 293 cells were used for transfection with pcDNA5/TO-derived vectors. These cells are derived from HEK 293 cells, from human embryonic kidney, and are stably transformed with pcDNA6/TR plasmid which causes constitutive expression of Tet repressor proteins. T-REx 293 cells were grown in DMEM supplemented with 10% FBS, 100 U penicillin, 100 µg streptomycin, 2 mM L-glutamine, and 50 µg/mL blasticidin. Cells transfected with pcDNA5/TO vectors were selected with 100 µg/mL hygromycin, and protein translation was induced by addition of 1 µg/mL doxycycline.

Cell transfections were through the lipofectamine method. The appropriate cell type was seeded to 80% confluency in a 6-well plate. One microgram of desired plasmid DNA and 10 µL of lipofectamine transfection reagent were added to 200 µL OptiMEM and incubated for 45 minutes at room temperature, followed by a further addition of 200 µL OptiMEM. Cells were washed extensively with phosphate buffered saline (137 mM sodium chloride / 8.1 mM sodium phosphate / 1.5 mM potassium phosphate / 3 mM potassium chloride, pH 7.4 – PBS) and their surface was covered in 400 µL OptiMEM. The transfection solution was added and cells were incubated at 37°C for 5 hours in 5% CO_2. Following this incubation period, 800 µL MEM or DMEM supplemented with 20% FBS, 100 U penicillin, and 100 µg streptomycin was added and cells were incubated

overnight at 37°C in 5% CO_2. Cells were then washed with PBS and the medium was replaced with the appropriate selection medium listed above. When distinct colonies could be identified, the cells were washed, trypsonized, and diluted in appropriate selection medium to 1 cell per 200 µL, and plated in 96-well plates with 100 µL per well. When independent, transformed cell colonies could be identified, they were transferred to 6-well plates, analyzed for protein expression (by FA-Ala-Lys assay, and/or immunoblot) and propagated.

To purify rCPN2, the protein was overexpressed in T-REx 293 cells. T-REx cells containing the pcDNA5/TO CPN2 plasmid were grown in DMEM supplemented with 10% FBS, 100 U penicillin, 100 µg streptomycin, 2 mM L-glutamine, 50 µg/mL blasticidin, and 100 µg/ml hygromycin. Cells were plated to 80% confluency in T-75 flasks, washed extensively with PBS and expression was induced with OptiMEM supplemented with 100 U penicillin, 100 µg streptomycin, 2 mM L-glutamine, 5 µg/ml blasticidin, 100 µg/ml hygromycin, 1 µg/ml doxycycline and ITS+ (6.7 µg/L sodium selenite / 110 mg/L sodium pyruvate / 10 mg/L insulin / 5.5 mg/L transferrin). Conditioned media was collected and cells were re-induced every second day for 8 days followed by recovery in DMEM.

2.3.12. Multi-angle laser light scattering (MALLS)

MALLS was used to determine the molecular mass of CPN and CPNc. Purified CPN was run over a Superose-6 gel filtration column attached in-line to a miniDawn TREOS multi-angle laser light scattering detector. Data was collected as a function of elution volume from the miniDawn TREOS, as well as the Optilab rEX refractometer and the WyattQUELS quasi elastic light scattering detector, and was amalgamated using the ASTRA program. The temperature was maintained at 37°C throughout each run.

BioRad molecular mass standards were used to standardize the equipment, and they eluted as expected. Purified CPN from a single preparation was split into three runs of 0.1 mL each, and was run at 20 mL/hr. Continuous sample analysis began following a 35 minute lag, and continued for 35 minutes; fractions of 0.5 mL were collected. Fractions containing CPN were pooled and activated by 10 nM plasmin in 5 mM $CaCl_2$ for 1 hour at 37°C. Plasmin was inhibited by 10 µM VFKCK and the reaction solution was concentrated to 0.1 mL by spin concentrator. CPNc was then analyzed by MALLS as

described for CPN, but as a single run of 0.1 mL. Molecular mass was determined by the ASTRA V program, using Zimm equations with a refractive index increment (dn/dc) of 0.179 (for both CPN and CPNc).

3. RESULTS

3.1. Contamination of fibrinogen by CPN

3.1.1. Preamble

In the course of examining the lysine removal from (DD)E, the initial cofactor activity of (DD)E was found to vary significantly from one experiment to another. Additionally, in the absence of added carboxypeptidase, what should be stable cofactor activity appeared to decrease over time. This effect was related to a decrease in the number of carboxy-terminal lysine residues: there was a decrease in the number of moles of lysine that could be proteolytically released per mole of (DD)E between consecutive determinations. Upon closer examination, the preparation of (DD)E appeared to contain an associated or contaminating carboxypeptidase activity, which could be traced back to the original fibrinogen source used for the synthesis of (DD)E. The carboxypeptidase activity contaminating various commercial sources of fibrinogen needed to be identified, quantified and removed.

3.1.2. Identification of the contaminating carboxypeptidase

The effects of a series of carboxypeptidase inhibitors on the contaminant activity toward the substrate FA-Ala-Lys were measured in the lysine detection assay (section 3.2.2.). MERGETPA and GEMSA both inhibited the contaminating activity, while PTCI had no effect (Figure 3.1). Treatment of fibrinogen with EDTA to chelate the active-site zinc ion also inhibited the activity. Of all potential contaminating carboxypeptidases, only CPN demonstrates this pattern of inhibition, as shown in Table 3.1.

Table 3.1: Inhibition patterns of human basic carboxypeptidases and the unknown fibrinogen contaminant.

	CPB	***TAFIa***	***CPN***	***Unknown fibrinogen contaminant***
MERGETPA	X*	X	X	X
GEMSA	X	X	X	X
EDTA	X	-	X	X
PTCI	X	X	-	-

* 'X' indicates inhibition; '-' indicates no inhibition

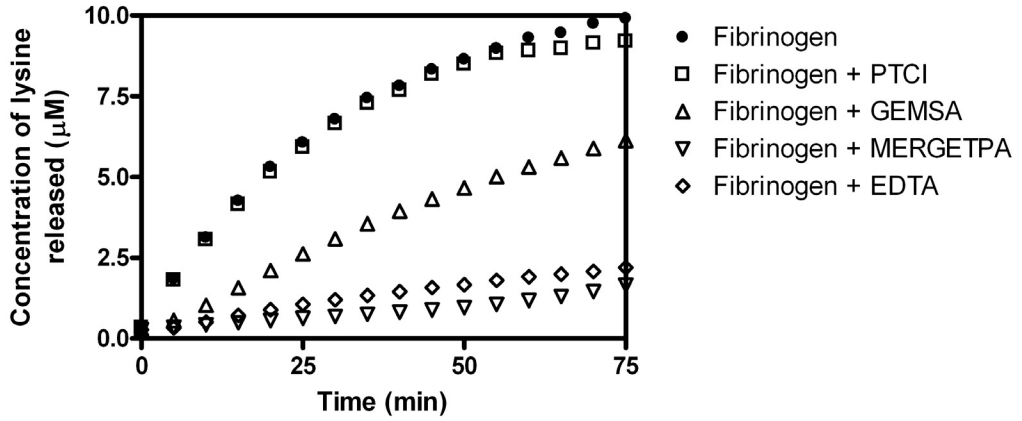


Figure 3.1: The inhibition of the fibrinogen contaminant by a series of carboxypeptidase inhibitors. Preparations of fibrinogen hydrolyze FA-Ala-Lys, as measured by the production of free lysine. Addition of PTCI (40 μM) does not alter this rate of hydrolysis. Treatment with GEMSA (200 μM), MERGETPA (200 μM) and EDTA (150 mM) all resulted in varying degrees of inhibition of the carboxypeptidase.

3.1.3. Determination of the concentration of the contaminating carboxypeptidase

The activity of the contaminating carboxypeptidase was measured in samples of fibrinogen from Calbiochem, Enzyme Research Laboratories, American Diagnostica and Sigma. Since this thesis is not an advertisement or an endorsement for the fibrinogen of one company over another, the fibrinogen stocks will be referred to as A, B, C, and D (not necessarily related to the order described above). Based on activity measurements with the FA-Ala-Lys chromogenic assay, the concentration of contaminating carboxypeptidase varied from one commercial source to another. These results are presented in Figure 3.2 and Table 3.2.

Table 3.2: Functional and antigenic quantitation of fibrinogen contaminant.

Fibrinogen source	***FA-Ala-Lys hydrolysis nM CPN per mg/mL fibrinogen (% contamination)***	***antiCPN ELISA nM CPN per mg/mL fibrinogen (% contamination)***
A	0.71 ± 0.09 (0.02%)*	0.21 ± 0.04 (0.007%)
B	0.75 ± 0.09 (0.02%)	0.20 ± 0.04 (0.007%)
C	(not detectable)	0.040 ± 0.007 (0.001%)
D	0.17 ± 0.03 (0.006%)	0.13 ± 0.01 (0.004%)

* n = 8

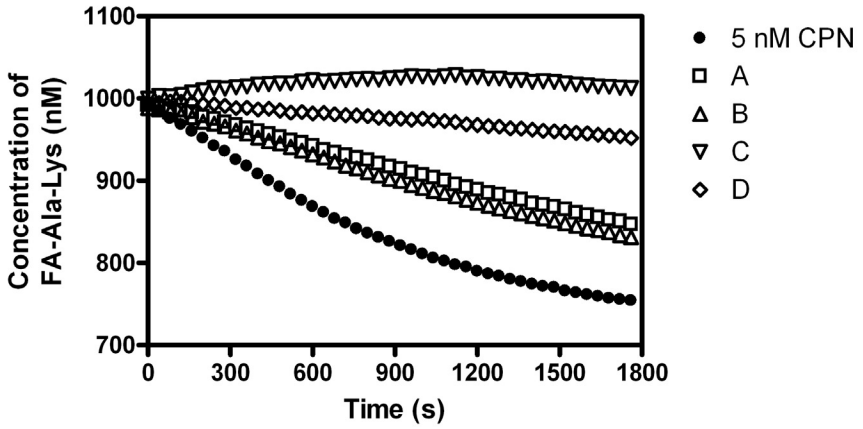


Figure 3.2: Quantitation of the concentration of contaminating carboxypeptidase in commercial fibrinogen sources. The carboxypeptidase substrate FA-Ala-Lys is hydrolyzed by fibrinogen in the absence of exogenous purified carboxypeptidase. A sample of the activity of 5 nM CPN is included for comparison purposes.

The antigenicity of the contaminating CPN was measured in samples of fibrinogen from sources A, B, C, and D. Based on measurements by antiCPN ELISA, the concentration of contaminating carboxypeptidase varied from one commercial source to another (Table 3.2). Additionally, for three of the four fibrinogen sources, the concentration of contaminant as determined by antiCPN ELISA differed from the concentration as measured by chromogenic assay.

Differing lots of fibrinogen purchased from the same commercial source (source B) also displayed a range of contamination, from 0.47 ± 0.03 to 0.77 ± 0.01 nM CPN per mg/mL fibrinogen (Table 3.3).

Table 3.3: Lot to lot variation of fibrinogen contamination.

Lot number	***FA-Ala-Lys hydrolysis*** ***nM CPN per mg/mL fibrinogen***
3132	0.74 ± 0.02*
3222	0.58 ± 0.03
3252	0.68 ± 0.09
3254	0.47 ± 0.03
3256	0.77 ± 0.01

* n = 4

3.1.4. Immunodepletion of fibrinogen and immunoblotting

Fibrinogen from several commercial sources was depleted of CPN by passage over antiCPN-Sepharose and the residual carboxypeptidase activity was measured by FA-Ala-Lys hydrolysis in the chromogenic assay. CPN-immunodepleted fibrinogen showed an 80% to 85% decrease in CPN activity in the chromogenic assay (Figure 3.3). Eluates from the immunodepletion experiments were subjected to antiCPN Western blotting which revealed the presence of the 83 kDa CPN regulatory subunit and the 48-55 kDa CPN catalytic subunit in samples from all four fibrinogen sources. Additionally, uncharacterized cleavage products were also detected (Figure 3.4).

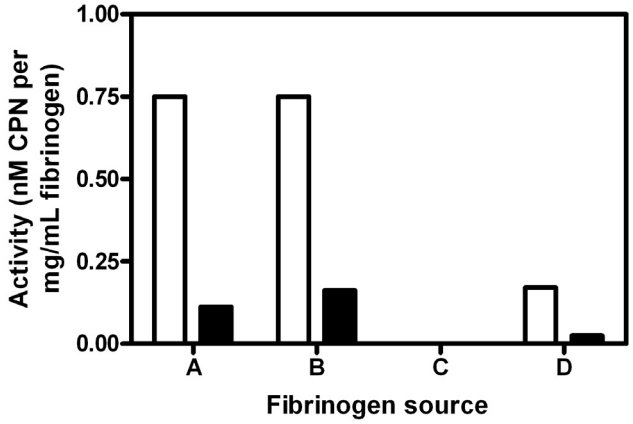


Figure 3.3: The effect of CPN-immunodepletion on fibrinogen contaminant activity. White bars depict activity prior to CPN-immunodepletion, and black bars following CPN-immunodepletion.

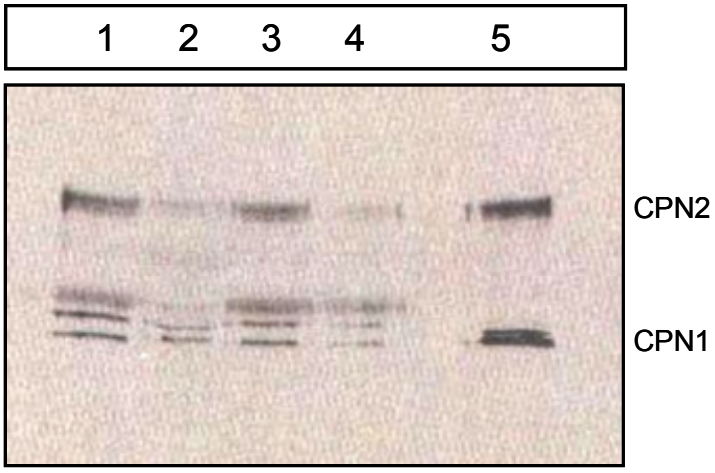


Figure 3.4: AntiCPN immunoblot of the eluates from CPN-immunodepletion of contaminated fibrinogen. Lane 1: fibrinogen from source A; lane 2: fibrinogen from source B; lane 3: fibrinogen from source C; lane 4: fibrinogen from source D; and lane 5: purified CPN.

3.1.5. Effects of the fibrinogen contaminant in clot lysis assays

Basic carboxypeptidases are antifibrinolytic. They remove carboxy-terminal lysine residues (and therefore plasminogen and tPA binding sites) from fibrin degradation products, thereby eliminating the plasminogen activation rate enhancement mediated by plasmic digestion. Fibrinogen stocks contaminated with varyious concentrations of CPN may display attenuated plasminogen activation in *in vitro* clot lysis assays, which could confound the results of these assays. The presence of active CPN could therefore account for the day-to-day variability in (DD)E quality that was observed initially. To determine the effect of the fibrinogen contamination, fibrinogen from source B was used as a representative sample for *in vitro* clot lysis assays (which monitor the clotting of fibrinogen and the lysing of the resultant fibrin over time).

The lower the tPA concentration in the clot lysis assay, the greater the effect of the contaminating CPN. In the absence of α_2-antiplasmin, during short duration lysis assays with tPA concentrations in the range of 400-800 pM, the contaminating CPN had little effect on lysis time. However, when the tPA concentration was lowered to 50 pM, such as during typical long-term lysis assays, the prolongation in lysis time caused by the contaminating CPN became significant. The contaminated fibrinogen demonstrated a nearly two-fold increase in lysis time compared to the CPN-depleted fibrinogen. Unexpectedly, in the presence of α_2-antiplasmin, this effect is not observed. The results of the experiments performed in the absence of α_2-antiplasmin are illustrated in Figure 3.5.

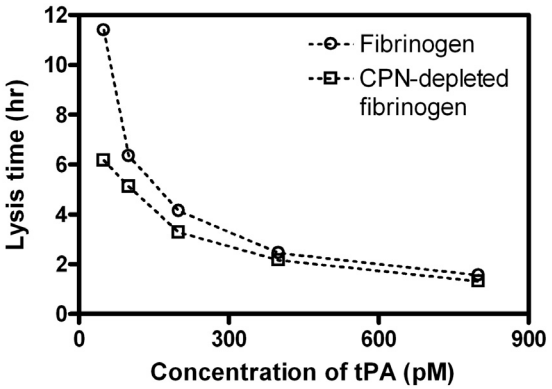


Figure 3.5: The effect the contaminating carboxypeptidase on clot lysis. Fibrinogen was CPN-depleted and compared to autologous parent fibrinogen in a clot lysis assay. The lysis time is defined as the time at which clot turbidity equals one-half of full-scale.

3.2. *The release of carboxy-terminal lysine residues from degraded fibrin by basic carboxypeptidases*

3.2.1. Preamble

Many assays have been developed to measure CPN activity. Early CPN assays were functional physiological assays; for example, CPN was identified by its ability to reduce the contractions of isolated muscle from rat uterus or guinea pig ileum stimulated with purified bradykinin or C3a (*192-194;218*). Subsequently, simpler methods were developed, particularly spectrophotometric assays, which measure the change in absorbance resulting from CPN cleavage of synthetic substrates (*205;206;209-211;213;406*). More complex assays were later developed based on the high-performance liquid chromatography separation of synthetic substrates from their cleavage products (*407-411*). Unfortunately, these assays rely on the activity of CPN toward synthetic chromogenic substrates, not natural CPN substrates.

Recently, a coupled enzymatic assay was developed to quantitate carboxypeptidase activity toward substrates with carboxy-terminal arginine residues (*412*). After the release of a carboxy-terminal arginine residue, the enzyme arginine kinase, in the presence of ATP, produces *N*-phospho-L-arginine and ADP. This ADP is then phosphorylated by pyruvate kinase in the presence of phosphoenolpyruvate. Finally, the pyruvate by-product in this reaction is reduced to lactate by lactate dehydrogenase and the concomitant oxidation of NADH can be measured at 340 nm in an absorbance spectrophotometer (Figure 3.6). When all enzymes and intermediary substrates are present in sufficient excess of CPN and its substrates, the overall reaction rate is dependent upon the release of arginine by CPN. This assay is convenient because it is quick, sensitive, and can measure CPN activity toward any substrate with a carboxy-terminal arginine residue. No such assay exists for measurement of carboxypeptidase activity toward substrates with carboxy-terminal lysine residues. Therefore, to examine the hydrolysis of substrates with carboxy-terminal lysine residues, such as (DD)E, a coupled assay needed to be developed.

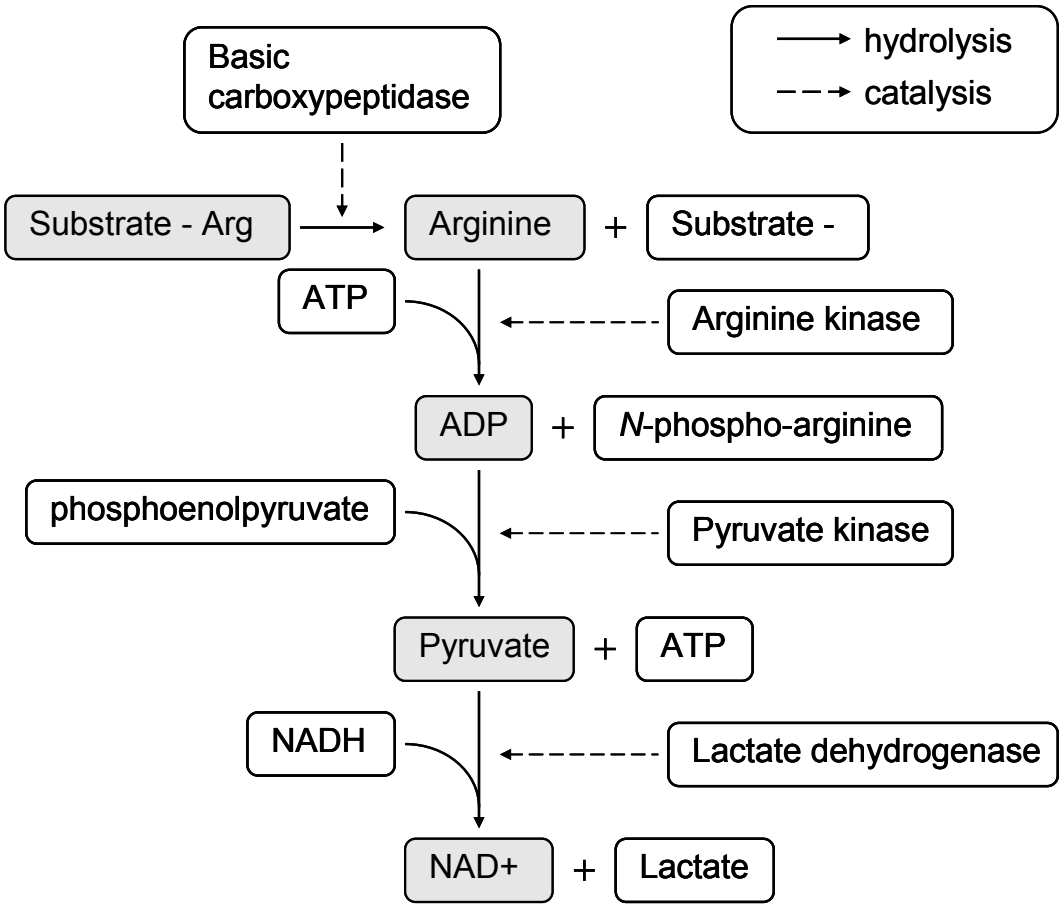


Figure 3.6: The arginine kinase assay. The NAD^+ generated by a series of enzymatic reactions can be measured as a function of time by absorbance spectrophotometer at 340 nm.

3.2.2. Development of the lysine detection assay

A novel coupled enzyme assay was created to measure the release of lysine by basic carboxypeptidases from substrates with carboxy-terminal lysine residues (Figure 3.7). The free lysines produced by carboxypeptidase activity are oxidized by lysine oxidase to produce 6-amino-2-oxohexanoate and ammonia with hydrogen peroxide as a by-product. Subsequently, amplex red is used as an electron donor in the reduction of hydrogen peroxide by horseradish peroxidase (HRP). The resulting oxidized product, resorufin, is a fluorescent compound which can be easily quantified. Conditions were determined empirically to ensure that the overall reaction rate is only limited by, and therefore directly sensitive to, carboxypeptidase activity.

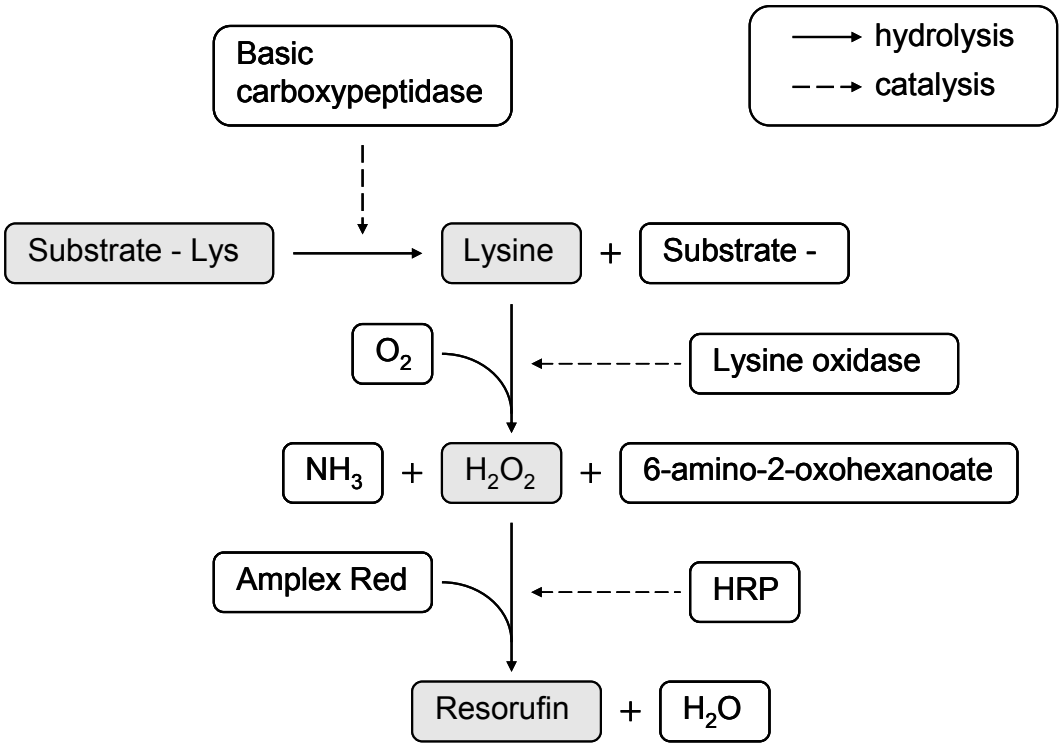


Figure 3.7: The lysine detection assay. The resorufin generated by a series of enzymatic reactions can be measured as a function of time by an absorbance spectrophotometer at 571 nm or fluorescence spectrophotometer with an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

Standard experiments are made to a final volume of 128 µL in a black microwell plate, by addition of equal volumes of assay components (final concentrations): 50 µM amplex red; 1 U/mL HRP with 0.1 U/mL lysine oxidase; substrate with carboxy-terminal lysine; and the desired carboxypeptidase. All components are diluted in HBSmT. Development of resorufin is monitored as a function of time at 37°C on a Spectramax Gemini plate reader by exciting at 560 nm and detecting at 590 nm (with a 570 nm cut-off). The excitation and emission spectra of resorufin are shown in Figure 3.8. One mole of resorufin is produced per mole of lysine released, and a lysine standard curve can be used to relate the measured absorbance to the concentration of lysine released. The lysine standard curve is linear up to approximately 3 µM, under the conditions described (Figure 3.9).

The coupling enzyme lysine oxidase is fairly specific for lysine residues. At very high concentrations, it will oxidize arginine; however under the conditions specified in the assay procedure, such concentrations of arginine should not be reached. When a lysine standard curve is supplemented with arginine, the curve remains nearly identical (Figure

3.10.). Due to the possibility of hydrolysis of alternate amino acids, or due to the presence of lysine or lysine analogues in the substrate or enzyme solutions, background fluorescence measurements are required. The substrate (DD)E demonstrates some endogenous fluorescence which was subtracted from the calculations following each experiment (Figure 3.11). Typical experiments (with the exception of kinetic measurements) used a maximum of 0.2 μM (DD)E in the lysine detection assay. The background fluorescence at this concentration of (DD)E is approximately 150 relative fluorescence units, while the quantitative release of lysine from this concentration of (DD)E represents approximately 11 000 relative fluorescence units. Although the background contributes to less than 1.5% of the signal, this background fluorescence was subtracted from the calculations following each experiment.

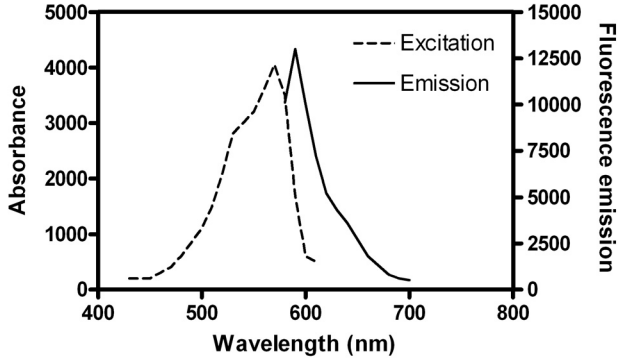


Figure 3.8: The excitation and emission spectra of resorufin. Resorufin has an excitation maximum of 563 nm and an emission maximum of 587 nm.

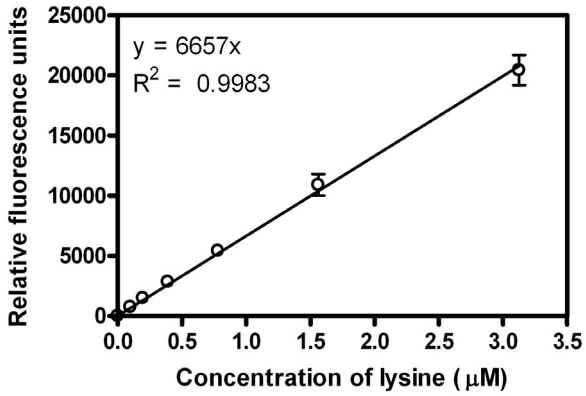


Figure 3.9: Lysine standard curve. The standard curve used for determining the relationship between relative fluorescence units and the concentration of free lysine (n = 6).

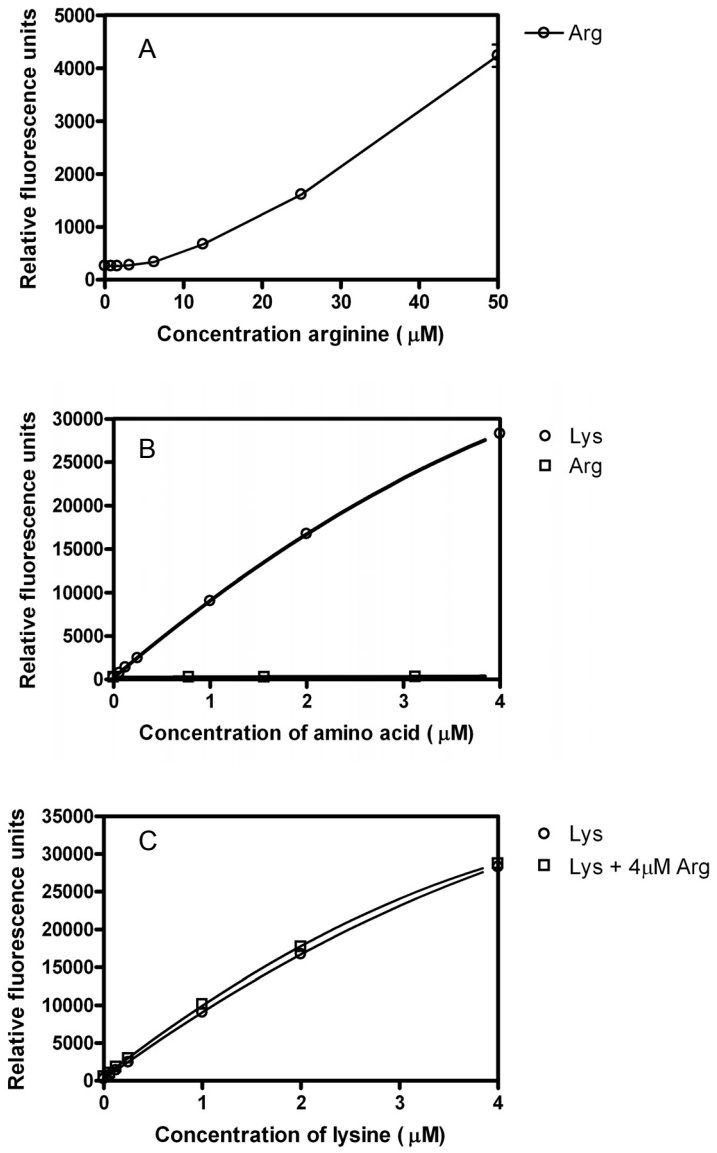


Figure 3.10: Arginine detection in the lysine detection assay. Panel A: an arginine standard curve. Note that arginine is only hydrolyzed in significant amounts at very high concentrations which should not be achieved during the reaction. Panel B: a comparison of lysine and arginine standard curves over the same range of concentrations. Panel C: a lysine standard curve in the presence and absence of 4 μM arginine.

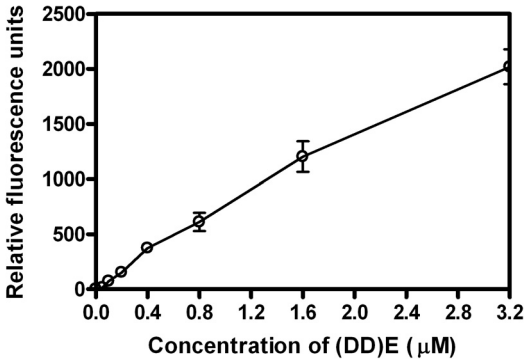


Figure 3.11: Background fluorescence of the carboxypeptidase substrate (DD)E. A standard curve of up to 3.2 μM (DD)E demonstrates some background fluorescence at the higher concentrations of (DD)E.

Due to the transient presence of H_2O_2 as a by-product during the lysine detection assay, the possibility of oxidation of the carboxypeptidase (which could reduce the apparent activity) was examined. All enzymes (CPN is demonstrated as a representative example) were unaffected by the presence of up to 10 μM H_2O_2 (6.25-fold higher than the possible concentration of lysine in a typical experiment), as measured by FA-Ala-Lys chromogenic assay (Figure 3.12, panel A). Only concentrations over 500 mM H_2O_2 produced a significant reduction in carboxypeptidase activity. The oxidation products NH_3 and 6-amino-2-oxohexanoate were also examined for potential inhibition of the carboxypeptidases. Concentrations as high as 40 μM of NH_3 and 6-amino-2-oxohexanoate (25-fold higher than the possible concentration of lysine in a typical experiment) had no effect on carboxypeptidase activity (Figure 3.12, panel C). All enzymes were unaffected by the presence of 10 μM lysine (6.25-fold higher than the possible concentration of lysine in a typical experiment) (Figure 3.12, panel B). Curiously, in the presence of high concentrations of lysine and lysine oxidase (which would produce some concentration of NH_3 and 6-amino-2-oxohexanoate), the carboxypeptidase activity was noticeably inhibited. Although none of these components individually, or the hydrogen peroxide produced during the reaction inhibit basic carboxypeptidase activity, a combination of these components does show small but definite attenuation of the rate of FA-Ala-Lys cleavage. Note that these reactions were performed in the absence of the reducing agent amplex red and the enzyme HRP, which determine the specificity of the oxidation-reduction reaction, and therefore the carboxypeptidase (or the substrate FA-Ala-Lys) may be an unfortunate bystander in the reduction of hydrogen peroxide.

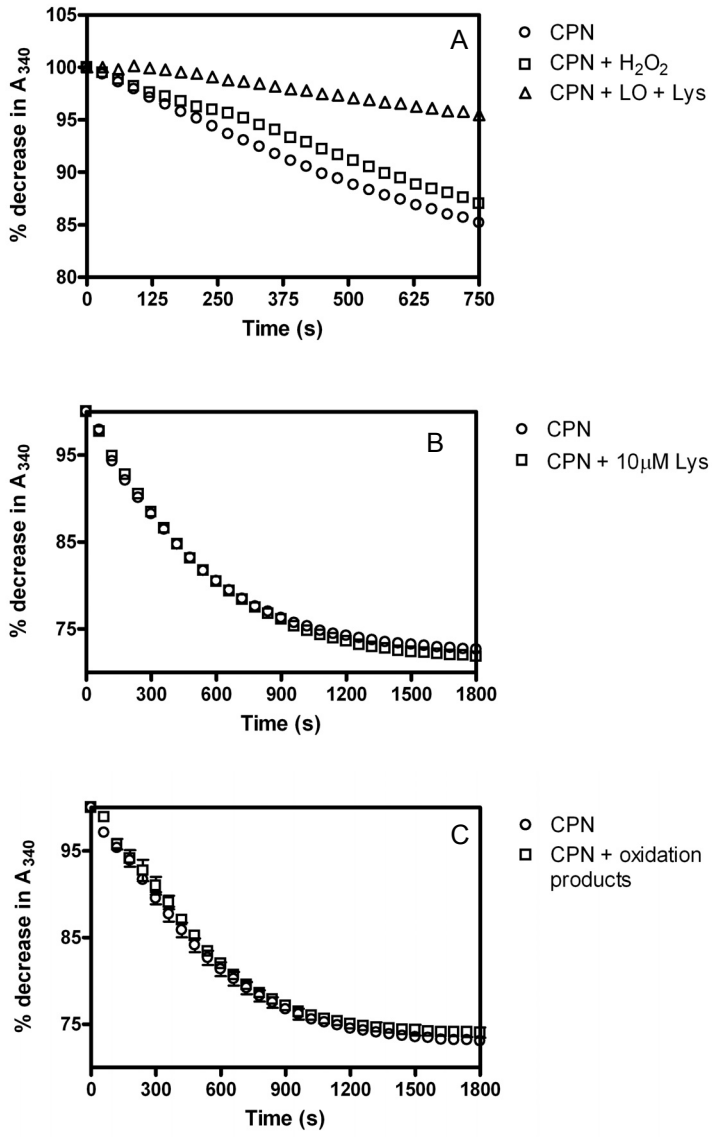


Figure 3.12: Effect of assay components on apparent carboxypeptidase activity. Carboxypeptidase activity is unaffected by the presence of H_2O_2, lysine, ammonia or 6-amino-2-oxohexanoate, but is decreased in the presence of lysine oxidase plus lysine. Panel A: addition of 500 mM H_2O_2 is required to noticeably decrease CPN activity. However, addition of 200 μM lysine plus 0.1 U/mL lysine oxidase significantly diminishes CPN activity. Panel B: 10 μM lysine does not affect the activity of CPN. Panel C: 40 μM of NH_3 and 6-amino-2-oxohexanoate do not affect the activity of CPN.

Fluorescent intensity of the resorufin signal does decrease with time, due to further oxidation of resorufin to the non-fluorescent compound resazurin (see discussion section 4.2.). The loss of signal is intensified by addition of HRP, but this can be abolished by addition of ascorbic acid, an antioxidant, which demonstrates that the nature of the reaction is indeed an oxidation-reduction process (Figure 3.13). To prevent confounding results all measurements made using the lysine detection assay should be end-point measurements, or alternatively all data for kinetic measurements should be obtained within the first few minutes of the reaction, and the concentration of amplex red should be in excess of the concentration of substrate.

The reproducibility of the assay is good, assuming careful attention is paid to ensure that all conditions are kept constant (the assay is temperature-, and buffer-dependent). The intra-assay variability for lysine detection is 4.73%; the inter-assay variability is 8.36%. For determination of carboxypeptidase activity, the intra-assay variability is 8.69% and the inter-assay variability is 12.75% (Figure 3.14)

The chromogenic FA-Ala-Lys assay (section 2.3.1.) is a relatively standard carboxypeptidase assay; however, extrapolating carboxypeptidase activity toward FA-Ala-Lys to other substrates is not always relevant or reasonable. This standard assay was compared to the lysine detection assay (also using the substrate FA-Ala-Lys) as a measure of accuracy. The lysine detection assay is much more sensitive than the chromogenic assay, therefore lysine samples for the chromogenic assay were prepared from 0.01-0.1 μM, and then an aliquot of each was diluted 1:10 (v/v) in HBSmT prior to detection in the lysine detection assay. Similarly, CPN samples for the chromogenic assay were prepared from 0.5-6 nM, and then an aliquot of each was diluted 1:5 (v/v) in HBSmT for the lysine detection assay. As shown in Figure 3.15, the two assays produced very similar results.

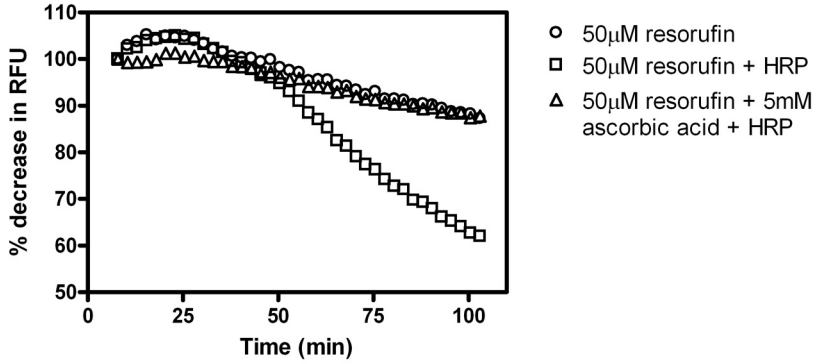


Figure 3.13: Loss of fluorescent signal intensity over time. Resorufin is slowly oxidized to the non-fluorescent compound resazurin. Therefore all reactions should include a concentration of amplex red in excess of the concentration of substrate. RFU represents relative fluorescence units.

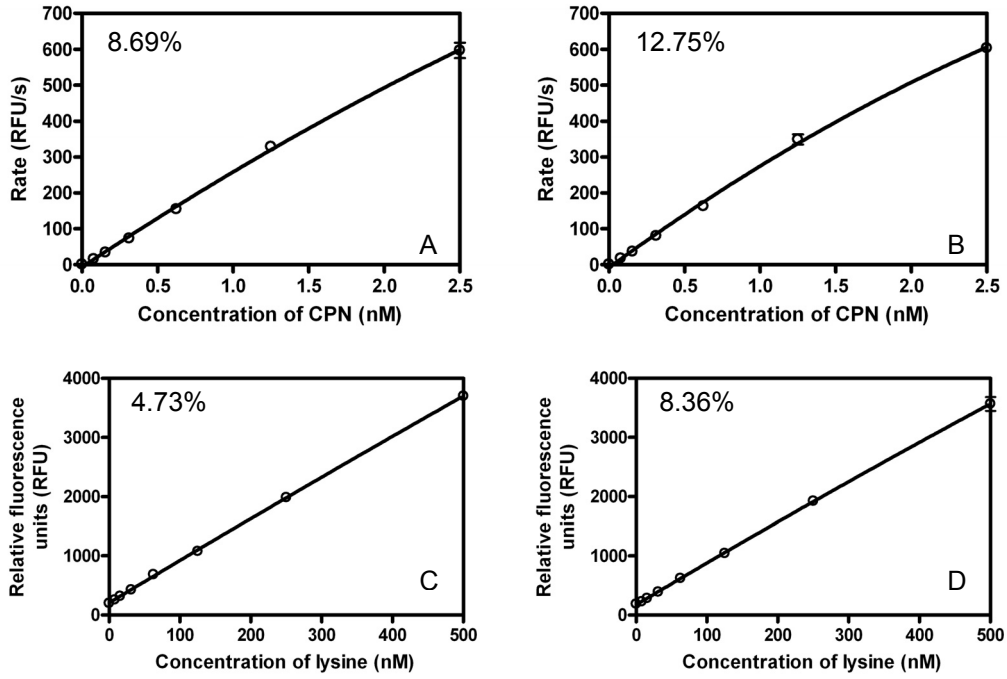


Figure 3.14: The inter- and intra-assay variability of the lysine detection assay. Panel A: intra-assay variation for carboxypeptidase detection; panel B: inter-assay variation for carboxypeptidase detection; panel C: intra-assay variation for lysine detection; and panel D: intra-assay variation for lysine detection. (n = 6 for each determination). Variation was determined by the standard deviation of all experimental determinations compared to the calculated values.

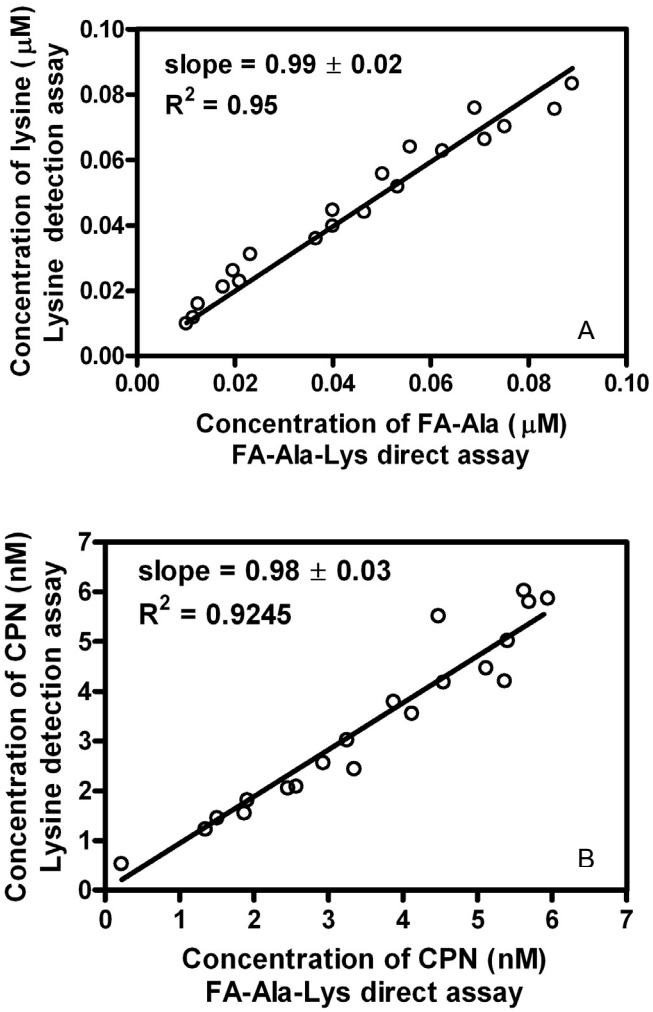


Figure 3.15: Comparison of lysine (or FA-Ala-Lys) and CPN detection between the chromogenic FA-Ala-Lys assay and the lysine detection assay. Panel A: a comparison for lysine (or FA-Ala-Lys) detection; and panel B: a comparison for CPN detection.

3.2.3. Determination of the kinetics of lysine release from (DD)E

Following the discovery that CPN contaminates preparations of fibrinogen and that this contamination can have a significant effect on *in vitro* clot lysis assays, the previously accepted lack of antifibrinolytic effect by CPN needs to be revisited. Antifibrinolysis mediated by basic carboxypeptidases is caused by removal of carboxy-terminal lysine residues from fibrin cofactor and its degradation products; therefore the rate of lysine release from fibrin degradation products is a measure of an enzyme's antifibrinolytic activity. With the novel lysine detection assay, the activity of a basic carboxypeptidase toward any substrate with a carboxy-terminal lysine residue, including (DD)E, can be measured.

The (DD)E purification procedure (as described in section 2.1.10.) was modified from existing procedures in order to obtain the highest-quality substrate possible. Fibrinogen purchased from commercial sources is contaminated with CPN (see section 3.1.), α_2-antiplasmin and TAFI. Therefore fibrinogen was depleted of these proteins by passage over polyclonal antiCPN-, antiTAFI- and antiα_2-antiplasmin-Sepharose columns. Fibrinogen was then clotted with thrombin in the presence of plasminogen and tPA and allowed to lyse until the visible clot had vanished. Following inhibition of the enzymes, the solution of degraded fibrin was separated by gel filtration to obtain only those products of the correct 250 kDa molecular mass. Fractions were then analyzed by SDS-PAGE with Coomassie blue staining (Figure 3.16 and Figure 3.17).

Although (DD)E is considered to be the terminal fibrin degradation product, plasmin continues to degrade (DD)E into (DD)E_2 and (DD)E_3. These products are composed of the same DD and E domains, but with small additional cleavages on several of the chains. The ultimate plasmin-cleaved product, (DD)E_3, does not promote plasminogen activation to the same degree as (DD)E_1 and (DD)E_2 and therefore should be avoided in pure (DD)E preparations. The difference in molecular mass between these three forms of (DD)E is not large enough to differentiate by gel filtration chromatography or SDS-PAGE; however, (DD)E_3 lacks A- and B-knobs and therefore the molecule dissociates into DD and E products. This product can be distinguished by non-reducing SDS-PAGE (Figure 3.16). Only fractions with little or no (DD)E_3 should be pooled and used for the remaining purification steps. Examples of good and poor (DD)E preparations are shown in Figure 3.17.

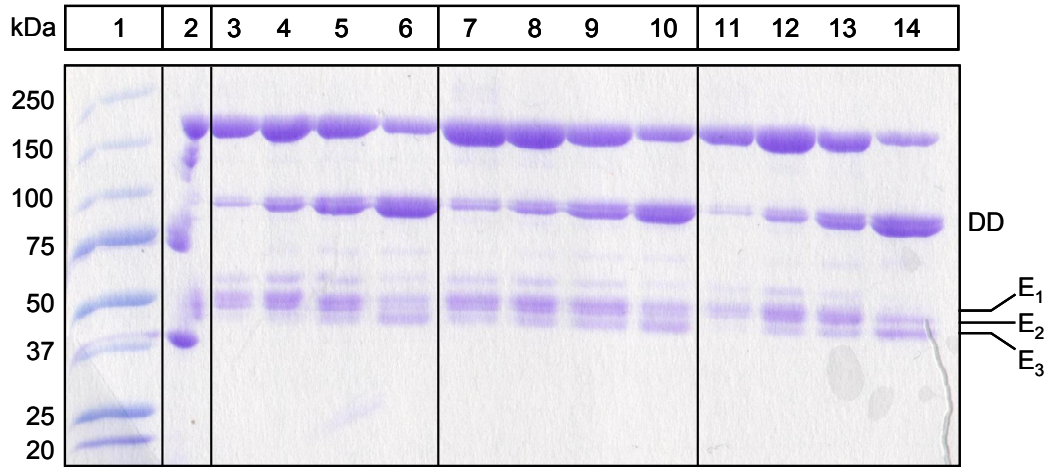


Figure 3.16: SDS-PAGE gel of (DD)E purification on Sephacryl S-200 chromatography column. Lane 1: molecular mass marker (250, 150, 100, 75, 50, 37, 25, 20 kDa); lane 2: a non-specific fraction (not used); lane 3 – lane 6: progressive fractions from one (DD)E preparation; lane 7 – lane 10: progressive fractions from a second (DD)E preparation; lane 11 – lane 14: progressive fractions from a third (DD)E preparation. Note the increasing intensity of the band at 100 kDa. This, in addition to the increasing intensity of the lowest band of the triplet at 50 kDa, indicates the presence of (DD)E_3.

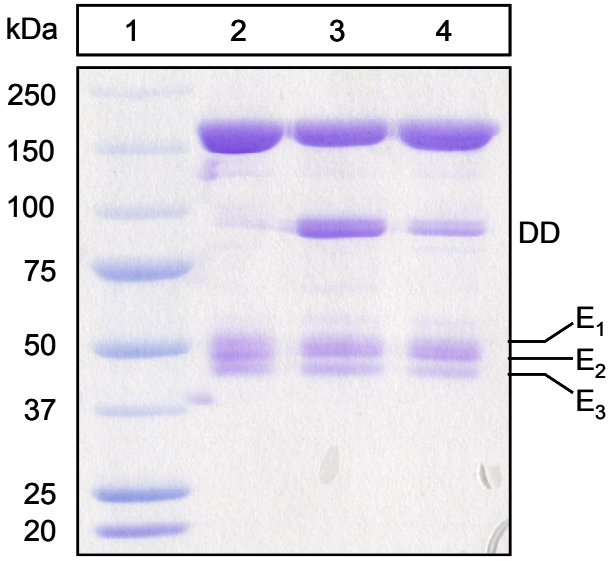


Figure 3.17: SDS-PAGE gel of purified (DD)E preparations. Lane 1: molecular mass marker (250, 150, 100, 75, 50, 37, 25, 20 kDa); lane 2: an excellent (DD)E preparation, with limited (DD)E_3; lane 3: a poor (DD)E preparation, with too much (DD)E_3; lane 4: a poor (DD)E preparation, with too much (DD)E_3.

The rate of lysine release from (DD)E by CPN, CPNc, CPB and TAFIa was determined to obtain a relative measure of their antifibrinolytic capabilities. As shown in Figure 3.18 and Table 3.4, CPN is equally active toward (DD)E as are CPB and TAFIa – both of which are generally recognized as antifibrinolytic enzymes. Furthermore, plasmin-cleaved CPNc has a catalytic efficiency approximately three-fold greater than that of the other enzymes (the number of active sites was normalized based upon an equivalent starting concentration of CPN used to create CPNc and used as untreated CPN). Note that only a single active site was considered when calculating the catalytic efficiency of CPN (see section 3.6.2.).

For comparison purposes, the differential activity of CPN and CPNc toward (DD)E and two small chromogenic substrates is compared in Table 3.5. The increased activity of CPNc mediated by plasmic cleavage appears to only affect activity toward larger, physiological substrates. All enzymes were measured under identical conditions at 37°C to simulate their physiological milieu. The spontaneous decay of TAFIa should not be a problem, as all measurements were obtained within 30 seconds, at which point standing TAFIa displays 96-98% of its initial activity; furthermore, TAFIa is stabilized by substrate, therefore zero decay can be assumed. TAFI activation was verified by SDS-PAGE, and the percent activation was used to determine the actual TAFIa concentration for kinetic determinations, since quantitative activation was not achieved (Figure 3.19).

Table 3.4: Kinetic constants for CPN, CPNc, CPB and TAFIa with substrate (DD)E.

	CPN	***CPNc***	***CPB***	***TAFIa***
K_M (µM)	1.6 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	0.72 ± 0.03
k_{cat} (s^{-1})	0.150 ± 0.006	0.43 ± 0.02	0.121 ± 0.005	0.069 ± 0.001
k_{cat}/ K_M ($\mu M^{-1}s^{-1}$)	0.094 ± 0.009	0.29 ± 0.03	0.10 ± 0.01	0.096 ± 0.005

* n = 20 for CPN, CPB and TAFIa; n = 7 for CPNc

Table 3.5: Kinetic constants for CPN versus CPNc.

	FA-Ala-Lys		***FA-Ala-Arg***		***(DD)E***	
	CPN	CPNc	CPN	CPNc	CPN	CPNc
K_M (µM)	250 ± 10	300 ± 50	340 ± 22	340 ± 27	1.6 ± 0.1	1.5 ± 0.1
k_{cat} (s^{-1})	44.4 ± 0.6	57 ± 3	38.2 ± 0.8	39 ± 1	0.150 ± 0.006	0.43 ± 0.02
k_{cat}/ K_M ($\mu M^{-1}s^{-1}$)	0.18 ± 0.01	0.19 ± 0.04	0.112 ± 0.009	0.11 ± 0.01	0.094 ± 0.009	0.29 ± 0.03

* n = 15 for CPN – FA-Ala-Lys; n = 12 for CPNc – FA-Ala-Lys; n = 9 for FA-Ala-Arg; n = 20 for CPN – (DD)E; n = 7 for CPNc – (DD)E

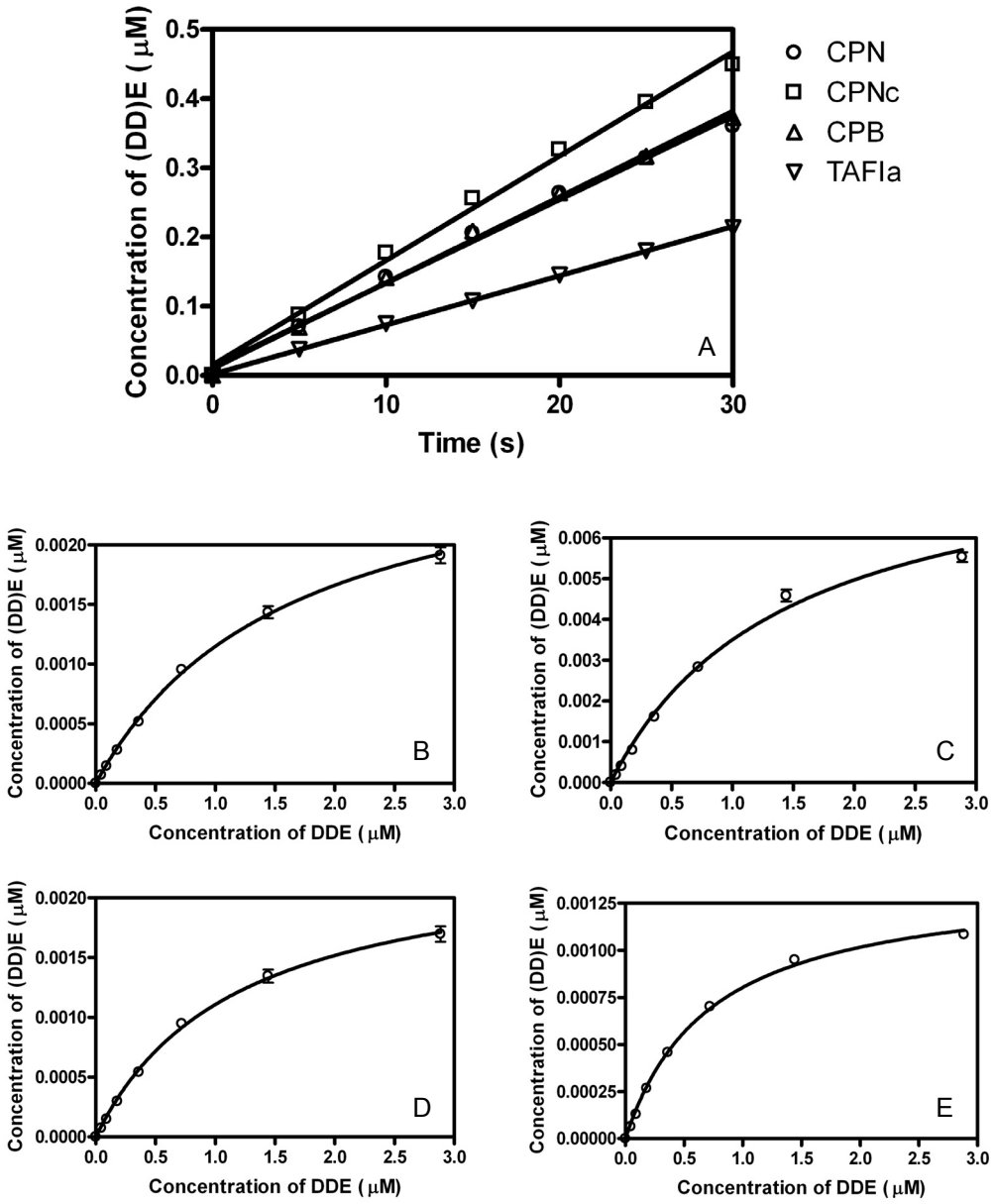


Figure 3.18: Determination of the kinetic constants for CPN, CPNc, CPB, and TAFIa for the substrate (DD)E. Panel A: the initial reaction rates for CPN, CPNc, CPB, TAFIa toward 722 nM (DD)E; panel B: Michaelis-Menten plot for CPN; panel C: Michaelis-Menten plot for CPNc; panel D: Michaelis-Menten plot for CPB; panel E: Michaelis-Menten plot for TAFIa.

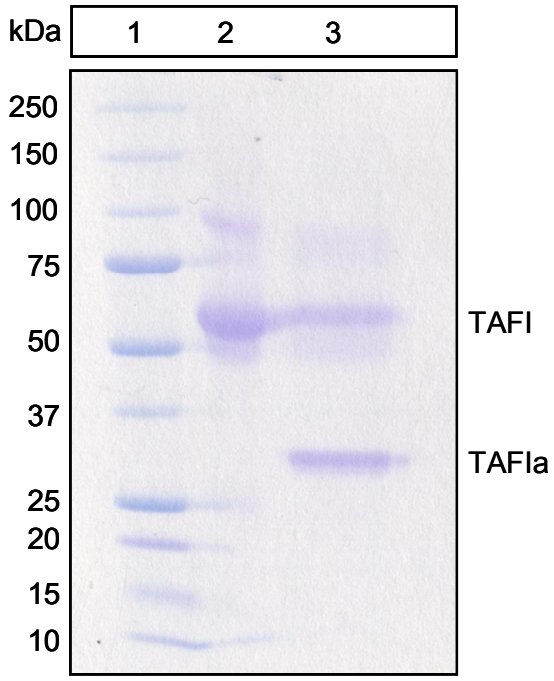


Figure 3.19: SDS-PAGE gel of TAFI activation. Lane 1: molecular mass marker (250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa); lane 2: TAFI; lane 3: TAFIa

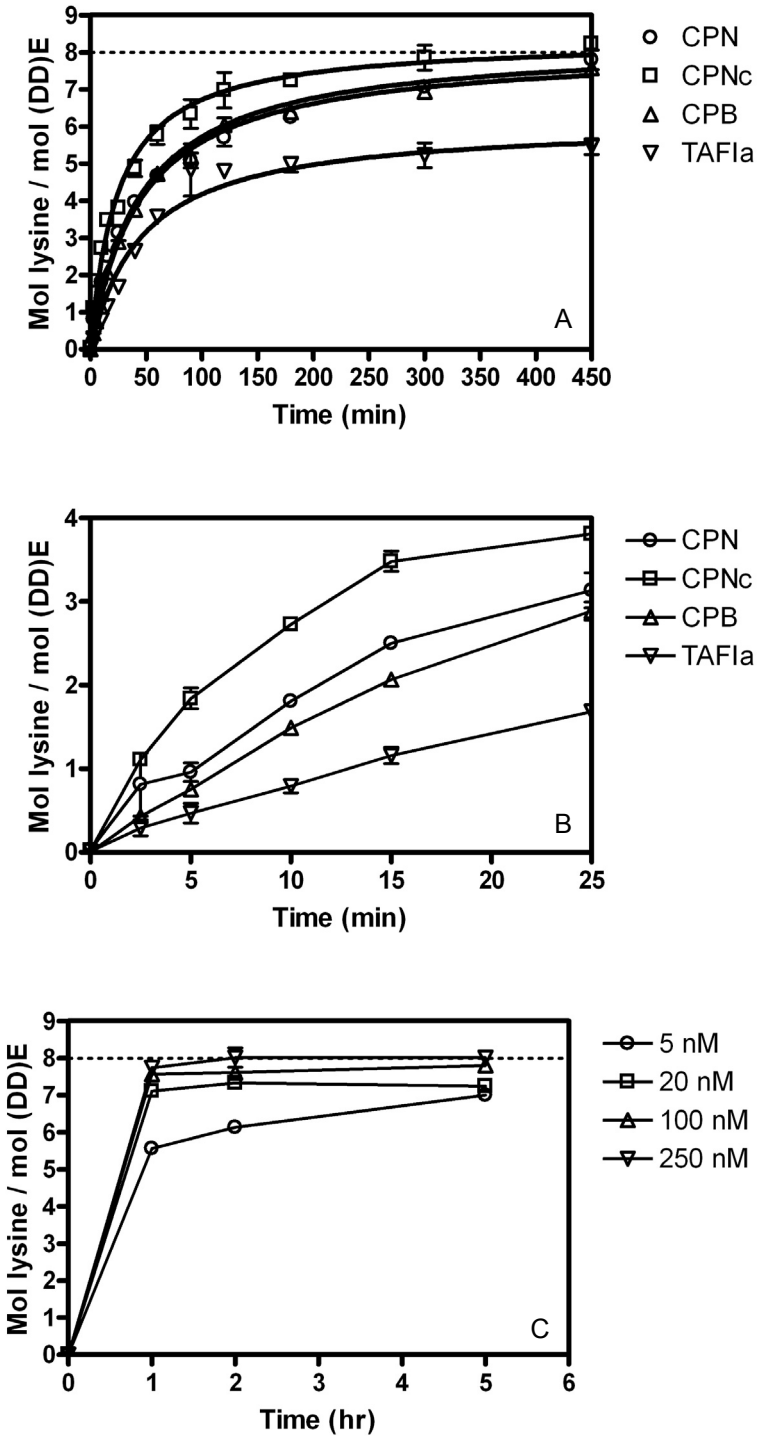


Figure 3.20: Determination of the maximum amount of lysine released by basic carboxypeptidases per (DD)E molecule. Panel A: room temperature incubation for 7.5 hours with 5 nM carboxypeptidase; panel B: the initial rates of lysine release from (DD)E differ with carboxypeptidase identity; panel C: (DD)E was incubated at 37°C with high concentrations of CPB to determine the absolute maximum lysine released from (DD)E. (n = 4)

3.2.4. Determination of the molar ratio of carboxy-terminal lysine:(DD)E

Despite the general assumption that CPN is not an antifibrinolytic enzyme, the kinetics demonstrated in section 3.2.3. clearly demonstrate the ability of CPN to remove carboxy-terminal lysine residues from degraded fibrin products. There are eight lysine residues on (DD)E that could potentially be removed by a basic carboxypeptidase (two each of α^{78}, β^{133}, γ^{62}, and αC^{206}) (*5;70-72*). To determine how many lysines are released per molecule of (DD)E, and if the same ratio of lysines:(DD)E is released by each of the basic carboxypeptidases CPN, CPNc, CPB and TAFIa, (DD)E was incubated for extended periods at room temperature with these enzymes.

The enzymes CPN, CPNc and CPB released a maximum of 8 lysine residues per molecule of (DD)E (Figure 3.20). TAFIa released fewer lysines, but due to the intrinsic instability of TAFIa, the enzyme had likely decayed before completing (DD)E hydrolysis. TAFIa appeared to release approximately 5 moles of lysine per mole of (DD)E before its activity dissipated. When (DD)E was digested for up to 5 hours with 250 nM CPB (50-fold greater concentration than in the previous experiments) at 37°C, no more than 8 lysine residues were released per molecule of (DD)E. Therefore it appears that CPN, CPNc and CPB all release a lysine residue from each of two α^{78}, β^{133}, γ^{62}, and αC^{206} chains of (DD)E (as indicated in Figure 1.2).

3.3. The relationship between cofactor activity and lysine release from fibrin degradation products

3.3.1. Preamble

TAFIa, as its full name implies, is an antifibrinolytic enzyme. CPB is considered to be an antifibrinolytic enzyme *in vitro*, although its absence from the bloodstream under normal physiological conditions rules out a role for this enzyme during fibrinolysis in most situations. CPN, despite its similar activity and substrate specificity to TAFIa, is not considered to be antifibrinolytic. Clots formed from TAFI-depleted plasma supplemented with TAFIa or CPB show decreased fibrinolysis over the low nanomolar range, while CPN does not affect fibrinolysis when supplemented with up to twice its plasma concentration (*166*). Similar results are found with whole blood clot lysis experiments (*90*). Furthermore, partially plasmin-digested whole blood clots treated with CPN released fewer than half of the specific plasminogen binding sites in the same amount of

time that TAFIa nearly abolished plasminogen binding (*90*). Yet, CPN clearly has the capacity to hydrolyze carboxy-terminal lysine residues from fibrin degradation products, and it does so to the same degree and with the same catalytic efficiency as CPB and TAFIa. Furthermore, plasmin-cleaved CPNc has a catalytic efficiency three-fold higher than the other enzymes. The discrepancy between the activity of CPN toward (DD)E and its lack of an effect on fibrinolysis could potentially be due to the relationship between the presence of carboxy-terminal plasminogen binding sites, and effective plasminogen activation. Perhaps not all lysine residues contribute equally to plasminogen activation, and though TAFIa removes fewer residues, these could be more relevant to plasminogen activation than those removed by CPN. Therefore this relationship was examined.

3.3.2. Determination of the relationship between lysine released from (DD)E and its cofactor activity

Plasminogen activation by tPA was examined in the presence and absence of (DD)E cofactor, and in the presence of CPB-treated (DD)E which does not retain any carboxy-terminal lysine residues (this is referred to as desLys-(DD)E). The rate of plasminogen activation, in the absence of α_2-antiplasmin, was determined as a function of plasmin activity toward the substrate S-2251, which upon plasmic cleavage increases in absorbance at 405 nm. DesLys-(DD)E promoted plasminogen activation to only 66% of the degree of untreated (DD)E (achieved half-maximal plasminogen activation 185 min faster than (DD)E-free control, compared to 545 min faster for native (DD)E; Figure 3.21). The absence of a complete attenuation of cofactor-mediated plasminogen activation following carboxypeptidase treatment (and a resulting rate of plasminogen activation similar to that in the absence of cofactor) may be due to plasminogen binding to internal lysine residues of the (DD)E molecule.

(DD)E was digested with CPN, CPNc, CPB or TAFIa for up to 10 hours, and the rate of plasminogen activation, mediated by the carboxypeptidase-treated (DD)E, was determined as above. In these experiments, the full scope of carboxypeptidase activity was examined, due to the presence of α_2-antiplasmin during plasminogen activation. As shown in Figure 3.22, the reduction in (DD)E cofactor activity caused by CPN-cleavage demonstrates a similar affect on plasminogen activation as with CPB-cleavage. CPNc also has comparable results. Over the first hour of incubation, TAFIa demonstrates parallel results, but due to its spontaneous inactivation over the 10-hour room temperature

incubation, TAFIa does not affect plasminogen activation to the same degree as CPN, CPNc and CPB.

To examine the direct relationship between lysine removal from (DD)E and loss of cofactor activity, a five-hour timecourse experiment was undertaken, with an aliquot from each timepoint measured for cofactor activity, and an identical aliquot measured for the concentration of lysine released. It appears that the first seven lysine residues removed from (DD)E play a role in plasminogen activation, with equivalent effects upon their removal (Figure 3.23). The role of the final lysine residue cannot be determined because the data points do not extend this far; however, it can be extrapolated that it also plays a similar role due to the linearity of the reaction (Figure 3.24). The identity of each lysine residue removed is unknown; therefore no conclusions as to the order of lysine removal can be made.

The aggregate results from the above experiment were plotted with the lysine released versus the percentage of delay in plasminogen activation to directly compare the effect of lysine released on plasminogen activation (Figure 3.24). The linear relationship between lysine released and plasminogen activation indicate that each carboxy-terminal lysine residue of (DD)E is equally responsible for the enhancement of plasminogen activation. Furthermore, CPN displays a relationship identical to that of CPB and TAFIa, which rules out the possibility that CPN releases alternate lysine residues which are not responsible for plasminogen activation rate enhancement.

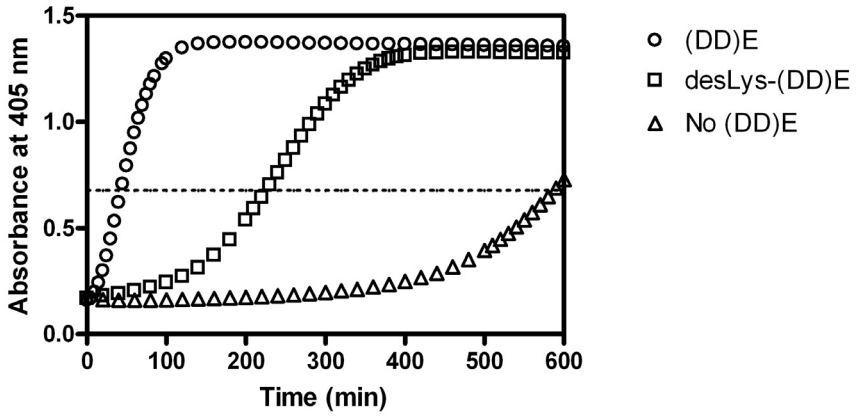


Figure 3.21: The effect of complete carboxypeptidase digestion on the cofactor activity of (DD)E. The time to half-maximal plasminogen activation is indicated by the dashed line.

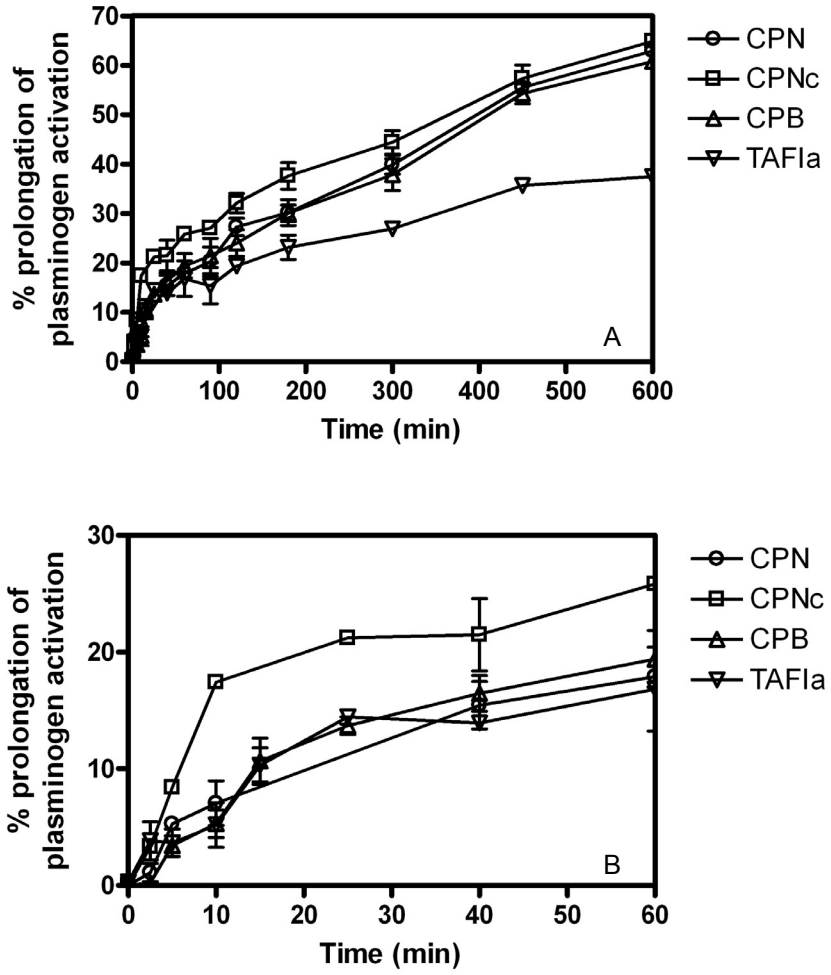


Figure 3.22: Reduction in (DD)E cofactor activity due to cleavage by basic carboxypeptidases. Panel A: the full 10-hour timecourse digestion of (DD)E by CPN, CPNc, CPB and TAFIa; panel B: an expanded view of the first hour.

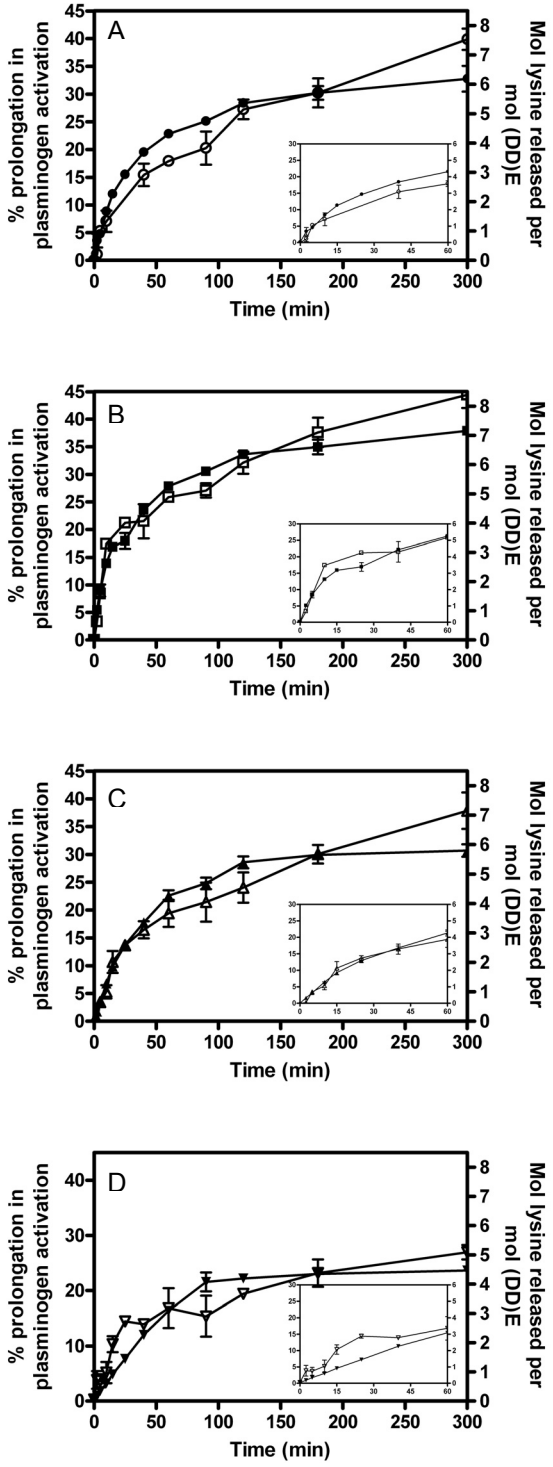


Figure 3.23: Examination of the relationship between lysine removal from (DD)E and loss of cofactor activity. Panel A: CPN-treatment of (DD)E; panel B: CPNc-treatment of (DD)E; panel C: CPB-treatment of (DD)E; panel D: TAFIa-treatment of (DD)E. For all plots, delay in plasminogen activation is represented by open symbols, and lysine concentration is represented by closed symbols.

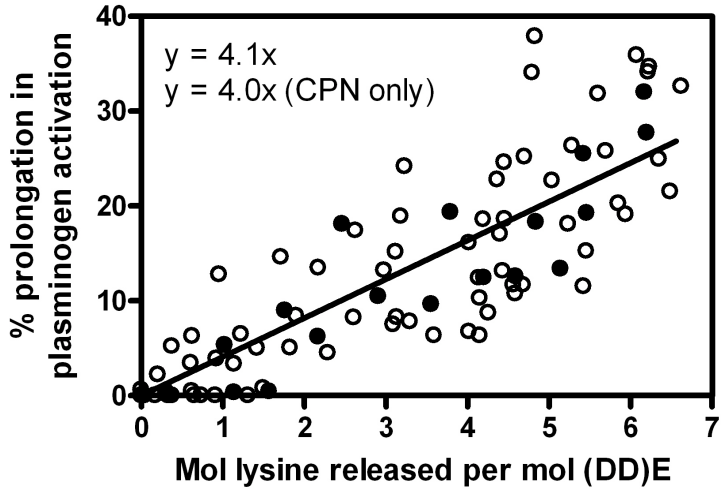


Figure 3.24: The linear relationship between lysine removal from (DD)E and loss of cofactor activity. Aggregate data from CPN, CPNc, CPB and TAFIa are represented. Data from CPN is represented by shaded circles and falls along a nearly identical best fit line to that of the data from the other enzymes (open circles).

3.4. Genetic engineering, expression and purification of recombinant CPN2

3.4.1. Preamble

To gain a better understanding of the activity, interactions and structure of CPN, a closer examination of the individual subunits of CPN is required. Separation of the CPN multimer by chemical methods into its components may have effects on its structure and activity. Additionally, isolation of the subunits after their separation can be tricky due to their tendency to interact hydrophobically, leading to a major loss of protein during purification. This also results in alteration of subunit activity. Therefore, recombinant subunits expressed in mammalian cell culture and secreted into the medium are a good alternative and provide simpler isolation procedures.

3.4.2. Cloning of rCPN2

cpn2 was amplified by PCR from pcDNA3.1+ CPN2 (courtesy of G.R. Lambkin) and was ligated into pEGFP-C1 between 5' *Eco*RI and 3' *Bam*HI restriction sites. This vector (pEGFP-C1 CPN2) was used for other purposes, following which, *cpn2* was amplified by PCR and ligated into pcDNA5/TO in between 5' *Eco*RI and 3' *Not*I restriction sites (Figure 3.25). The plasmid was multiplied via *E. coli* DH5α, isolated, and used to transfect T-REx 293 mammalian cells by the lipofectamine method. Transformed cells were selected by hygromycin resistance and cultures derived from single isolated cells were generated.

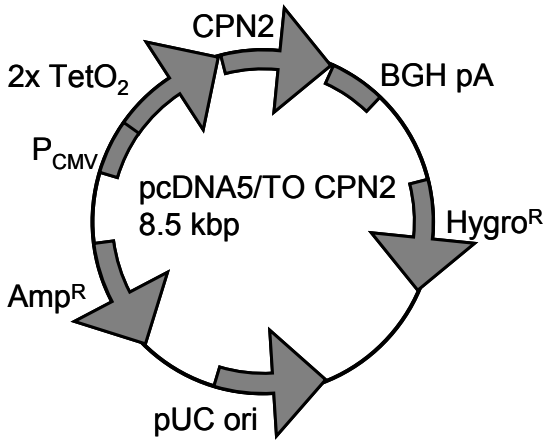


Figure 3.25: Vector map of pcDNA5/TO CPN2. *cpn2* is inserted in the MCS between *Eco*RI and *Not*I sites under the control of the P_{CMV} immediate-early promoter and the Tet operon.

3.4.3. Purification of rCPN2

rCPN2 was overexpressed in T-REx 293 cells in serum-free media. Cells were plated to 80% confluency in T-75 flasks, washed extensively with PBS and expression was induced with OptiMEM supplemented with 100 U penicillin, 100 µg streptomycin, 2 mM L-glutamine, 5 µg/mL blasticidin, 100 µg/mL hygromycin, 1 µg/mL doxycycline and ITS+ (6.7 µg/L sodium selenite / 110 mg/L sodium pyruvate / 10 mg/L insulin / 5.5 mg/L transferrin). Conditioned media was collected and cells were re-induced every second day for up to 8 days followed by recovery in DMEM. Despite the various media supplements, cells were unable to survive and secrete sufficient quantities of rCPN2 when grown for longer than 8 days in serum-free media.

Three hundred millilitres of conditioned media was centrifuged to remove precipitate, and diluted 1:3 with HB with 0.001% Tween20. This solution was loaded onto Q-Sepharose, and rCPN2 was eluted in HB with 0.5 M NaCl. rCPN2-containing fractions were determined by A_{280} and dot blot using Sheep antiCPN (Figure 3.26). rCPN2-containing fractions were pooled, diluted 1:3.33 in HB and loaded onto an antiCPN-Sepharose column. rCPN2 was eluted with 0.2 M glycine pH 2.5 directly into 1 M Tris pH 8.0 to neutralize the acid.

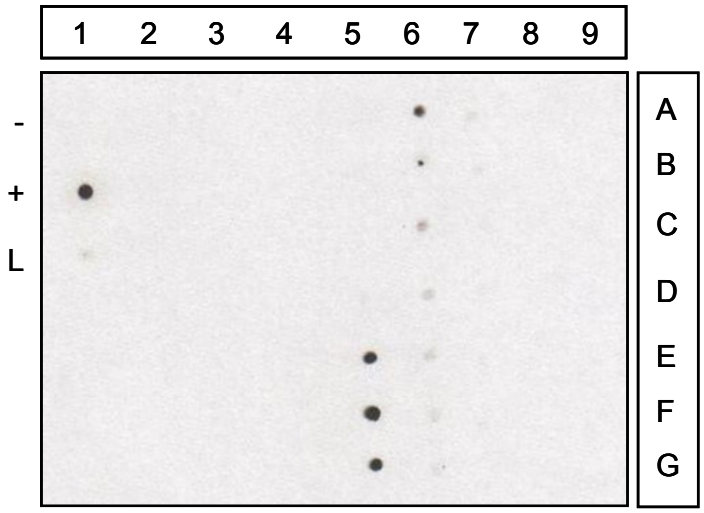


Figure 3.26: Dot blot of rCPN2 purification. This is a representative example of a dot blot of rCPN2 during purification. 1-2 µL of each fraction is dotted onto a nitrocellulose filter, and blotted as a typical immunoblot using polyclonal antiCPN. Column 1 contains the controls: negative control (buffer only), positive control, and load sample (top to bottom). Each subsequent dot represents a fraction (wash or elution), in order from top to bottom (A to G) , left to right (columns 2-9). Fractions containing rCPN2 are identified (5E - 7F) and pooled for the subsequent purification step.

rCPN2-containing fractions were pooled, diluted 1:3 with HB plus 0.001% Tween20 and loaded onto Q-Sepharose column. rCPN2 was eluted in HB containing 0.5 M NaCl. rCPN2-containing fractions were pooled and concentrated by Amicon spin concentrator with a 30 000 Da molecular mass cut-off, followed by buffer exchange with HB to decrease the concentration of NaCl to 50 mM.

Protein concentration was verified by A_{280} using a using a $\varepsilon_{1\%}$ of 14.8 and a molecular mass of 83 000 Da, and by antiCPN ELISA. Purity was determined by SDS-PAGE followed by Coomassie blue staining and immunoblotting (Figures 3.27 and 3.28).

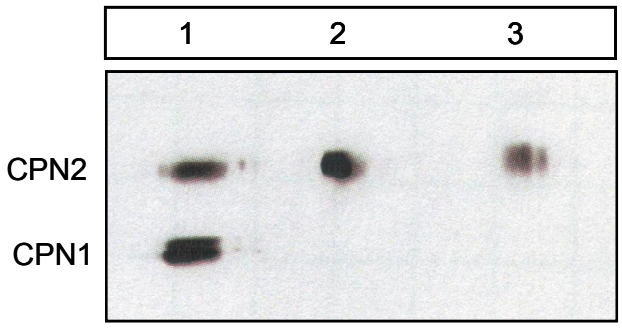


Figure 3.27: AntiCPN immunoblot of purified rCPN2. Lane 1: plasma purified CPN (reduced); lane 2: purified rCPN2 (reduced); lane 3: purified rCPN2 (non-reduced).

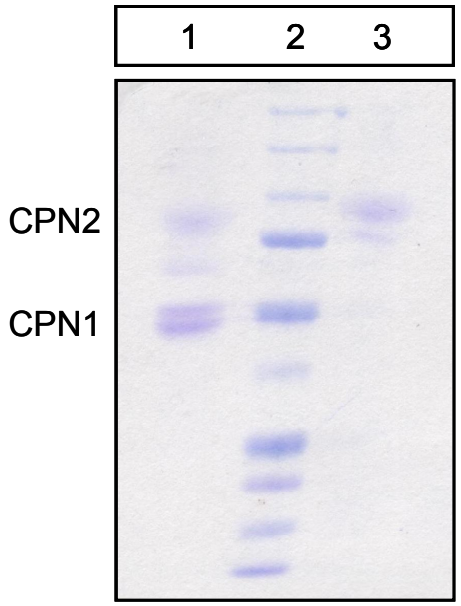


Figure 3.28: SDS-PAGE gel of purified rCPN2. Lane 1: plasma purified CPN; lane 2: molecular mass marker (250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa); lane 3: purified rCPN2.

3.5. Regulation of CPN by excess CPN2

3.5.1. Preamble

There is a curious discrepancy between the ability of CPN to remove carboxy-terminal lysine residues from (DD)E, causing an antifibrinolytic effect during *in vitro* experiments using purified components, and its apparent lack of an antifibrinolytic effect *in vivo* and during experiments using plasma as a source of CPN. The initial suspicion is that there is a specific CPN inhibitor or activity modulator present in plasma which inhibits the activity of CPN toward (DD)E or other fibrin degradation products. Due to several theories (in the Bajzar Lab) about the identity of this inhibitor, plasma immunodepletion experiments were carried out, followed by more definitive experiments using recombinant proteins.

3.5.2. Inhibition of CPN by plasma

A stationary-phase plasminogen activation assay was performed including incubations of (DD)E with buffer only, with 5 nM CPN and with 5% normal human plasma (NHP; which contains endogenous 5 nM CPN, final concentration). Five nanomolar purified CPN caused a significant prolongation in the rate of plasminogen activation, whereas NHP did not. Therefore it appears that purified CPN is antifibrinolytic, yet CPN in NHP is not. To determine if there is an inhibitor present in plasma that affects the ability of CPN to interact with or proteolyze (DD)E, NHP was added to purified CPN and the effect on CPN activity was examined. Titration of NHP (0.039-20% of final volume) into exogenous 5 nM purified CPN decreased the antifibrinolytic activity of CPN, and addition of greater than 2.5% NHP eliminated the activity of the exogenous CPN altogether (Figure 3.29).

In contrast, activity measurements of NHP with the substrate FA-Ala-Lys, demonstrate that the CPN endogenous to NHP is indeed present, and is active toward this small chromogenic substrate (Figure 3.30).

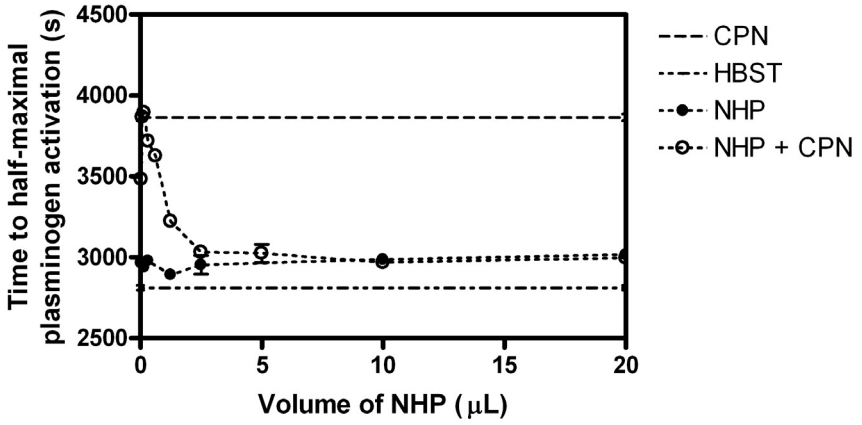


Figure 3.29: Inhibition of CPN antifibrinolytic activity by plasma. The rate of plasminogen activation with cofactor (DD)E was measured in the presence of buffer alone or 5 nM CPN controls (shown as dashed horizontal lines), a dilution series of NHP (closed symbols) and a dilution series of NHP in the presence of 5 nM CPN (open symbols). The ability of CPN to delay plasminogen activation is decreased by the addition of NHP.

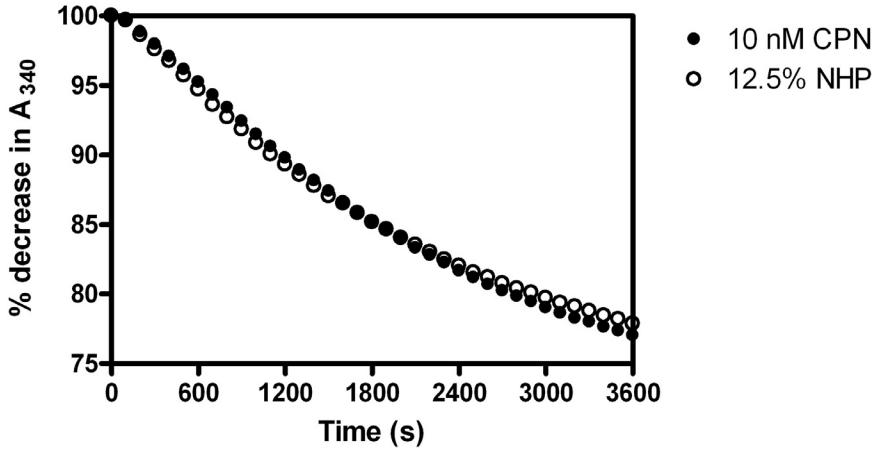


Figure 3.30: The activity of NHP toward FA-Ala-Lys. The decrease in absorbance at 340 nm associated with hydrolysis of the carboxy-terminal lysine of FA-Ala-Lys is measured with the addition of purified CPN or NHP.

3.5.3. CPN-depleted plasma does not inhibit the antifibrinolytic activity of CPN

The effect of NHP on exogenous CPN is confounded due to the presence of endogenous CPN in NHP; therefore to determine the inhibition of NHP on exogenous CPN only, NHP was depleted of CPN by passage over an antiCPN-Sepharose column. A stationary-phase plasminogen activation assay was then performed with NHP or CPN-depleted NHP (NHPdCPN) titrated into exogenous 5 nM purified CPN. NHPdCPN had little effect on the antifibrinolytic activity of exogenous CPN (Figure 3.31). Compared to untreated NHP, which quickly inhibited the activity of CPN toward (DD)E, passage over antiCPN modified the inhibitory activity of NHP, likely through elimination the CPN inhibitor.

3.5.4. Inhibition of CPN by rCPN2

The Bajzar Lab has previously demonstrated an excess of CPN2 circulating in plasma, separate from CPN activity (unpublished work; section 4.4). These subunits may be responsible for the inhibition of CPN by NHP (and their co-depletion by antiCPN-Sepharose would explain the lack of inhibition of CPN by NHPdCPN). Therefore, for more definitive proof of the role of CPN2 in modifying the substrate specificity of CPN, the inhibitory activity of the purified rCPN2 toward CPN was examined.

rCPN2 was titrated into 5 nM purified CPN in a stationary-phase plasminogen activation assay. Recombinant CPN2 inhibited the activity of CPN in a concentration-dependent manner (Figure 3.32). Addition of 20 nM rCPN2 eliminated the antifibrinolytic activity of 5 nM CPN. This effect can be directly attributed to inhibition of CPN by rCPN2, as rCPN2 alone had no effect on plasminogen activation.

Finally, if CPN2 is indeed the inhibitor of CPN present in NHP, then rCPN2 should have little effect on the activity of CPN against the small chromogenic substrate FA-Ala-Lys, as NHP is fully active in the chromogenic assay. Indeed, this is the case. Up to a 4-fold molar excess of rCPN2 was unable to exert any inhibitory effect on CPN against FA-Ala-Lys (Figure 3.33).

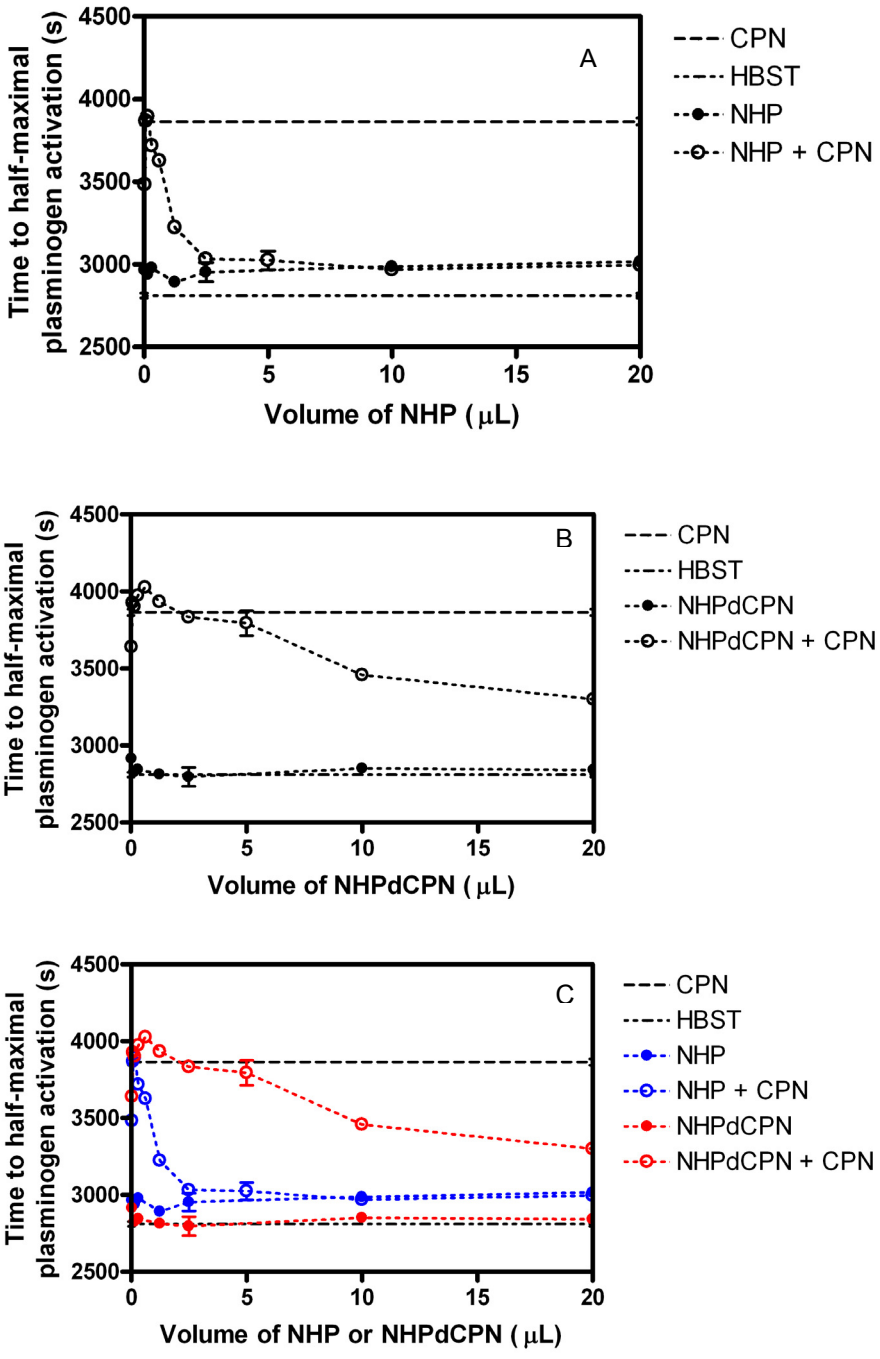


Figure 3.31: CPN-immunodepletion of NHP decreases its inhibition toward CPN antifibrinolytic activity. The rate of plasminogen activation with cofactor (DD)E was measured in the presence of buffer alone or 5 nM CPN controls (shown as dashed horizontal lines), NHP dilution series in the presence or absence of 5 nM CPN (panel A), or CPN-depleted NHP (NHPdCPN) dilution series in the presence or absence of 5 nM CPN (panel B). Panel C represents a combined plot of these data. The inhibition of CPN caused by NHPdCPN is significantly less than that caused by untreated NHP.

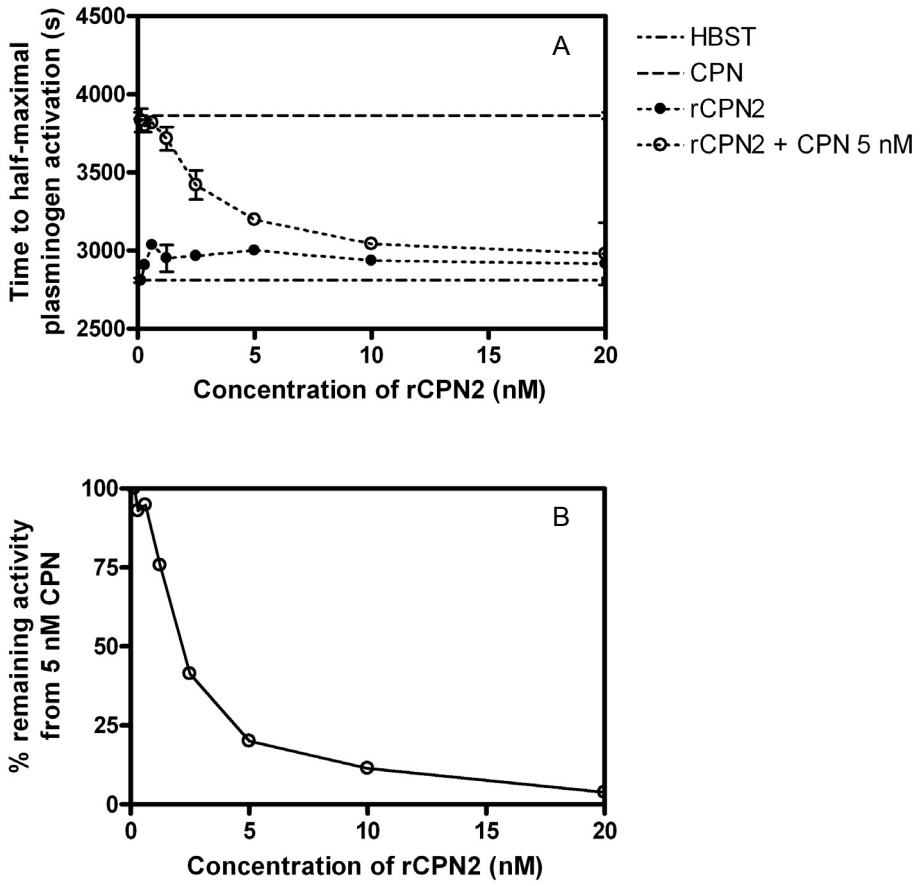


Figure 3.32: Inhibition of CPN antifibrinolytic activity by rCPN2. Panel A: the rate of plasminogen activation with cofactor (DD)E was measured in the presence of buffer alone or 5 nM CPN controls (shown as dashed horizontal lines), a dilution series of rCPN2, or a dilution series of rCPN2 in the presence of 5 nM CPN. Panel B: the decrease in CPN antifibrinolytic activity caused by rCPN2 inhibition.

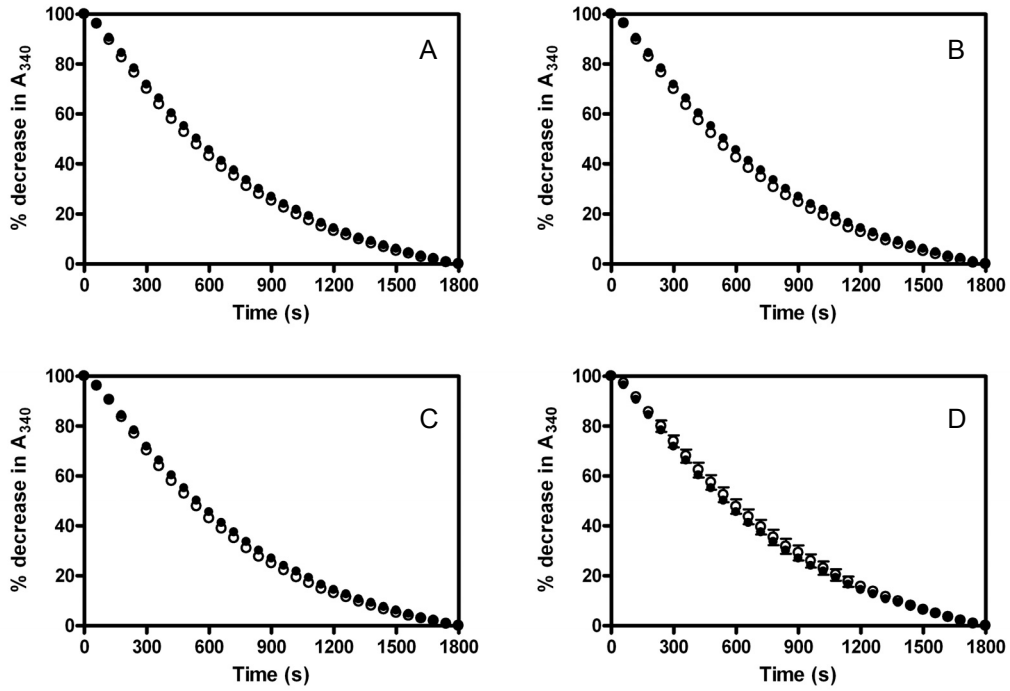


Figure 3.33: The absence of inhibition by rCPN2 on CPN activity toward FA-Ala-Lys. Twenty nanomolar CPN was added to rCPN2 and activity was measured against the small chromogenic substrate FA-Ala-Lys. Panel A: 5 nM rCPN2; panel B: 20 nM rCPN2; panel C: 40 nM rCPN2; and panel D: 80 nM rCPN2. Closed symbols represent uninhibited CPN, open symbols represent CPN + rCPN2.

3.6. The structure of CPN

3.6.1. Preamble

To understand the mechanism of CPN2 inhibition of multimeric CPN (toward certain substrates) the structure of CPN needs to be clarified. There is not yet a crystal structure of multimeric CPN, although a structure for the isolated recombinant catalytic subunit has been determined. However, this model, as well as some current information regarding structural aspects of CPN is confusing and inconsistent. One such questionable result relates to the molecular mass and stoichiometry of multimeric CPN. The generally accepted structure of CPN is of a heterotetramer, containing two each of the regulatory and catalytic subunits (*188;192;195;201-205*). The molecular mass is generally accepted to be 280 kDa with individual results ranging from 290 kDa (native PAGE) to 300-325 kDa (sedimentation ultracentrifugation) (*188;192*). Based upon these data, the best estimation of molecular stoichiometry is two regulatory subunits of 83 kDa and two catalytic subunits of 48-55 kDa (totalling 269 kDa). Unfortunately these methods of molecular mass determination are not reliable for proteins of unusual structure or those containing a significant amount of carbohydrate, and have a tendency to conclude an increased apparent molecular mass (*413*), which could lead to a false estimate of stoichiometry. CPN2 is 28% carbohydrate by mass, leading to a total of 17.3% carbohydrate for the tetramer, which is more than enough to cause errors in molecular mass determinations by these methods.

Additional evidence of a potentially false estimate of stoichiometry is the zinc content of CPN. Plummer *et al.* determined the zinc content to be 0.31 ng Zn/mg CPN, which leads to the unexpected ratio of 0.78 mol Zn/mol CPN when using the commonly accepted molecular mass for CPN (*188*). Since zinc is an essential cofactor for CPN, and if CPN is thought to have two catalytic active sites, it would be expected to have 2 mol Zn/mol CPN. For comparison, the monomeric CPB contains 0.96 mol Zn/mol CPB. Furthermore, a Hill plot (used to determine the cooperativity of substrate binding) for CPN with benzoyl-Gly-Lys has a slope of 1.02, and for benzoyl-Gly-Arg has a slope of 1.04 (*220*). This indicates a lack of cooperativity (positive or negative) between the two active sites of CPN, and is also consistent with the presence of only a single active site. In order to clarify these issues, and to determine the correct stoichiometry of CPN, the exact molecular mass needed to be determined.

3.6.2. Determination of the molecular mass of CPN

The methods for molecular mass determination previously used (gel filtration, PAGE) can be influenced unpredictably by the Stoke's radius of the protein, the carbohydrate content, and any interactions with the matrix support. Also, the less sensitive interference sedimentation ultracentrifugation was used in place of absorption sedimentation ultracentrifugation, which can result in unreliable molecular mass determination. In order to determine the correct stoichiometry of CPN, an accurate molecular mass was determined by multi-angle laser light scattering (MALLS)

MALLS is a preferred method for determining molecular mass because it is an absolute method: it provides results that are not dependent on molecular mass standards, column calibration, or molecular conformation. The intensity of light scattered by a protein solution is directly proportional to the product of its mass-average molecular mass and its concentration. The angle of scattering is a function of the average square radius of the protein. Absolute molecular mass is determined from light scattering using three values: the concentration, the specific refractive index increment (dn/dc) and the amount of scattered light at each angle.

Purified CPN was passed over a Superose-6 gel filtration column and the elution fractions were directly passed into a MALLS detector. CPN was determined to have a molecular mass of 220 kDa (Figure 3.34). This molecular mass cannot accommodate two 83 kDa regulatory subunits and two 48-55 kDa catalytic subunits; however, two regulatory subunits in complex with a single catalytic subunit yields a predicted molecular mass of 218 kDa. If the zinc content of CPN is re-evaluated, taking this new molecular mass into consideration, there is 0.97 mol Zn/mol CPN. This would be consistent with a single catalytic subunit in the multimeric CPN.

The sample of CPN from these initial analyses was recovered and cleaved with plasmin. The reaction was allowed to go to completion, to obtain activated two-chain CPNc. This solution was concentrated and the molecular mass determination by MALLS was repeated. CPNc was determined to have a molecular mass of 176 kDa (Figure 3.34). This is consistent with a trimeric CPN (of two regulatory subunits and a single catalytic subunit) which has been cleaved at the known plasmin-sensitive sites.

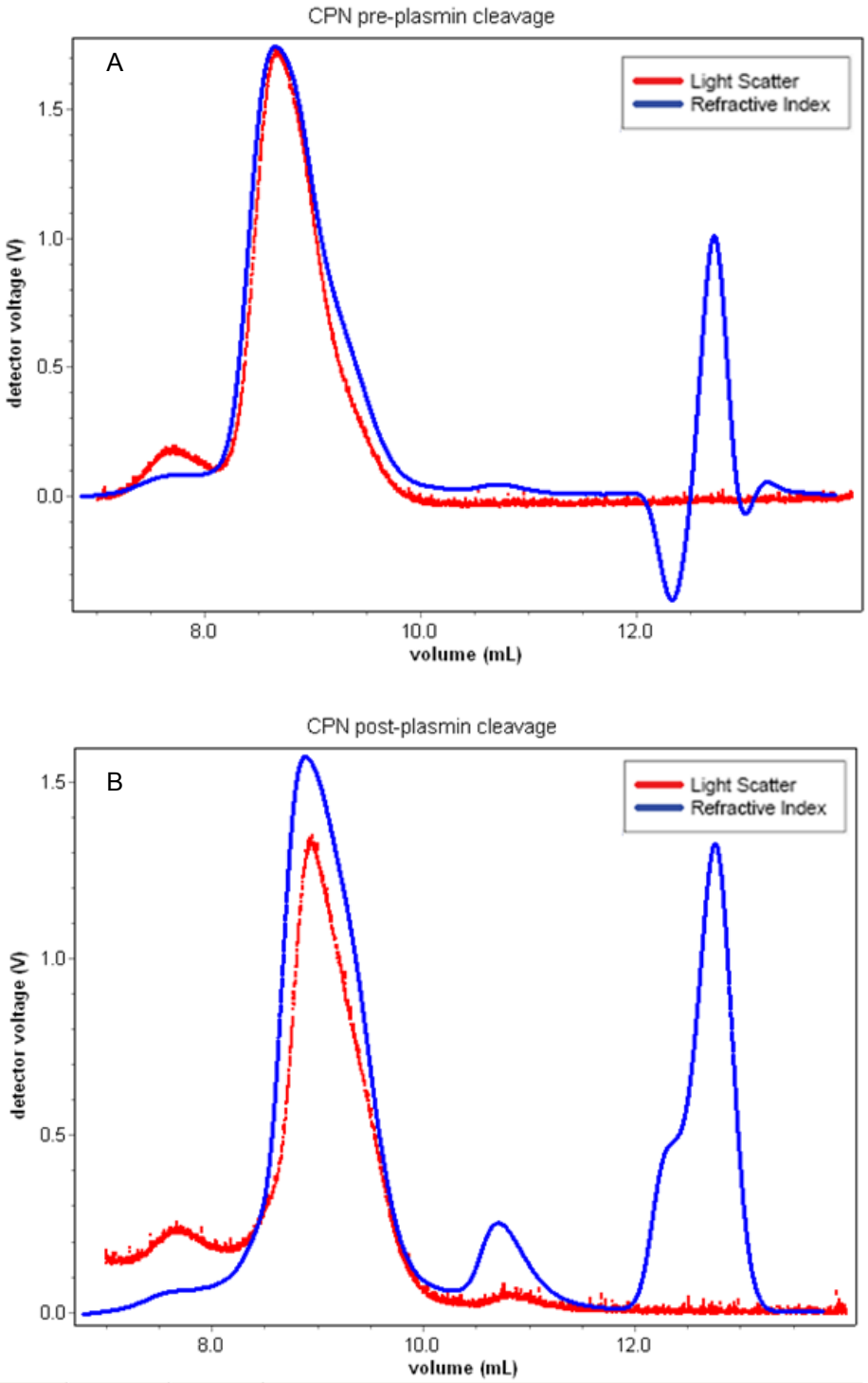


Figure 3.34: MALLS molecular mass determinations for purified CPN and CPNc. Panel A: CPN was determined to have a molecular mass of 220 kDa; panel B: CPNc was determined to have a molecular mass of 176 kDa. The red line indicates light scatter and the blue line the refractive index. The spike in refractive index beyond 12 mL is not protein-containing.

4. DISCUSSION

After re-evaluation of the activity, regulation, structure and stoichiometry of CPN, we show that CPN is a trimeric enzyme, composed of a single catalytic subunit and two regulatory subunits. Plasmic cleavage enhances the activity of CPN toward certain substrates, and can therefore be considered as a form of activation of the enzyme. This activation is not required to induce CPN activity toward all substrates; in some cases, plasmic cleavage has no effect on the rate of catalysis, while in other cases, it results in a dramatic increase in the catalytic efficiency.

CPN is also regulated by a circulating inhibitor / modulator of its activity. This negative regulation is due to an interaction with a protein that is either derived from the CPN2 regulatory subunit, or exists as this subunit in its entirety. Interaction of CPN with its inhibitor affects only certain activities of CPN. It is likely due to the presence of this inhibitor in plasma that the antifibrinolytic activity of CPN was not previously identified. CPN can now be defined as an antifibrinolytic enzyme that is positively regulated by plasmic activation, and negatively regulated by a circulating inhibitor.

4.1. CPN contaminates commercial stocks of fibrinogen

The terminal fibrin degradation product (DD)E is a soluble cofactor for plasminogen activation, and is often used *in vitro* as a fibrin surrogate for determination of the rate of cofactor-dependent plasmin generation in solution. This circumvents the issues surrounding non-classical kinetic interpretations of surface-type reactions on the fibrin gel-matrix. (DD)E cofactor activity, in the absence of an added basic carboxypeptidase, should be stable over time, and the initial cofactor activity should not vary between experiments. Preparations of (DD)E from commercial sources of fibrinogen were found to vary significantly from one experiment to another, and this cofactor activity appeared to decrease over time. This effect was related to a decrease in the number of carboxy-terminal lysine residues per molecule of (DD)E and therefore a contaminating basic carboxypeptidase was suspected. The contamination could be traced back to the original source of fibrinogen used to synthesize (DD)E.

The effects of a series of carboxypeptidase inhibitors on the contaminant activity were determined: MERGETPA, GEMSA and EDTA all inhibited the contaminating activity, while PTCI had no effect. This pattern of inhibition is consistent only with CPN (and not

CPB or TAFIa). Following the development of an antiCPN ELISA and a functional assay, the extent of CPN contamination among various commercial sources of fibrinogen was evaluated. The concentration of CPN contaminating the fibrinogen varied from 0.040 ± 0.007 to 0.21 ± 0.04 nM CPN per mg/mL by antiCPN ELISA, or from 0 to 0.75 ± 0.09 nM CPN per mg/mL by chromogenic activity assay, depending on the initial commercial source of the fibrinogen. Differing lots of fibrinogen purchased from the same source also displayed a range of contamination, from 0.47 ± 0.03 to 0.77 ± 0.01 nM CPN per mg/mL fibrinogen.

For some sources of fibrinogen, there was a discrepancy between the concentration of contaminating CPN measured by activity assay and that measured by antiCPN ELISA. In the case of fibrinogen from source C, the situation is quite simple. No activity was detected by chromogenic assay, but CPN was detected by ELISA. The activity of the contaminating CPN was probably destroyed during the purification process, but the protein remained physically associated with the fibrinogen. This hypothesis is consistent with the immunoblot of the fibrinogen from source C, which indicated the presence of both CPN1 and CPN2 subunits. On the other hand, the situation with fibrinogen from sources A and B is more difficult to explain: in these two cases, the activity measured by chromogenic assay is greater than that measured by antiCPN ELISA. It is possible that some of the CPN associated with these fibrinogen stocks is in the more active, two-chain form, which would suggest an apparent increased activity when compared to a standard curve created from purified single-chain CPN. Not all CPN in these fibrinogen samples is of the two-chain form, as demonstrated by immunoblot, but perhaps that only a small concentration of two-chain CPN (undetectable by immunoblot) may have a significant effect on the overall activity of CPN. An additional cause for the inconsistency could be the use of purified CPN as a standard curve for samples of fibrinogen. If CPN co-purifies with fibrinogen due to a physical association that masks epitopes recognized by the polyclonal antiCPN antibodies, then the apparent concentration as measured by ELISA would be lower than the true value. Furthermore, if indeed some of the contaminating CPN is of the two-chain form, this may also mask epitopes recognized by antibodies raised against single-chain CPN.

The basis for co-purification or association of CPN and fibrinogen has not yet been elucidated; however, both CPN and fibrinogen are considered to be ‘sticky’ proteins

because they have a tendency to associate with themselves or other proteins through hydrophobic interactions. The contamination of fibrinogen with CPN may simply be due to a mutual association of proteins occurring during the purification process, as other hydrophobic partners are removed from the solution. CPN, like fibrinogen, is a large molecule, and their purification procedures share similar size-based purification steps and differential precipitations in salt solutions. Moreover, contamination of fibrinogen is not unusual; α_2-antiplasmin is also known to contaminate commercial sources of fibrinogen. Then again, it is also possible that the association occurs *in vivo* and plays some yet unknown role. The interaction between CPN and fibrinogen is not likely covalent, although it is possible. CPN contamination can be reduced by passage over an antiCPN-Sepharose column, and depletion of CPN does not lead to depletion of fibrinogen protein, as measured by A_{280} pre- and post-depletion. However, it is likely that due to the small quantities of CPN associated with fibrinogen (maximally 0.77 nM CPN / 2.94 μM fibrinogen), that the loss of fibrinogen during immunodepletion would be minimal and therefore go unnoticed.

The variable degree of CPN contamination in the four sources of fibrinogen is likely due to the differing fibrinogen purification procedures used by the companies. Most companies do not provide details of their purification procedures, therefore the purification steps which tend to promote or hinder CPN association cannot be determined. Since CPN contamination may profoundly affect investigations of the functional roles of fibrinogen or its products, researchers must implement strategies for depleting the CPN, depending on their use of the fibrinogen. However, the magnitude of this issue cannot be understated as nearly every investigation of the binding interactions or cofactor activity of fibrinogen or its products would be affected by contaminating CPN, particularly where plasmin is generated. Furthermore, the effects of inhibitors of fibrinolysis could be grossly exagerated in the pressence of CPN.

The antiCPN immunoblots of the eluates from CPN-immunodepletion of fibrinogen revealed the presence of the regulatory and catalytic subunits of CPN in samples from all four fibrinogen sources. Additionally, uncharacterized cleavage products of an intermediate molecular mass (approximately 55-65 kDa) were detected. Unfortunately, an accurate estimation of the molecular mass of these fragments is not possible using a single percentage SDS-PAGE gel. These products could be due to proteolytic cleavage

that occurred *in vivo*, or during the purification process. To distinguish between these possibilities further examination is required; however, exact identification of these fragments is not required for their successful removal and rendering of fibrinogen CPN-free, therefore further studies were not continued.

Basic carboxypeptidases are antifibrinolytic because they remove plasminogen and tPA binding sites from partially degraded fibrin, and inevitably CPN contamination leads to attenuated plasminogen activation during *in vitro* clot lysis assays. The lower the concentration of tPA, the greater the effect of the contaminating CPN on fibrinolysis. When the tPA concentration was 50 pM or below, such as during typical long-term lysis assays (when concentrations of tPA are frequently as low as 0.5 pM), the CPN-contaminated fibrinogen demonstrated a nearly two-fold increase in lysis time compared to CPN-depleted fibrinogen. Presumably, the contaminating CPN has a greater effect at lower concentrations of tPA because the rate of plasminogen activation decreases with decreasing concentrations of tPA. When the rate of plasmin generation is low, CPN is able to maintain a rate of carboxy-terminal lysine removal equivalent to the rate of carboxy-terminal lysine generation by plasmin; CPN therefore removes plasmin binding sites and slows plasmin action in addition to slowing plasminogen activation. In contrast, when the rate of plasmin generation is high, plasmin degrades fibrin more quickly and CPN is overwhelmed and cannot attenuate plasminogen activation to the same degree.

Unexpectedly, in the presence of α_2-antiplasmin, the antifibrinolytic effect of CPN was not observed. It would be expected that the contaminating CPN would have a greater effect in the presence of α_2-antiplasmin: as CPN removes plasmin binding sites, plasmin would be released into solution and easily inhibited by α_2-antiplasmin, halting all fibrin degradation until more plasmin was generated. The reason for the absence of an effect could be related to the relative concentrations of the components in the clot lysis assay: α_2-antiplasmin causes a large delay in the rate of clot lysis, and any small additional delay caused by the contaminating CPN may be so minute that its effect is negligible at the concentrations of tPA examined. If tPA concentrations below the typical range are used, an antifibrinolytic effect by the contaminating CPN may be seen in the presence of α_2-antiplasmin.

The effect of CPN contamination in fibrinogen cannot be adequately predicted or accommodated in any single experiment. Baseline experiments can give an indication of the degree of antifibrinolytic activity of the contaminant in a fibrinogen stock, but since the concentration of contaminant varies by commercial source and by lot, the concentration would have to be determined for each lot of fibrinogen purchased. Additionally, as the concentration of tPA is decreased, the effect of the contaminant becomes more evident, and as the concentration of inhibitors (such as α_2-antiplasmin), or other carboxypeptidases (such as TAFIa) are altered, the effect of the contaminant will also change. Therefore the clot lysis assay conditions cannot be changed without repeating lengthy and expensive control experiments; ultimately the best solution to deal with CPN contamination is to deplete fibrinogen prior to use, as done in the Bajzar Lab.

4.2. Development of the lysine detection assay

Most physiological carboxypeptidase substrates do not exhibit a measurable spectral change upon release of the carboxy-terminal residue, and extrapolating catalytic rates for complex substrates based on the rates for non-physiological chromogenic substrates is questionable. Therefore cleavage is often assessed indirectly, by monitoring a change in function associated with the proteolysis. However, some multi-subunit substrates have multiple carboxy-terminal basic residues, and cleavage of a subset of these residues may alter the enzyme cleavage rates. Moreover, the functional determination itself may interfere with enzymatic activity. To better evaluate the hydrolysis of physiological carboxypeptidase substrates with carboxy-terminal lysine residues, a coupled fluorogenic assay was developed and optimized to quantitatively follow lysine release.

This lysine detection assay has two steps. First, the free lysine residues produced by carboxypeptidase activity are oxidized by lysine oxidase to produce 6-amino-2-oxohexanoate and ammonia, with hydrogen peroxide as a by-product. Subsequently, amplex red is used as an electron donor in the reduction of hydrogen peroxide by HRP. The resulting oxidized product, resorufin, is a fluorescent compound which can be easily quantified (see Figure 4.1). Conditions were determined empirically to ensure that the overall reaction rate is limited by carboxypeptidase activity. This real-time assay is quick, reliable, quantitative, and easy; and it can be used with any substrate containing a carboxy-terminal lysine residue, including fibrin degradation products.

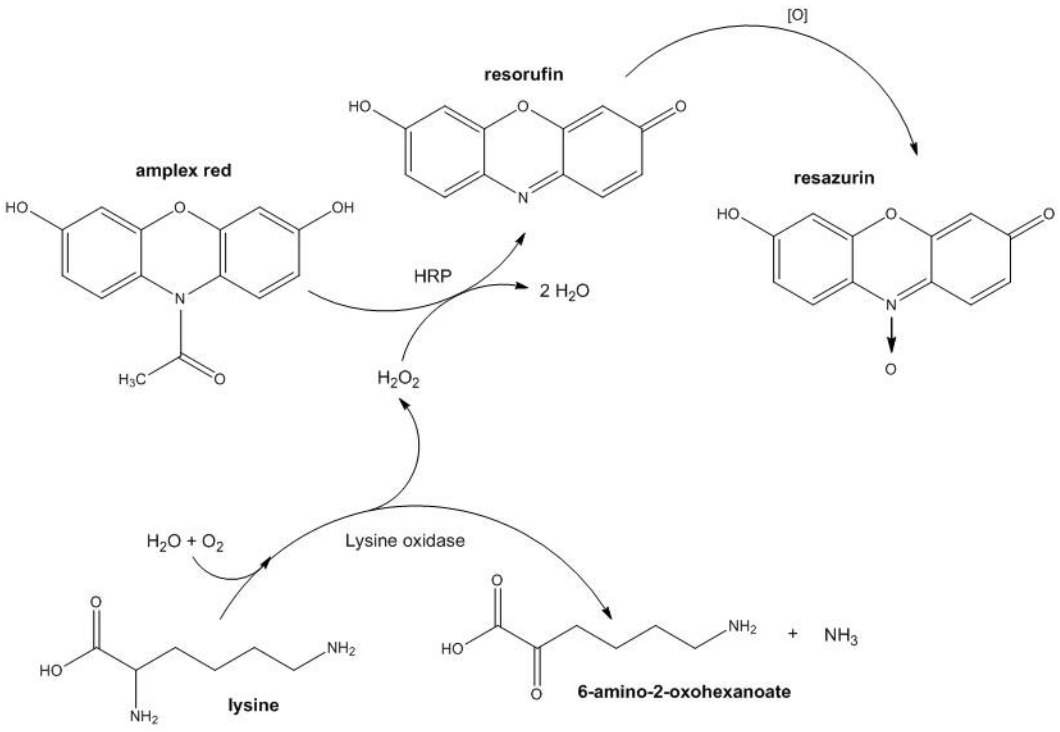


Figure 4.1: The chemical reactions of the lysine detection assay. Lysine is oxidized by lysine oxidase to 6-amino-2-oxohexanoate and ammonia with hydrogen peroxide as a by-product. This is used to oxidize amplex red to the fluorescent compound resorufin. If the reaction is allowed to continue, resorufin is further oxidized to resazurin, a clear non-fluorescent product.

The coupled lysine detection assay is consistent and reliable assuming adherence to the optimized assay conditions. The assay was optimized for signal and costs, and the ideal buffer was determined to be HBSmT (0.02 M HEPES / 0.15 M NaCl / 0.001% Tween80, pH 7.4) for use with basic carboxypeptidases. Tween80 has been found by the Bajzar lab to be ideal for use with CPN, CPB and TAFIa; therefore for the sake of comparison to other assays, this detergent was used in the lysine detection assay as well. A ten-fold increase in the stated concentration of Tween80 interferes with assay detection (data not shown) but the absence of detergent causes the enzymes (including the coupling enzymes) to adhere to the sides of the wells, and results in an apparent decrease in enzyme activity. A compromise between these factors determined the optimal concentration of Tween80 for the assay. Additionally, for the sake of comparison to the plasminogen activation assay, Hepes buffer was chosen for the lysine detection assay because some other common biochemical buffers are known to interfere with plasminogen activation.

Under the conditions described, a large range for lysine detection (three-orders of magnitude) is achieved, which is ideal because concentrated samples require fewer dilutions for detection while lysine in dilute samples will still be detectable. The detection of CPN is linear between 6 pM and 10 nM over a large range of FA-Ala-Lys concentrations. However, the linearity of reaction depends greatly on the identity of the carboxypeptidase and substrate; therefore differing enzymes and differing substrates may display a much different range, depending on the specific activity of the reaction.

The coupling enzyme lysine oxidase is fairly specific for lysine, however it does display some reactivity toward other substrates. Assigning a relative activity of 100% toward lysine, lysine oxidase shows 18% activity toward ornithine, 8% activity toward phenylalanine, 6% activity toward arginine, 5% activity toward tyrosine, and 4% activity toward histidine (*414*). Due to the potential reactivity toward other amino acids, proper background controls are necessary, including titrations of enzyme alone and substrate alone. Assuming that proper background controls are included in each experiment (particularly when not using purified sample components), the only remaining concern is with the oxidation of arginine, which may also be released by basic carboxypeptidases from the carboxy-terminus of substrates. When measured in the lysine detection assay, substrates with both lysine and arginine residues at the carboxy-terminus may indicate a falsely high concentration of lysine due to the contribution by arginine. However, in the experiments performed by the Bajzar lab using this assay, the substrates chosen had only lysine residues at the carboxy-terminus. In the case that a substrate has both carboxy-terminal lysine and arginine residues, the arginine kinase assay (section 3.2.1.) may be used to account for the contribution of the arginine.

The final product of the coupled lysine detection assay, resorufin, is a fluorescent compound. Its excitation maximum is 563 nm and its emission maximum is 587 nm (*415*). Resorufin has been used extensively as a biochemical probe. The most common use is in the measurement of hydrogen peroxide production during physiological (phagocyte function) or pathological (oxidative stress) situations in cells. The non-fluorescent amplex red (*N*-acetyl-3,7-dihydroxyphenoxazine) is added to cell preparations and the production of H_2O_2, or the activity of enzymes which produce H_2O_2, can be measured indirectly via the oxidation of amplex red to form resorufin (7-hydroxy-3-iso-phenoxazin-3-one) (*415-419*). The conversion of amplex red to resorufin has been well

characterized, and was found to have a one-to-one stoichiometry, which means that one molecule of amplex red requires one molecule of H_2O_2 to produce one molecule of resorufin (and H_2O) (*415;419*). The oxidation of amplex red to resorufin by HRP / H_2O_2 is an irreversible reaction, so every H_2O_2 molecule should result in productive conversion of amplex red (*419*). The reaction was also determined to be quite specific for HRP / H_2O_2 (*415*).

The original use of resorufin as an oxidation state probe for the detection of peroxidase activity was in the resorufin-resazurin system (*420;421*). With the addition of hydrogen peroxide, the fluorescent resorufin is oxidized by peroxidase into the clear, non-fluorescent compound resazurin (7-hydroxy-3-iso-phenoxazin-3-one 10-oxide). This reaction is possible even in the amplex red-resorufin system; therefore the fluorescence change accompanying the oxidation of amplex red to resorufin can be counteracted by the further oxidation into resazurin (see Figure 4.1). Note however, that this reaction is significantly (30-fold) slower than the amplex red to resorufin transformation, therefore if data from kinetic experiments are taken over only a very short period of time, or the end-point measurements are read immediately after addition of amplex red, the production of resazurin is minimal (*415;422*). Furthermore, due to the kinetics of the reaction, resazurin is only produced when the concentration of H_2O_2 is greater than the concentration of amplex red. When the concentration of carboxypeptidase substrate is kept lower than the concentration of amplex red, then the formation of resazurin (and the concomitant decrease in fluorescence of resorufin) is negligible (*415;417*). Furthermore, to satisfy Michaelis-Menten kinetics, in the experiments detailed here the initial rates of hydrolysis were always determined when less than 5% of the substrate was consumed.

This assay is extremely sensitive and to obtain the greatest reproducibility, great care should be used during sample and reagent preparation. The stock solution of amplex red should be solubilized at a high concentration in DMSO and only diluted to the appropriate concentration in assay buffer immediately prior to the experiment to prevent degradation or spontaneous oxidation. Lysine oxidase and HRP should be kept on ice, and also diluted in assay buffer only immediately prior to use (enzymes are susceptible to decay at ambient room temperature). Assay buffer and assay plates should be pre-warmed to the appropriate temperature to prevent lag times during kinetic measurements. Assay

buffers should be made fresh daily to prevent contaminations, and assay plates should be pre-washed in assay buffer prior to the experiment for the same reason.

The lysine detection assay correlates well with the chromogenic FA-Ala-Lys assay for both carboxypeptidase and lysine (or FA-Ala) detection. The measurements of CPN activity and lysine concentration were nearly identical by both assays, but note that CPN samples were diluted 1:5 (v/v) and the lysine / FA-Ala samples were diluted 1:10 (v/v) prior to analysis in the lysine detection assay due to its greater sensitivity.

4.3. CPN hydrolyzes carboxy-terminal lysine residues from (DD)E and attenuates plasminogen activation

Antifibrinolysis mediated by basic carboxypeptidases is caused by the removal of carboxy-terminal lysine residues from fibrin cofactor and its degradation products. TAFIa is known to be antifibrinolytic, and CPB is generally accepted to be an antifibrinolytic enzyme *in vitro* (but not *in vivo* due to its pancreatic compartmentalization). In contrast, CPN is not currently recognized as antifibrinolytic, despite being present in plasma at a high concentration, being a constitutively active, and exhibiting the substrate specificity of a basic carboxypeptidase similar to TAFIa. Due to the discovery that the contaminating CPN in fibrinogen stocks has a significant effect on *in vitro* clot lysis assays, a potential role for CPN in fibrinolysis was rigorously investigated.

With our novel lysine detection assay, the kinetics of lysine release from (DD)E by TAFIa, CPB, CPN and plasmin-cleaved CPNc were determined to obtain a relative measure of their antifibrinolytic capabilities. The data show conclusively that CPN is as active toward (DD)E as are the antifibrinolytic enzymes CPB and TAFIa. Furthermore, plasmin-cleaved CPNc has a catalytic efficiency approximately three-fold greater than that of the other enzymes. There are eight carboxy-terminal lysine residues on (DD)E that could potentially be removed by a basic carboxypeptidase (two each of α^{78}, β^{133}, γ^{62}, and αC^{206}); CPB released all eight lysine residues per molecule of (DD)E, as did CPN and CPNc. TAFIa released approximately five moles of lysine per mole of (DD)E prior to decaying due to its intrinsic instability. The identity of each removed lysine residue is unknown; therefore no conclusions as to the order of lysine removal can be made. CPN and CPNc evidently have the capacity to hydrolyze carboxy-terminal lysine residues from

fibrin degradation products, and they do so to the same extent and with the same, or greater, catalytic efficiency as CPB and TAFIa.

Many researchers believe that plasmic cleavage of CPN results in the formation of two heterodimers, and that this is the cause of the increased activity following plasmic cleavage. They hypothesize that there may be greater activity when the two active subunits are separated, allowing them to act independently. In tetrameric CPN, only a single catalytic subunit at a time may be involved in actively cleaving any one of the multiple substrates constrained by the three dimensional lattice of the fibrin meshwork. However, this does not explain the increase in activity toward unconstrained and independently mobile soluble substrates, such as (DD)E, because both catalytic subunits may simultaneously hydrolyze substrate molecules. This also does not explain the three-fold increase in activity, because release of one catalytic subunit should only cause a two-fold increase (by freeing the second catalytic subunit). It is worth noting that the increase in catalytic efficiency due to plasmic cleavage is almost exclusively a result of an increase in the first order catalytic rate constant, and is consistent with either an increased number of active sites or greater activity associated with an equal number of active sites. Furthermore, the antifibrinolytic activity of CPN increases eight-fold following plasmic activation whereas doubling the CPN concentration only increases lysis time less than two-fold, which also counters these hypotheses. The increase in activity could therefore be explained by a different type of steric constraints. Plasmic cleavage is known to release an approximately 13 kDa peptide from the carboxy-terminus of CPN2. This region is not part of the LRR, and forms its own separate globular domain. Removal of the 13 kDa peptide from CPN2 could increase access of the large (DD)E molecule to the active site of CPN1, resulting in an increased catalytic efficiency (Figure 4.2). However, without knowledge of the inter-subunit interactions and tertiary structure of CPN, this hypothesis is largely speculative.

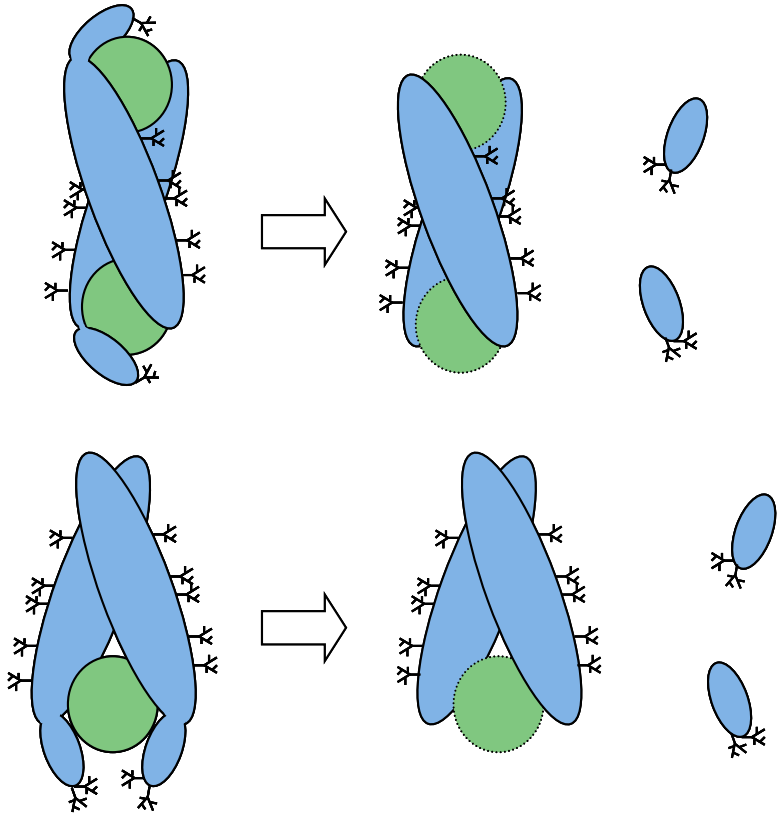


Figure 4.2: Plasmic activation of CPN. Digestion of CPN with plasmin releases the 13 kDa docking peptide from CPN2, which may increase access to the active site groove of CPN1 by relieving steric constraints. Two models are depicted. The first is plasmic cleavage of the generally accepted tetrameric structure of CPN, and the second is plasmic cleavage of the new trimeric structure of CPN. Plasmic digestion of either multimeric form results in the same increase in access to the active site groove of CPN1. CPN1 is coloured in light grey, CPN2 is coloured in dark grey, and the carbohydrate is depicted as stick figures. Plasmic cleavage of CPN1 is depicted by a dashed border.

Although TAFIa is a known antifibrinolytic enzyme, it appears to have the smallest effect on plasminogen activation compared to CPN, CPNc and CPB (at saturating levels). TAFIa demonstrates an initial catalytic efficiency identical to that of CPN and CPB, but due to its intrinsic instability, the enzyme is unable to remove as many carboxy-terminal lysine residues per molecule of (DD)E, and therefore it is unable to decrease the cofactor activity of (DD)E to the same degree as its counterparts. Unfortunately, *in vitro* experiments do not always accurately model the *in vivo* situation. Within the circulation, TAFI may be continuously activated by thrombin/thrombomodulin or plasmin during the coagulation/fibrinolytic cascades. Therefore the concentration of active enzyme may remain quite stable if the rate of formation equals the rate of decay, and the overall effects of TAFIa activity may be similar to those of CPB and CPN until all of the TAFI is

activated and/or decays. Furthermore, the requirement for activation by coagulation and fibrinolytic components localizes TAFIa activity to the clot, unlike CPN, which is freely active throughout the circulation. On the other hand, the *in vivo* situation is further complicated as TAFI must be activated, whereas CPN is already active. TAFI circulates at an average concentration of 100 nM, so even if all of the TAFI was activated, it would maximally produce a concentration of active carboxypeptidase equivalent to CPN. Since both enzymes are similarly active toward (DD)E (and by inference toward partially degraded fibrin) it seems unlikely that TAFIa could outperform CPN, unless there is additional regulation of the antifibrinolytic activity of CPN.

(DD)E depleted of all of its carboxy-terminal lysine residues loses approximately one third of its plasminogen activation cofactor activity compared to native (DD)E. The remaining two thirds of the cofactor-mediated enhancement of plasminogen activation may be due to plasminogen binding to internal lysine residues of the (DD)E molecule. Depletion of carboxy-terminal lysine residues from (DD)E can be accomplished by incubation with CPB, TAFIa, CPN or CPNc; and the extent of reduction in (DD)E cofactor activity caused by CPN- or CPNc-digestion is similar to that caused by CPB-digestion. Over the first hour of incubation, TAFIa-digestion also demonstrates parallel results, but due to its spontaneous inactivation, TAFIa ultimately does not affect plasminogen activation to the same extent. Each carboxy-terminal lysine residue of (DD)E is equally responsible for the enhancement of plasminogen activation, as demonstrated by the linear relationship between lysine released and plasminogen activation. CPN displays a linear relationship identical to that of CPB and TAFIa, which rules out the possibility that CPN releases alternate lysine residues which are not responsible for the rate enhancement of plasminogen activation.

Based on the activity toward (DD)E of CPN and CPNc in comparison to CPB and TAFIa, CPN clearly demonstrates antifibrinolytic activity. CPN has similar a catalytic efficiency toward (DD)E as CPB and TAFIa, it releases the same amount of lysine, and it has a similar ability to attenuate plasminogen activation. So why does it not appear to play a role in fibrinolysis *in vivo*? The main difference between these experiments and those concluding the absence of an antifibrinolytic effect is the presence of plasma. The above experiments are performed using purified proteins exclusively. In contrast, the experiments which contradict these findings were performed using plasma as a source of

CPN, or with exogenous purified CPN added to plasma. This strongly suggests the presence of a selective CPN inhibitor in plasma.

4.4. CPN is inhibited by excess CPN2 in plasma

Normal human plasma contains an endogenous CPN activity (equivalent to approximately 100 nM of the purified enzyme), which can be measured using the small chromogenic substrate FA-Ala-Lys. Other small synthetic substrates have also been used to measure CPN activity in plasma with similar results (*205;206;209-211;213;406*). However, unlike the purified protein, the endogenous CPN in plasma is not active toward the large physiological substrate (DD)E.

The release of carboxy-terminal lysine residues from (DD)E is simultaneous with the loss of cofactor activity for tPA-induced plasminogen activation. Five nanomolar purified CPN digests the cofactor (DD)E, resulting in a significant attenuation in its ability to promote plasminogen activation. In contrast, 5% NHP (which contains 5 nM endogenous CPN) does not. Therefore, it appears that purified CPN is antifibrinolytic, yet CPN in NHP is not. When NHP was titrated (0.039 - 20% of final volume) into exogenous 5 nM purified CPN, it decreased the antifibrinolytic activity of CPN in a concentration-dependent manner; addition of greater than 2.5% NHP eliminated the antifibrinolytic activity altogether. This suggests the presence of a selective CPN inhibitor (or activity modulator) naturally present in plasma. Whether this inhibitor is specific for CPN activity toward fibrin degradation products, such as (DD)E, or acts generally toward all other proteinacious substrates is unknown.

To analyze only the effects of the exogenous CPN (without the confounding effects of CPN in plasma), NHP was depleted of endogenous CPN by passage over an antiCPN column. The ability of CPN-depleted NHP (NHPdCPN) to inhibit the antifibrinolytic activity of CPN was measured. NHPdCPN or untreated NHP were titrated into exogenous 5 nM purified CPN. In contrast to NHP, which quickly inhibited the activity of CPN, NHPdCPN had little effect. The small remaining inhibitory activity of NHPdCPN was consistent with the residual CPN activity following immunodepletion, which suggests that both the inhibitor and CPN were removed from plasma to the same extent.

Members of the Bajzar Lab have demonstrated the presence of free CPN2 in NHP (unpublished results). NHP was depleted using a polyclonal antiCPN1 antibody, which recognizes only the catalytic subunit (even within the context of multimeric CPN), and removes all CPN1 (CPN2-bound or any free CPN1). Activity analysis and immunoblots indicated the complete removal of CPN1; however, immunoblots using a polyclonal antiCPN antibody (which recognizes both CPN1 and CPN2) demonstrated the persistent presence of CPN2 subunits remaining in the CPN1-depleted plasma. Furthermore, the inhibitory activity toward CPN was still present in the depleted plasma.

Conceivably CPN2 is expressed and secreted in excess of CPN1 as a mechanism to ensure that all CPN1 is complexed to a regulatory subunit. However, the function of the residual free CPN2 is unknown. If these free CPN2 subunits can recognize CPN1 subunits that are already part of a complex with CPN2, they may bind to this complex in some manner and limit access of substrates to the active site of CPN1. Small chromogenic substrates may retain access to CPN1, while larger substrates such as (DD)E may be denied. If indeed this is the case, the physiological role of CPN2 may be more complex than originally assumed. The mode and location of interaction of CPN2 with multimeric CPN may be important in determining the substrate specificity and mechanism of activity modulation by CPN2.

Five nanomolar CPN is inhibited by 2.5% NHP. If free CPN2 is an inhibitor, and if it binds to multimeric CPN in a 1:1 stoichiometric fashion, there must be minimally 200 nM of inhibitor in plasma, and the binding affinity must be extremely tight. But, depending on the affinity of free CPN2 for multimeric CPN, the concentration of circulating inhibitor might be significantly higher. However, if the binding is not stoichiometric, then the concentration could vary significantly from this value, although again, it would be dependent on the affinity of the interaction. NHPdCPN displays a greatly decreased ability to inhibit CPN antifibrinolytic activity; NHPdCPN is an approximately 13-fold poorer inhibitor of CPN. The minimal residual inhibition of NHPdCPN is likely due to incomplete depletion of CPN2.

4.5. CPN is inhibited by excess recombinant CPN2

For more definitive proof of the role of free CPN2 in modifying the substrate specificity of multimeric CPN, the inhibitory activity of the purified recombinant CPN2 (rCPN2) toward multimeric CPN was examined. rCPN2 was overexpressed in T-REx 293 cells in serum-free media. Interestingly, an inducible expression system was required for adequate expression of rCPN2 because constitutive expression systems led to poor expression or secretion of the protein, and had apparently lethal effects on the cells. Constitutive expression of rCPN2 in BHK-21, Cos-7, or HEK-293 cells (the cells from which T-REx 293 are derived) yielded low rCPN2 concentrations in the conditioned media, had an absolute requirement for serum supplementation, and frequently lead to unhealthy and apoptotic cells after only a few passages. The same was true for expression of rCPN1. Co-expression of rCPN1 and rCPN2 did not appear to improve the situation, although this was not rigorously investigated. Therefore an inducible system was chosen, in order to rest the cells in between bouts of overexpression.

rCPN2 was purified from conditioned media by ion exchange and affinity chromatography. The first step of the purification procedure was anion exchange on a Q-Sepharose column. rCPN2 eluted from the column in 0.5 M NaCl, identical to the conditions used to elute multimeric CPN from Q-Sepharose. Although many unrelated proteins also elute under these conditions, it does support the theory that the external surface of CPN is primarily composed of the CPN2 subunits, and they therefore greatly influence the purification of the enzyme. Note that Tween20 was added to the starting material to increase recovery by decreasing non-specific protein binding to the purification apparatus.

The majority of the purification was achieved by antiCPN column chromatography. The polyclonal antibody is highly selective for CPN2, and does not recognize other proteins in the load sample. rCPN2 was expressed in serum-free media specifically to avoid contamination with bovine CPN subunits, which could co-purify with recombinant human CPN2. Although harsh, elution with 0.2 M glycine, pH 2.5 was considered acceptable due to the lack of any specific catalytic activity of CPN2 that could be destroyed under these conditions. Elution fractions were collected into and neutralized with 1 M Tris pH 8.0 to prevent denaturation of the protein. Ideally, the inhibitory activity of rCPN2 (toward CPN antifibrinolytic activity) would have been followed

throughout the purification to determine the change in its specific activity; however, it is due to this antiCPN affinity chromatography purification step that the specific activity could not be followed. The Tris-glycine buffer used at this step is not compatible with the plasminogen activation assay; therefore the inhibitory activity of rCPN2 could not be measured, and the specific activity could not be determined.

In the final steps of the purification procedure, rCPN2 was concentrated on a Q-Sepharose column, and further concentrated and desalted by spin concentration (the final concentration of NaCl was reduced to 50 mM). The concentration of rCPN2 was determined by A_{280} (using an extinction coefficient calculated by amino acid analysis) and by antiCPN ELISA. In all preparations of rCPN2, the protein concentration as determined by ELISA was always one-half of the concentration as determined by A_{280}. The most likely reason for this discrepancy is that the concentration of rCPN2 as determined by A_{280} was the correct concentration for rCPN2 monomers while the concentration as determined by ELISA was correct for rCPN2 dimers. The standard curve for the ELISA is made up from multimeric CPN, which contains two CPN2 subunits per multimer. Therefore the signal detected for a certain concentration of CPN would indicate twice the concentration of monomeric CPN2. It is currently unknown whether rCPN2 circulates in monomeric, dimeric or higher multimeric forms, but it is highly possible that rCPN2 subunits spontaneously form dimers in solution, due to the significant hydrophobicity of the protein, and the complementary binding sites.

Purified rCPN2 titrated into 5 nM purified CPN inhibited the antifibrinolytic activity of CPN in a concentration-dependent manner. Addition of 10 nM rCPN2 abolished the activity of 5 nM CPN. This effect can be directly attributed to inhibition of CPN by rCPN2, as rCPN2 alone had no effect on plasminogen activation. As is the case in plasma, rCPN2 had no effect on the activity of CPN toward a small chromogenic substrate. Up to a 4-fold molar excess of rCPN2 was unable to exert any inhibitory effect on CPN against the substrate FA-Ala-Lys.

The ratios of inhibition are interesting, but not entirely straightforward. Ten nanomolar rCPN2 is required to inhibit 5 nM CPN. This could be due to extremely tight binding between the proteins and an inhibitory stoichiometry of 1 CPN:2 rCPN2; this could be due to tight binding and an inhibitory stoichiometry of 1 CPN:1 rCPN2; or this could be

due to even tighter binding (sub-nanomolar K_d) and a stoichiometry of several CPN molecules to one rCPN2 (for example, perhaps rCPN2 causes several CPN molecules to associate and this blocks access to their catalytic sites). It cannot be clearly determined from these data if inhibition is caused by a quantitative and stoichiometric interaction accounting for consumption of the 10 nM inhibitor or less than a quantitative amount of inhibitor interacting with the enzyme in a manner governed by a sub-nanomolar K_d.

4.6. CPN is a trimer of two CPN2 subunits and a single CPN1 subunit

The interpretation of the data collected over the years regarding the structural aspects of CPN may be flawed or inaccurate; particularly with respect to the molecular mass and the stoichiometry of the subunits which compose CPN. The generally accepted structure of CPN is of a 280 kDa heterotetramer, composed of two 83 kDa regulatory and two 48-55 kDa catalytic subunits. Unfortunately, the methods of molecular mass determination that have been used (PAGE, gel filtration, and sedimentation ultracentrifugation) are influenced by the Stoke's radius of the protein, the carbohydrate content, and any interactions that the protein may have with the matrix support. The combination of these effects can unpredictably alter the apparent molecular mass. MALLS, however, is an ideal method for determining the molecular mass of a protein: its analyses are independent of conformation, the carbohydrate content can be accounted for, there is no matrix on which the protein may differentially interact, and no molecular mass standards are required.

CPN was determined by MALLS analysis to have a molecular mass of 220 kDa. This molecular mass is consistent with a stoichiometry of two regulatory subunits in complex with only a single catalytic subunit [(2 x 83 kDa) + (1 x 55 kDa) = 221 kDa]. This new stoichiometry is in agreement with the previously reported Hill plots of 1, and with the zinc content of 0.97 mol Zn/mol CPN. This is also consistent with unpublished ultracentrifugation results from the Bajzar Lab. Using sedimentation velocity ultracentrifugation (which, unlike gradient density ultracentrifugation used previously for CPN determination, is sensitive to both mass and shape of the molecule: therefore the shape was established independently), the molecular mass of CPN was determined to be 197 kDa. This result is also most consistent with a trimeric structure for CPN. Furthermore, plasmin-cleaved CPNc was determined by MALLS to have a molecular mass of 176 kDa. This is also in agreement with a trimeric CPNc that has been cleaved at

known plasmin-sensitive sites [(2 x 72 kDa) + (1 x 48 kDa) = 192 kDa]. Since most of the mass was accounted for and the peak was symmetrical (yielding a uniform molecular mass across the peak) it is unlikely that an alternative structure could satisfy the data.

Unpublished work from the Bajzar Lab shines some light on the possible quaternary structure of CPN and the potential locations of inter-subunit interactions. CPN2 is an LRR protein. This structural motif is commonly seen in proteins involved in protein-protein binding and is thought to mediate binding interactions. The structure of many LRR proteins have been determined by x-ray crystallography and all are predicted to have very similar horseshoe-like structures. The Nogo receptor-1 is an LRR protein that dimerizes through interactions of the concave faces of its LRR regions. Using the crystal structure of the Nogo receptor-1 as a template (*423*), the sequence of the core LRR region of CPN2 was threaded onto the structure using Modeller 8v2 (Figure 4.3). The subunits were then docked to form CPN2 dimers using XFIT. Interaction of the subunits through the LRR regions effectively buries the highly hydrophobic regions of CPN2 from the exterior solvent.

This structure does not include the catalytic CPN1 subunit, nor does it include the carboxy-terminal regions of CPN2 that lie outside of the LRR. The crystal structure of the catalytic subunit has been solved, but the remaining carboxy-terminal portions of CPN2 are still of unknown conformation. The carboxy-terminus of CPN2 beyond the LRR is approximately 88 residues in length and is predicted to be highly flexible and solvent-accessible, and is thought to form its own globular domain. This region is cleaved from CPN2 by plasmin and is referred to as the 13 kDa docking peptide. The sequence of this peptide is predicted to have a globular structure according to DISOPRED2, IUPred, GlobProt and Dompred prediction software. Since this region does not contain a sequence for any well defined structural motif, the sequence was sent by the Bajzar Lab to Robetta (a full-chain protein structure prediction server) for *ab initio* folding experiments. Although not perfect at predicting structures, *ab initio* experiments are extremely powerful at identifying regions lacking defined secondary and tertiary structure. All attempts at *ab initio* folding indicated that the 13 kDa peptide indeed contains a defined secondary structure, and several potential domain structures were suggested.

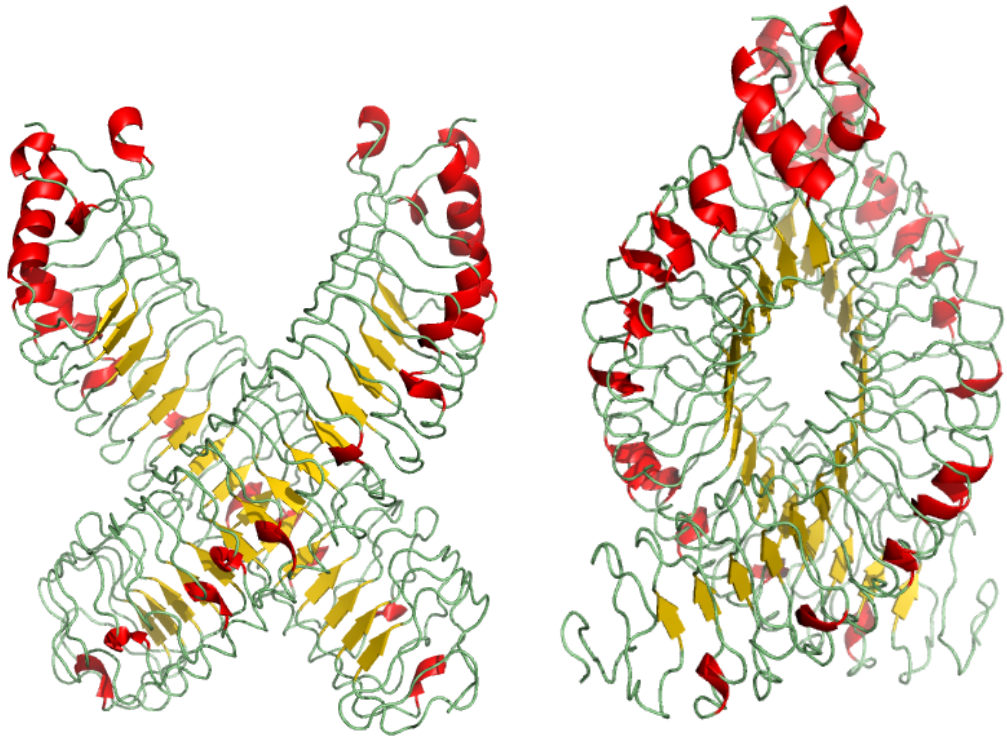


Figure 4.3: Molecular modeling of the LRR region of CPN2 and potential interactions of the LRR dimers of CPN2. The Nogo receptor-1 structure was obtained from PDB 1OZN. This structure is shorter than the CPN2 LRR, therefore the amino-terminal capping domain plus the LRR were used to model the amino-terminal portion of CPN2, and then a second copy of the LRR plus the carboxy-terminal capping domain were used to model the carboxy-terminal portion of CPN2. The dimers were docked based on energy minimizations and not based on any current crystal structure. The structure on the right is rotated 90 degrees around the y-axis with respect to the identical structure on the left. This figure was generated with PyMOL software.

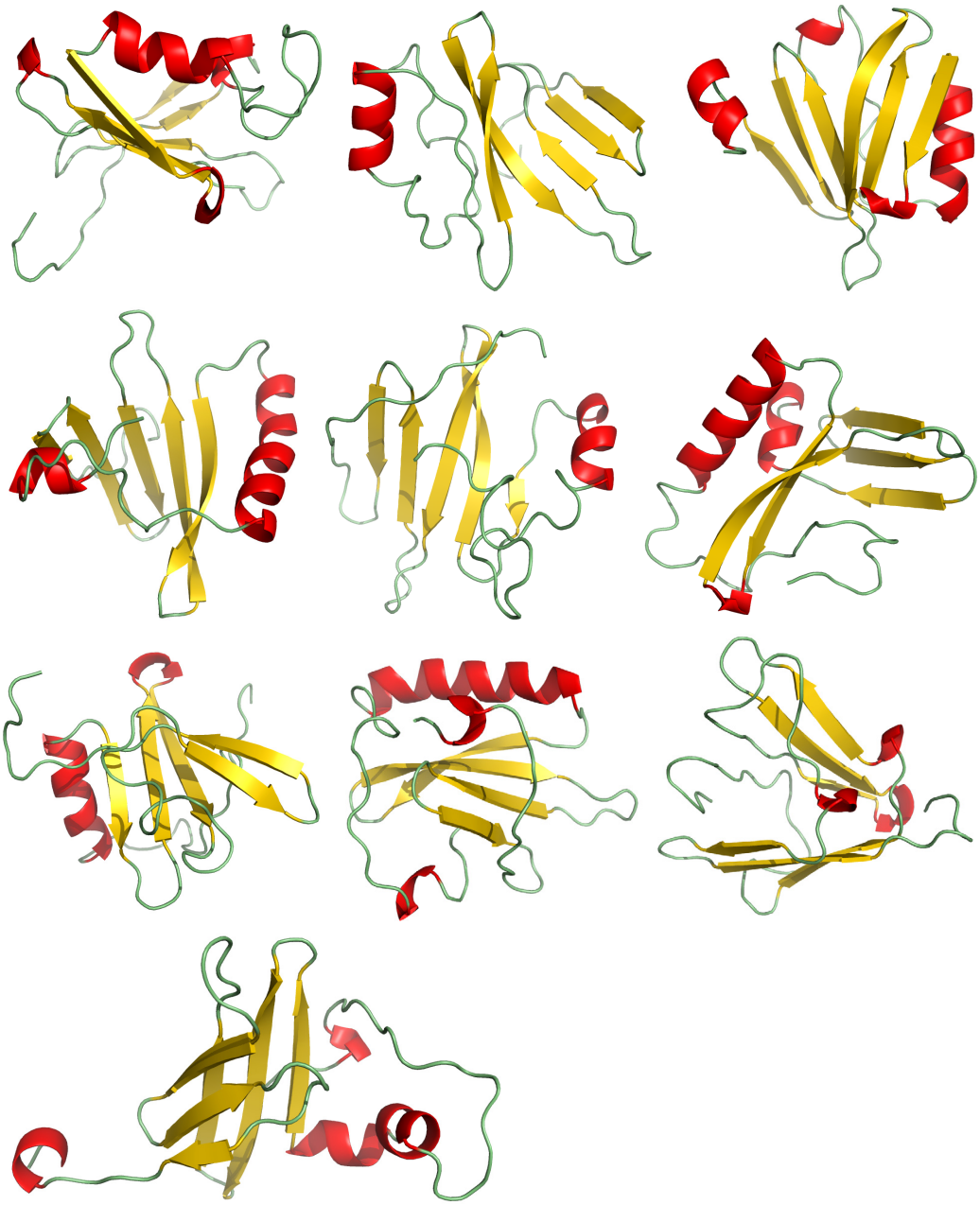


Figure 4.4: Potential structures of the 13 kDa CPN2 docking peptide as predicted by *ab initio* folding. Ten potential structures of the 13 kDa docking peptide. This figure was generated with PyMOL software.

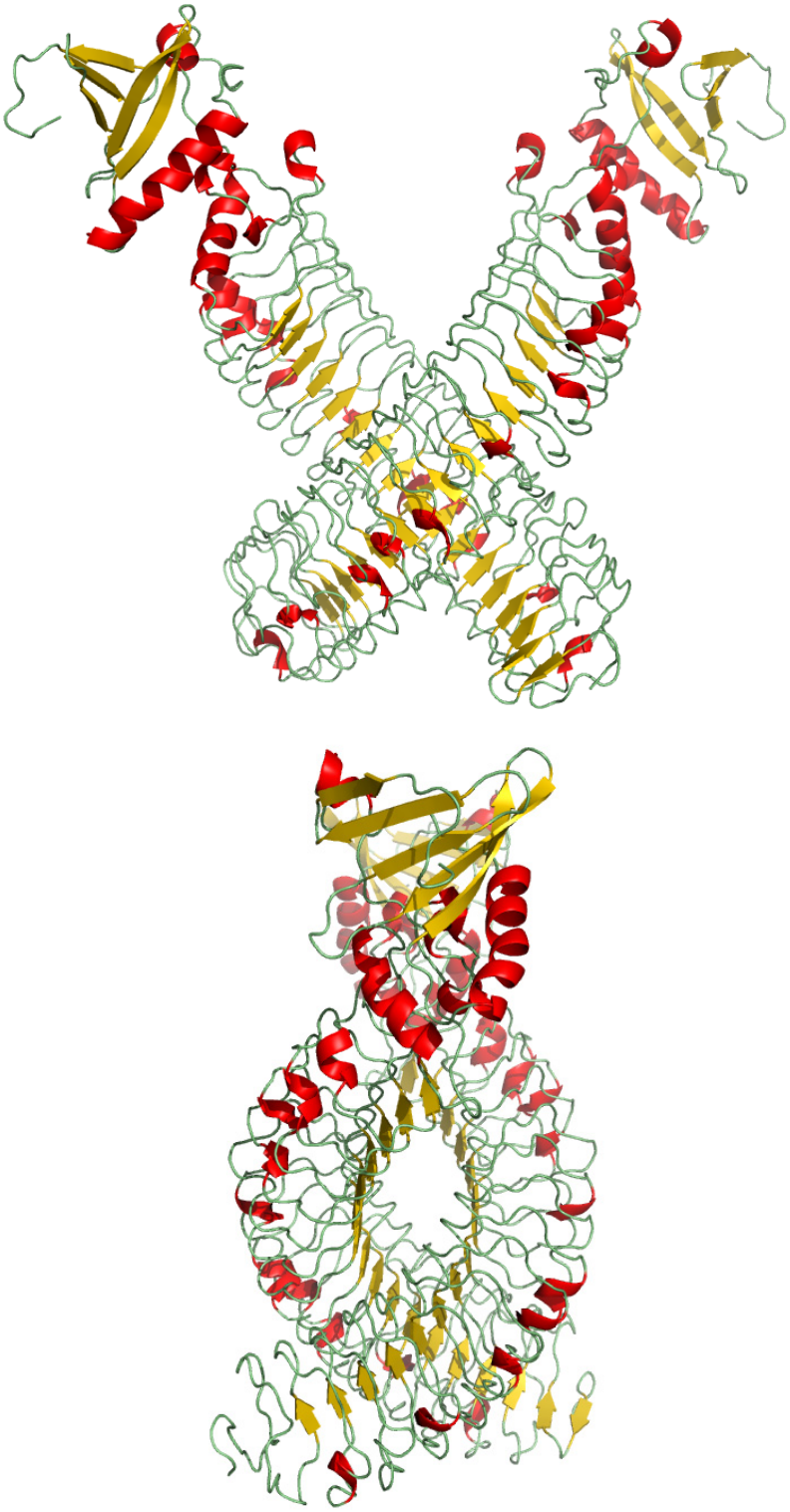


Figure 4.5: Molecular modeling of dimers of the full-length CPN2 subunits. One model of the 13 kDa docking peptide developed by *ab initio* folding was docked onto the molecular model of dimeric CPN2 LRR. The structure on the bottom is rotated 90 degrees around the y-axis with respect to the identical structure on the top. This figure was generated with PyMOL software.

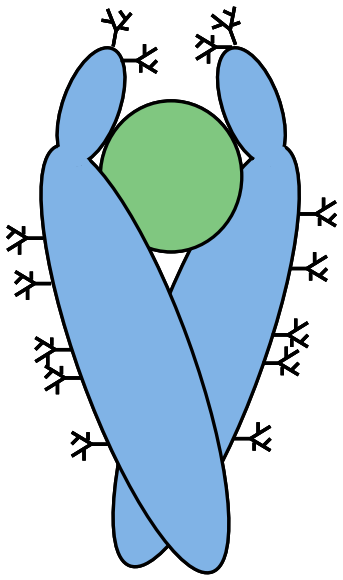


Figure 4.6: Hypothetical structure of the CPN trimer. A potential mode of interaction of the CPN1 and CPN2 subunits. CPN1 is coloured light grey, CPN2 is coloured dark grey, and the carbohydrate is depicted as stick figures.

Ten predicted structures were returned, each containing a high content of β-sheets, with or without variable regions of α-helix (Figure 4.4). One predicted structure of the 13 kDa carboxy-terminal peptide of CPN2 was docked onto the predicted dimeric structure of CPN2 to produce a potential model of the CPN2 dimers with which a single CPN1 subunit would interact (Figure 4.5). CPN1 was not docked into this structure and the mode of interaction of the subunits is still purely hypothetical; however, a diagram of the potential interactions of CPN1 and CPN2 in the CPN trimer is shown in Figure 4.6.

Many researchers believe that plasmic activation of CPN is caused by cleavage of the heterotetramer into two heterodimers. The subsequent two-fold increase in (free) enzyme (active site) concentration is thought to produce the increase in CPN activity associated with proteolytic cleavage. However, not only does this hypothesis not adequately describe the increase in catalytic efficiency of CPN, but based on the new trimeric structure of CPN, this is an impossible situation.

The evidence upon which these hypotheses were derived was a series of experiments by Quagraine *et al.* (*198*). These researchers examined CPN by gel filtration, immunoblot, and activity analysis (with the small chromogenic substrate dansyl-Ala-Arg) before and after incubation with plasmin. They found that the untreated CPN eluted as a single peak from gel filtration, and that this peak fraction matched the activity fraction. Treatment of CPN with plasmin resulted in four peaks eluting from gel filtration, none of which matched the elution volume of the untreated CPN.

The first peak demonstrated CPN activity and contained the 48 kDa catalytic subunit and the 72 kDa cleaved regulatory subunit (identified by immunoblot). The second peak was identified by immunoblot as the 13 kDa fragment of CPN2, and the third peak was identified as the isolated 48 kDa CPN1 subunit. The fourth peak did not possess any CPN activity, nor did it contain CPN subunits by immunoblot. Upon continued digestion with plasmin, gel filtered CPN had identical results, with the exception that the 48 kDa subunit was further cleaved by plasmin into the 21 kDa and 27 kDa two-chain form.

Based on these results, and the generally accepted tetrameric structure of CPN, it was thought that cleavage by plasmin splits CPN into two heterodimers (peak 1), and that these may dissociate, freeing the CPN1 subunit (peak 3). The authors describe 50% of the

CPN activity to reside in the heterodimer, and 50% of the activity to reside in the free CPN1 subunit. The researchers support this with immunoprecipitation experiments that demonstrate that CPN1 is co-precipitated with CPN2 following plasmic cleavage, and that 50% of the CPN activity precipitates with the regulatory subunit, while 50% remains in the supernatant. Finally, they also show that purified recombinant CPN1 can associate with the plasmin-cleaved CPN2 (72 kDa fragment) into heterodimers that remain associated throughout gel filtration. These data were used to support the hypothesis that CPN dissociates into two heterodimers upon plasmic cleavage.

However, these results are also consistent with plasmic cleavage of trimeric CPN, causing the release of a single CPN2 subunit, and producing a heterodimer (CPN1 and CPN2) plus an isolated CPN2. This heterodimer may then dissociate as described above, freeing the catalytic subunit. Such a hypothesis was never before considered due to the misleading assumption of a tetrameric structure of CPN.

Although this new description does not account for the absence of an isolated CPN2 subunit eluting from the gel filtration column, the authors of the experiment were also unable to account for this lack of the isolated CPN2 subunit following dissociation of their described heterodimers to release isolated CPN1 and CPN2 subunits. It is possible that due to interactions between the highly hydrophobic and glycosylated 72 kDa CPN2 with the column matrix, that it eluted from gel filtration at an unexpected time, such as in the uncharacterized peak 4. Poor specificity of the antibody (particularly if the CPN2 subunits associated hydrophobically, hiding epitopes) could have prevented the recognition of this protein in the late-eluting peak. Due to inconsistencies with the antibodies, inconsistencies with the activity measurements (a significant amount of activity is lost during the gel filtration procedure, and is not explained by the authors), and the inability to identify an entire subunit, any conclusions drawn from these experiments are dubious. Therefore the derived hypotheses are purely speculative, and require further investigation to be considered legitimate.

4.7. The regulation of CPN is a complex process

CPN appears to be regulated by two methods: by free CPN2 and by proteolytic cleavage by plasmin. These mechanisms have been demonstrated in plasma-based and in plasma-free systems. The exact process of regulation is incompletely understood, but suggests

that CPN activity cannot be simply interpreted by a single functional determination. The concentration of inhibitor in plasma is unknown, the stoichiometry of inhibition is unknown, and the relative degree of activity modulation by plasmin versus CPN2 is unknown. Additionally, the plasmin cleavage sites, and the effect of plasmic cleavage on stoichiometry (as described in section 4.6.) are not known. Many important details regarding the regulation of CPN have been elucidated here; however, these provide only an initial framework upon which hypotheses can be created, and do not provide any conclusive determinations.

A plausible hypothesis must account for the following results:

1. plasma contains 100 nM CPN, an unknown concentration of free CPN2 and potentially various forms of plasmin-cleaved CPN subunits
2. the CPN contained within normal plasma does not have antifibrinolytic activity
3. plasma inhibits the antifibrinolytic activity of purified exogenous CPN
4. plasma depleted of all forms of CPN no longer inhibits the antifibrinolytic activity of purified exogenous CPN
5. recombinant CPN2 inhibits the antifibrinolytic activity of purified exogenous CPN (in a plasma-free system)
6. plasmin cleaves CPN1 into a two-chain molecule, and cleaves CPN2 into a truncated LRR protein and a docking peptide
7. recombinant CPN2 is strongly inhibitory toward CPN at low concentrations, indicating a low K_d; yet through current plasma-derived purification protocols, free CPN2 does not seem to co-purify with multimeric CPN despite its presence in the circulation, indicating a high K_d

Many scenarios may be imagined to explain these results; three of these are described below. Initially, only the role of CPN2 will be considered (the additional role of plasmic cleavage of CPN1 will be considered subsequently). CPN2 is involved in the regulation of CPN as an inhibitor and also, when in its native form, as a steric modifier of CPN activity, which is released upon plasmic cleavage.

In scenario A (Figure 4.7), native CPN (containing the unprocessed 83 kDa CPN2) has minimal activity (toward some substrates, such as fibrin degradation products). Plasmic cleavage releases the 13 kDa docking peptides from both CPN2 molecules of the trimer

(leaving behind the 72 kDa CPN2 subunits). This cleavage reaction increases access of substrates to the active site groove of CPN1, thereby increasing activity. This cleaved form of CPN can be considered as the 'active' form of CPN, and it has full activity toward substrates such as fibrin degradation products. Since CPN isolated from plasma is of the native (83 kDa subunit-containing) trimeric form, then the K_d for the 83 kDa CPN2 toward CPN1 must be low. In contrast, the K_d for the 72 kDa CPN2 may be high, because this form is not identified in carefully purified plasma preparations. Therefore an equilibrium may be attained between the trimeric active form of CPN (containing the 72 kDa CPN2) and free, dissociated CPN1 and 72 kDa CPN2 subunits. If the plasmic activation of CPN is due to release of steric constraints through removal of the 13 kDa docking peptide, then both the active trimeric CPN, and the free CPN1 would have full activity.

If the inhibitory CPN2 exists as a dimer of 83 kDa subunits, then inhibition could be through binding to the free CPN1 subunit. Since the trimeric active CPN and the free subunits are in equilibrium, the inhibitory CPN2 would deplete free CPN1 from the circulation, and restore the original, native CPN trimer. Due to the low K_d of interaction between CPN1 and the 83 kDa subunits, this inhibition would be strong. It is also possible that during instances of excessive plasmin activity, the inhibitory CPN2 subunits may be cleaved. This would deplete the inhibitor in the circulation, and provide a secondary role for plasmin in addition to its role in activating CPN.

Scenario B (Figure 4.8) is quite similar to scenario A. Again, native CPN is considered to have low activity, and the 83 kDa CPN2 subunits are thought to demonstrate a low K_d (tight binding) with the CPN1 subunit. Following plasmic cleavage, both of the 13 kDa docking peptides are released from CPN2. The remaining 72 kDa CPN2 subunits display a high K_d toward CPN1, therefore CPN1 is easily able to dissociate from the trimer. It is only in the free, unbound form that CPN1 is fully active. This differs from scenario A, in which CPN1 is fully active both in the free and in the active trimeric form. In this scenario, the inhibitory CPN2 exists as a dimer of 83 kDa subunits, and inhibition is through interaction with the free CPN1 subunit. As in scenario A, inhibition of free CPN1 results in restoration of the original, native CPN trimer. Additional modulation of CPN1 activity could be achieved through the instability of isolated CPN1 and its increased rate of clearance through glomerular filtration.

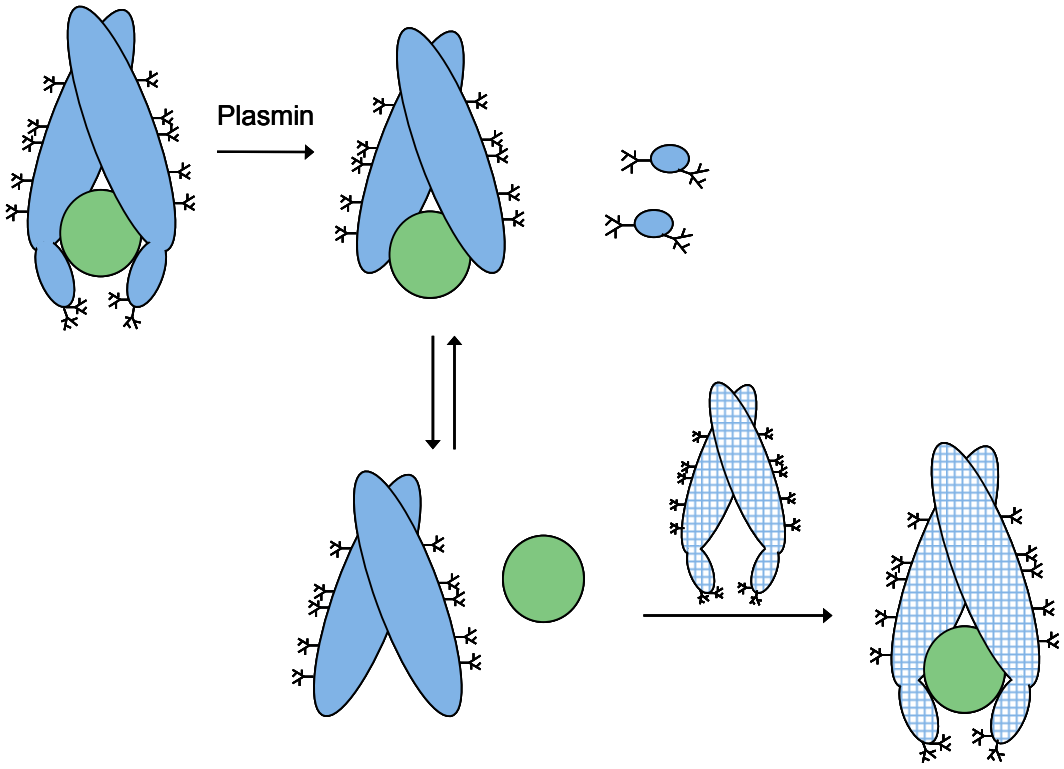


Figure 4.7: Regulation of CPN activity, scenario A. Full-length CPN with minimal activity is cleaved by plasmin to create the active enzyme. This trimeric enzyme is in equilibrium with the isolated CPN1, which also possesses full activity. Free CPN1 can be inhibited by circulating full-length CPN2 subunits, restoring the original trimeric enzyme. CPN1 is coloured in green, CPN2 is coloured in blue, and inhibitory CPN2 subunits are cross-hatched.

Scenario C (Figure 4.9) differs significantly from scenarios A and B, and it considers the possibility of a heterodimeric form of CPN, as suggested by the likely misinterpreted results of Quagraine *et al.*. In this scenario, plasmin releases the docking peptide from only a single CPN2 subunit. Assuming that the 83 kDa CPN2 has a low K_d toward CPN1, and that the 72 kDa CPN2 has a high K_d toward CPN1, then the 72 kDa CPN2 subunit would dissociate from the trimer, leaving behind a heterodimeric CPN (containing a full-length 83 kDa CPN2 subunit and an uncleaved CPN1 subunit). This heterodimeric CPN is the active enzyme. In this scenario, the inhibitory CPN2 exists as a monomer of the 83 kDa form. Due to the low K_d of the full-length CPN2, it would be strongly inhibitory toward heterodimeric CPN. As with the other scenarios, inhibition by CPN2 would result in restoration of the original, native CPN trimer.

In addition to the regulation described in the above scenarios, the role of plasmic cleavage of CPN1 must also be considered. Plasmin cleaves CPN1 from a 55 kDa protein to a 48 kDa protein. This cleavage has little to no effect on the activity of the enzyme; however, prolonged plasmic cleavage results in a two-chain form of CPN1, due to cleavage at the Arg^{218}-Arg^{219} bond. This two-chain form of CPN (which would necessarily also possesses cleaved regulatory subunits) displays increased catalytic efficiency toward some substrates, particularly large physiological substrates (Table 3.5). The increased activity is thought to be due to increased access to the active site groove through removal of an interfering peptide chain (Figure 4.10)

Due to the cleavage of both the 83 kDa CPN2 and the 55/48 kDa CPN1 by plasmin, it is difficult to determine which cleavage reaction is responsible for the increased catalytic efficiency (or the relative contributions of the two). However, the proteolysis of the two subunits does not appear be simultaneous – the regulatory subunit is more rapidly cleaved. Moreover, the increased CPN activity appears to be associated with the release of the 13 kDa docking peptide, rather than the formation of two-chain CPN1 (*166*). Yet these conclusions are based on immunoblot experiments, and it is possible that only a small amount of two-chain CPN1 is required to have a large effect on catalytic efficiency, and that this concentration is too low to be detected by immunoblot; additionally, sub-maximal CPN activation has not been examined. Therefore, due to the possibility of increased activation of CPN by plasmin through cleavage of the CPN1 subunit, the scenarios of CPN regulation may need to be modified.

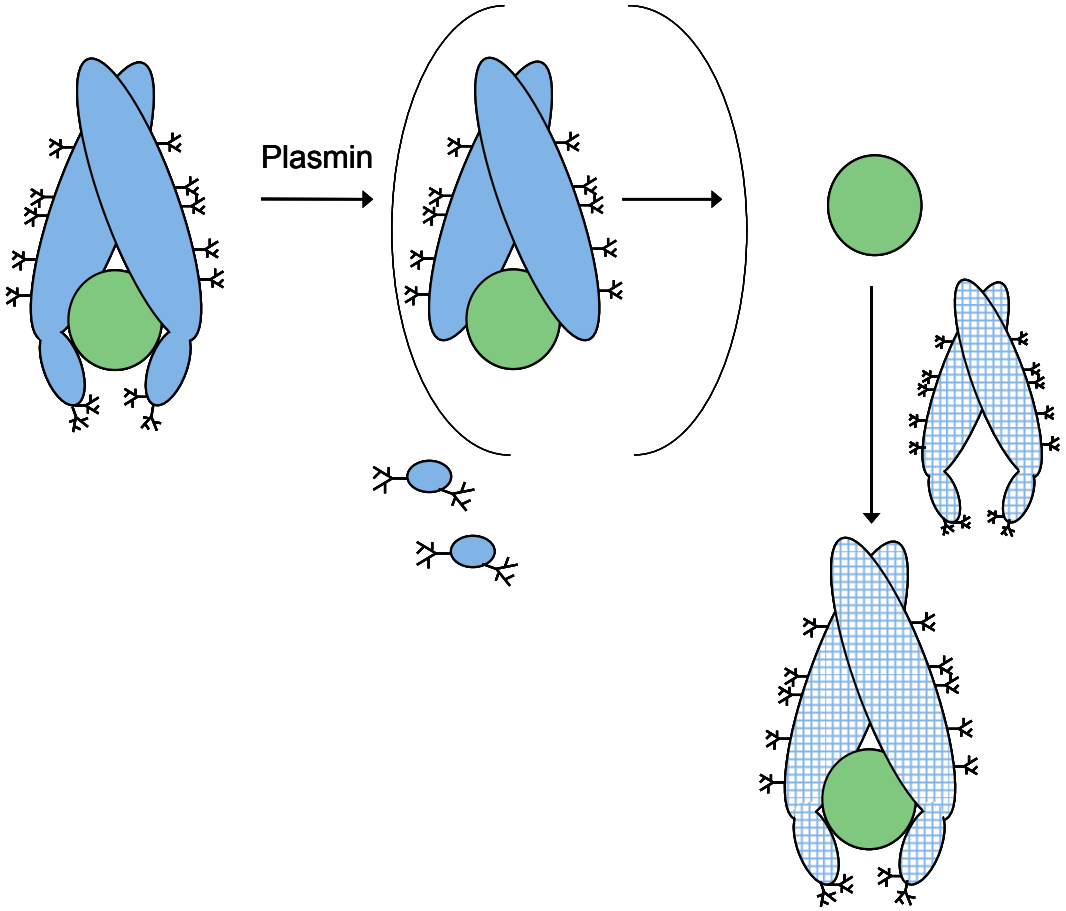


Figure 4.8: Regulation of CPN activity, scenario B. Full-length CPN with minimal activity is cleaved by plasmin, resulting in dissociation of the enzyme and release of free, fully active CPN1. Active CPN1 can be inhibited by circulating full-length CPN2 subunits, restoring the original trimeric enzyme. CPN1 is coloured in green, CPN2 is coloured in blue, and inhibitory CPN2 subunits are cross-hatched.

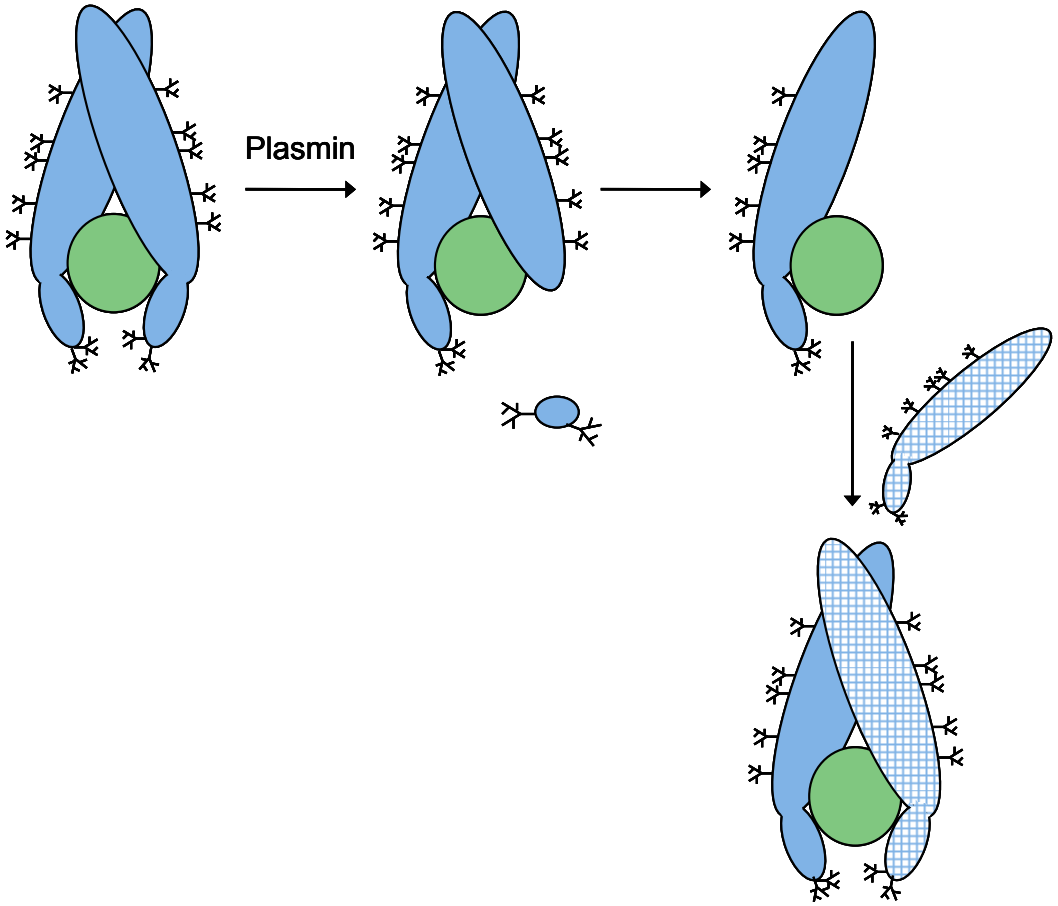
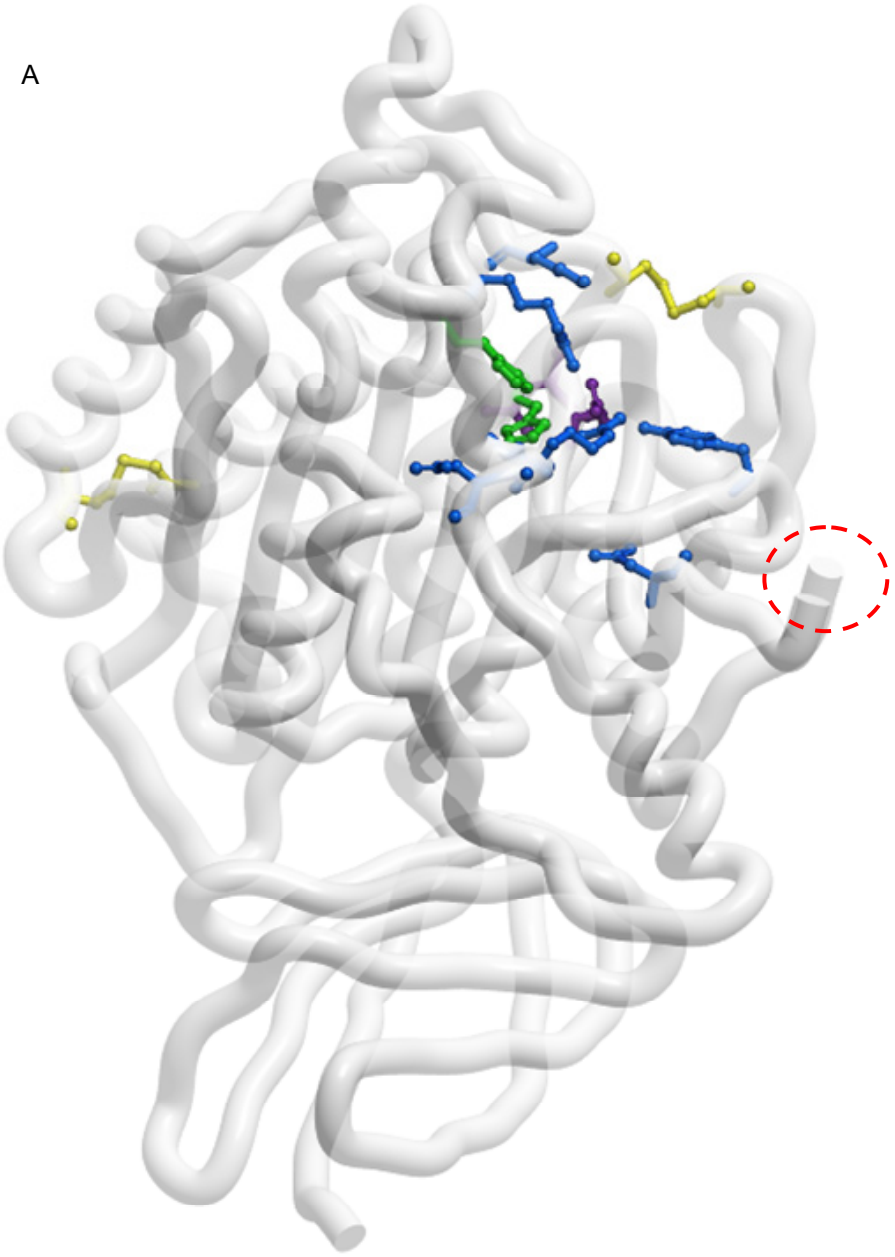


Figure 4.9: Regulation of CPN activity, scenario C. Full-length CPN with minimal activity is cleaved by plasmin, resulting in release of the 13 kDa docking peptide of a single CPN2 subunit. The cleaved form of CPN2 dissociates from the molecule, resulting in the active heterodimeric CPN. The heterodimer can be inhibited by circulating full-length CPN2, restoring the original trimeric enzyme. CPN1 is coloured in green, CPN2 is coloured in blue, and inhibitory CPN2 subunits cross-hatched.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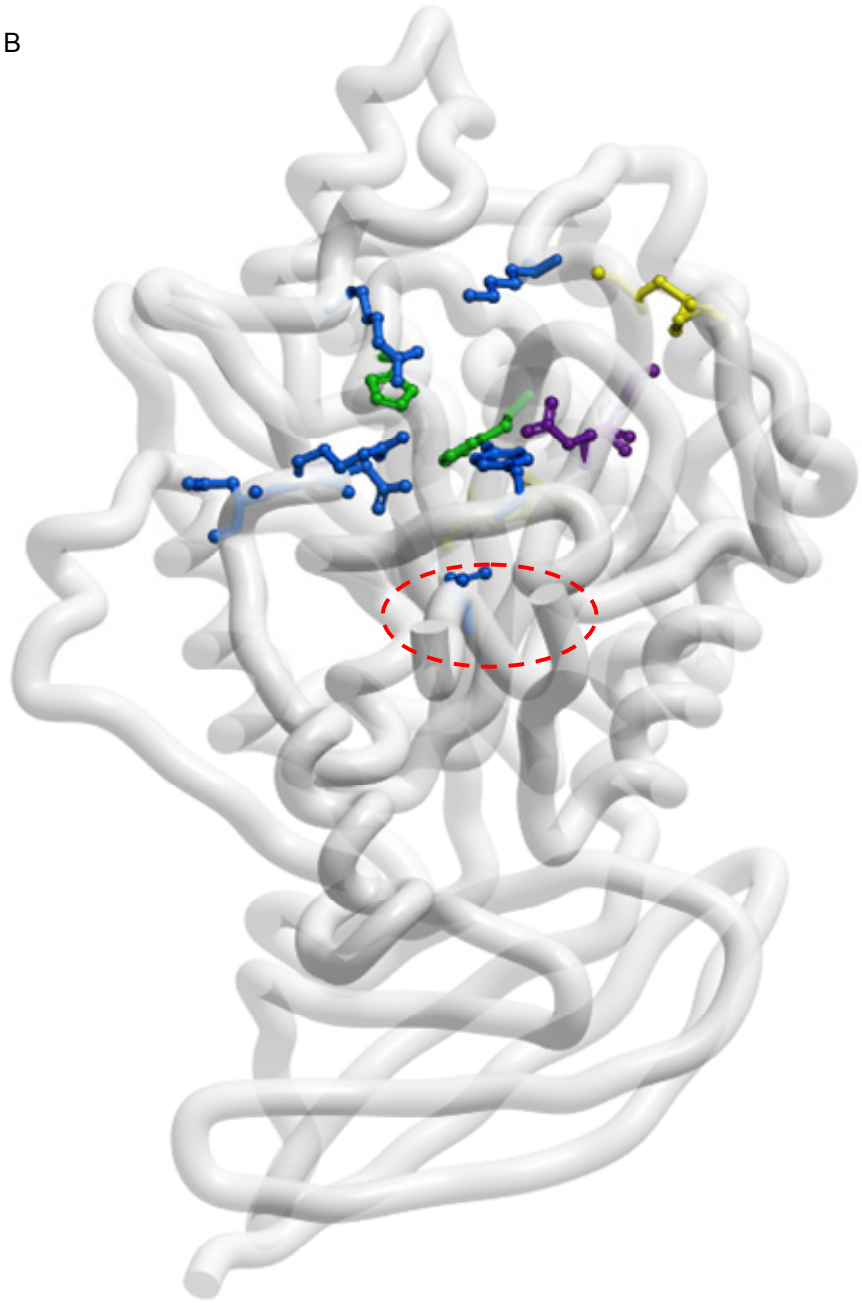

Figure 4.10: Plasmic cleavage of CPN1 may increase access to active site groove. Side (panel A) and front (panel B) views of the plasmin-cleaved two-chain form of the catalytic CPN1 subunit. Electron density of the residues surrounding the plasmin cleavage site (Arg^{218}-Arg^{219}) could not be defined, therefore residues 212-220 (inclusive) are not represented. This suggests a looser, more flexible conformation of these residues. The ends of the defined residues (211 and 221) are circled in red and are in close proximity to the active site groove. Active site residues involved in zinc coordination, substrate binding and catalytic activity are coloured in green, blue and purple, respectively. Disulfides are coloured in yellow. This figure was generated with ICM software. The CPN1 structure was obtained from PDB 2NSM.

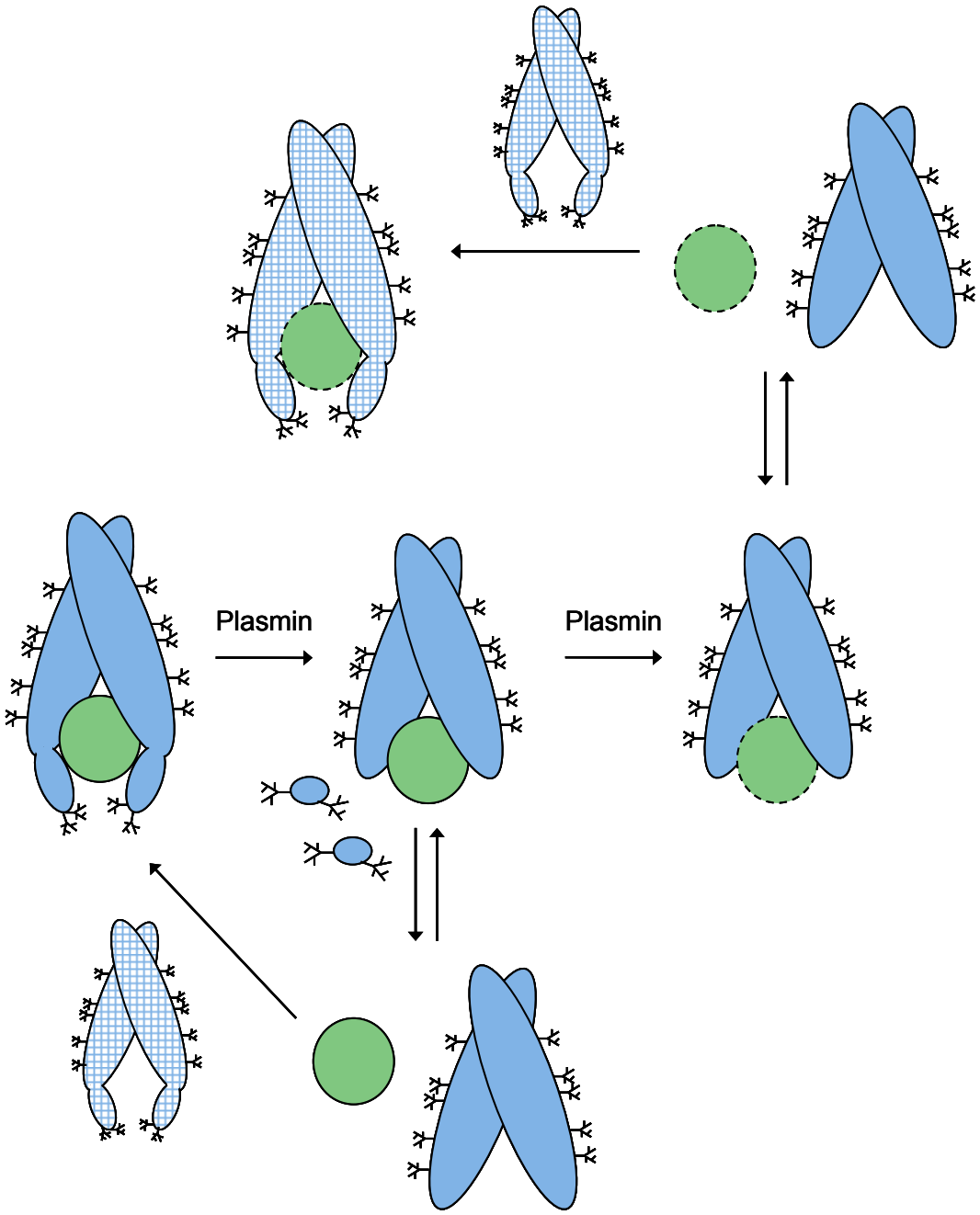

Figure 4.11: Regulation of CPN activity, scenario A with plasmic cleavage of CPN1. Full-length CPN with minimal activity is partially cleaved by plasmin to create an enzyme with intermediate activity, or is cleaved fully to create an enzyme with maximal activity. Either cleaved form of the trimeric enzyme is in equilibrium with isolated CPN1, which also possesses intermediate or maximal activity, for single- or two-chain CPN1 respectively. Free single-chain CPN1 can be inhibited by circulating full-length CPN2 subunits, restoring the original trimeric enzyme; and free two-chain CPN1 can be inhibited to produce a molecule with low or no activity. CPN1 is coloured in green, CPN2 is coloured in blue, inhibitory CPN2 subunits are cross-hatched, and activated CPN1 is outlined with dashed lines.

As an example, consider the effect of CPN1 cleavage in scenario A (Figure 4.11): as before, initial plasmic cleavage results in the release of the 13 kDa docking peptides. This form of CPN may display enhanced activity compared to the native trimer, but full activity is only achieved following plasmic cleavage of the CPN1 subunit. Due to the higher K_d of the 72 kDa form of CPN2, the trimeric CPN (containing either single-chain or two-chain CPN1) easily dissociates into its constituents. In the case of two-chain CPN, this free CPN1 may be equally active as the trimeric two-chain CPN (as described in scenario A). Inhibition of single-chain CPN1 by dimeric 83 kDa CPN2 produces the native CPN molecule, while inhibition of two-chain CPN1 results in a new molecule: plasmin-cleaved CPN1 within a dimer of non-plasmin-cleaved CPN2. The activity may be inhibited in this molecule, or it may be intermediate to that of native CPN and fully truncated CPN.

All of the above scenarios are possible mechanisms for the regulation of CPN; however, this does not discount alternative mechanisms of regulation which may also be consistent with all of the data presented to date. Much further research is required to accurately determine the biological roles of activation and inhibition of CPN.

4.8. CPN plays a role in fibrinolysis

Although the current state of general knowledge regarding CPN is that it is not an antifibrinolytic enzyme, the research presented here demonstrates otherwise. Furthermore, this work can explain why all other experiments to date have indicated a lack of a fibrinolytic role for CPN.

The rate of plasminogen activation, which heavily influences the rate of fibrinolysis, is enhanced in the presence of carboxy-terminal lysine residues on fibrin and fibrin degradation products. Removal of these residues greatly reduces the cofactor-mediated plasminogen activation rate enhancement. CPN efficiently removes carboxy-terminal lysine residues from the terminal fibrin degradation product (DD)E, and it can be extrapolated that CPN also removes carboxy-terminal lysine residues from all fibrin degradation products, based on the attenuation of fibrinolysis caused by the CPN contamination of fibrinogen. CPN removes the lysine residues *in vitro* with the same catalytic efficiency as CPB and TAFIa. Furthermore, plasmin-cleaved CPNc has a three-fold increased catalytic efficiency compared to these enzymes. The number of carboxy-

terminal lysine residues present on (DD)E directly correlates to its cofactor activity, and this relation is true for (DD)E digested by CPN, CPNc, CPB and TAFIa. Therefore CPN (and especially when in its plasmin-cleaved CPNc form) is a potent antifibrinolytic enzyme *in vitro*.

The situation *in vivo*, however, is different. Circulating free CPN2 may act as a modulator of CPN activity toward large physiological substrates such as (DD)E, and potentially other fibrin degradation products. Therefore in blood or plasma, the activity of CPN toward these substrates is altered from its activity in purified, isolated form. Experiments *in vitro* demonstrate an inhibition of CPN antifibrinolytic activity by this free CPN2 subunit, although the mechanism of inhibition has yet to be determined. This inhibition explains the inconsistencies between the *in vitro* and *in vivo* experiments.

There have been several experiments that concluded a lack of antifibrinolytic activity by CPN. When purified CPN was added to whole blood clots bathed in plasma, clot lysis was only very minimally delayed; and after 1.5 hours of incubation, the extent of clot lysis was identical in the presence or absence of purified exogenous CPN (*90*). Furthermore, addition of a CPN-specific inhibitor to clots formed from plasma did not potentiate fibrinolysis (*165;166*). Although experiments (such as these) performed in plasma are physiologically relevant, because *in vivo* CPN would be under the regulation of the plasma inhibitor, unfortunately, the actual ability of CPN to attenuate fibrinolysis is not demonstrated, but rather the overall fibrinolytic capacity is examined. Additionally, the extent of inhibition by circulating CPN2 is unknown, as the concentration of this inhibitor has not yet been determined. Similar problems exist with the experiments that compared the removal of carboxy-terminal lysine residues from fibrin clots by CPN and TAFIa, because these studies were performed in whole blood, and therefore CPN2 was also present and capable of inhibiting CPN activity (*90*).

Interestingly, both purified and plasma CPN constitutively remove the carboxy-terminal lysine residues of plasminogen receptors on leukocytes (*399-401*). Circulating CPN2 does not appear to inhibit the activity of CPN toward these substrates. Therefore CPN in a plasma environment can attenuate plasminogen activation on the cell surface, but not on the fibrin surface. This demonstrates the role of CPN2 as a modulator of CPN activity, as opposed to a role as a strict inhibitor.

Although CPN added to clots formed from TAFI-depleted plasma did not result in an appreciable attenuation of clot lysis unless excessive (supra-physiological) concentrations of CPN were added, much lower concentrations of CPNc produced a significant attenuation of clot lysis (*166*). The antifibrinolytic effect of CPNc is dependent upon the concentration of tPA, and follows a threshold mechanism (*165;166;181;402*). Curiously, the large increase in activity of CPN toward fibrin following plasmic cleavage is not paralleled by an increase in activity toward small chromogenic substrates (*166*). The changes in the activity of CPN caused by plasmic cleavage may be due to steric effects. The release of the carboxy-terminal docking peptide from CPN2 may increase access to the active site groove of CPN1 (Figure 4.2). The magnitude of the effect caused by plasmin would therefore vary, depending on the size of the substrate. Smaller substrates, such as FA-Ala-Lys, would have little steric interference in the presence of the docking peptide; therefore its removal would have minimal effect on the activity of CPN toward these types of substrates. In contrast, larger substrates, such as fibrin degradation products, would have limited access to the active site of native CPN; therefore plasmic cleavage could result in greater access, and greater activity of CPN toward these substrates.

The clinical relevance of examining CPN2-mediated inhibition of CPN and CPNc antifibrinolytic activity is that a patient with a high concentration of CPNc may demonstrate a lower rate of fibrinolysis compared to a patient with a lower concentration of CPNc, but with an equivalent total concentration of CPN. The CPN assays developed to date examine the concentration of CPN as a function of CPN activity, but only toward small synthetic substrates that are unaffected by the plasmic activation of CPN. Current methods of CPN determination may indicate that two people have identical CPN activity, and antigenic assays may concur, however the fibrinolytic state of these patients may be dramatically different, even if all other coagulation and fibrinolytic enzymes are the same.

Currently, the role of plasmic activation of CPN during fibrinolysis is unclear. CPN is activated by plasmin to CPNc *in situ* during clot lysis; however, this activated CPN does not have the same antifibrinolytic effects as does pre-activated CPNc. Walker *et al.* hypothesized that plasmin cannot activate CPN locally to antifibrinolytic concentrations (*166*). Furthermore, it is also possible that plasmin is less able to activate CPN in the

presence of bound CPN2, therefore the degree of CPN activation may be lower than that expected based on activation kinetics *in vitro* in the absence of CPN2. Therefore the physiological relevance of plasmin-cleaved CPNc in attenuating plasminogen activation is unknown, but it has been suggested that CPN may demonstrate a low-level but long-term antifibrinolytic activity, to complement the activity of TAFIa, which demonstrates a high-level short-term antifibrinolytic activity (*166*).

4.9. The quaternary structure and regulation of CPN is clinically relevant

The currently used methods for the determination of CPN activity are inadequate, as described in section 1.1.4.4.1.; furthermore, determination of the activity of CPN toward a single substrate may not be sufficient to describe the true activity of the enzyme. As mentioned in section 4.7., the existing methods of CPN determination may indicate that two patients have identical CPN activity, and antigenic assays may concur, however the fibrinolytic state of these patients may be dramatically different, even if all other coagulation and fibrinolytic enzymes are the same. Similarly, the activity of CPN toward various other physiological substrates may vary widely between these two patients. The difference in activity could be related to regulation by plasmic cleavage and/or inhibition by circulating CPN2. These mechanisms may also be temporal forms of regulation, and the activity measured in a certain person may vary with time, depending on their physiological state. A better understanding of the forms and activity of CPN toward all of its substrates, and an improved method of CPN determination could lead to important, clinically-relevant discoveries.

An ideal CPN assay (which to date has only been partially developed in the Bajzar Lab) would include antigenic determination of the concentration of CPN, including separate measurements using antibodies with epitopes selective for plasmin-cleaved CPNc and for isolated CPN2. These would determine the total CPN concentration, as well as the concentrations of CPNc and the CPN2 inhibitor. Subsequently, the activity of CPN would be measured using substrates toward which CPN has variable activity depending on its activated/inhibited state. Currently, the activity of CPN toward physiological and synthetic substrates based on its activated/inhibited state is unknown, therefore these types of kinetic calculations would need to be determined in order to interpret the results of this hypothetical assay. Ideally the substrates chosen for the assay would be suitable for an easy, accurate, kinetic method (possibly by using the coupled lysine detection and

arginine kinase assays). To overcome the common error of previously developed assays, the absolute activity of CPN should be determined through measurement of the patient plasma/serum samples, as well as measurement of autologous samples which have been variously spiked with purified CPN, CPNc and CPN2 in order to neutralize any inconsistencies due to endogenous substrates present in the samples.

This method could be used to compare healthy control patients to those with diseases and disorders, such as those listed in section 1.1.4.4.1.. From the point of view of fibrinolysis, it would be valuable to measure the concentration and activity of CPN in patients suffering heart attack, stroke, pulmonary embolism, or deep vein thrombosis. There may be a correlation between the CPN activity (or the degree of plasmic activation, or the concentration of inhibitor) to the rate or degree of recovery of these patients. If this theory holds true, potential therapeutics could be developed to alter the activity of CPN during the treatment of certain disorders and illnesses.

Additionally, this could change the procedures for blood donor screening, blood collection, or blood product transfusion. It was recently discovered that storage of platelet concentrates and bedside leukoreduction of platelet concentrates transiently generates bradykinin and desArg-bradykinin, which can lead to severe hypotension in the recipient (*424*). This is particularly a problem with patients undergoing ACE inhibitor therapy (which decreases the rate of bradykinin degradation). Adverse reactions do not occur in most patients, but in those that do, the reaction is unpredictable. Currently there is no definitive knowledge on which patients will develop problems, and therefore no techniques to screen for patients which might develop transfusion-induced hypotension. The differential outcomes could be due to variable form, activity or concentration of CPN or its inhibitor in these patients, all of which would alter the rate of degradation of bradykinin. If this is true, patients could be screened for CPN and CPN2 concentration and activity, and this could identify patients at risk for hypotensive transfusion reactions. Similar reactions could also occur with respect to other CPN substrates, for example, in patients suffering massive hemorrhage and receiving blood transfusions, the rate of fibrinolysis may be attenuated if the blood products contain large concentrations of active CPN or lower than normal concentrations of its inhibitor.

Also, additional blood screening could be performed, or additional medications could be given to patients undergoing cardiopulmonary bypass. During extracorporeal circulation, blood oxygenators can cause the activation of complement, producing the anaphylatoxins C3a, C4a and C5a (*425*). These inflammatory mediators are inactivated by CPN, therefore the concentration, activity and degree of inhibition of CPN may affect the inflammatory state of the patient. This would be particularly important for patients requiring non-autologous blood transfusions during the procedure. The blood products could be screened for CPN activity toward the anaphylatoxins to ensure adequate inactivation kinetics.

4.10. Future directions

The work presented here sheds some light onto the structure, activity and regulation of CPN, as well as increasing the understanding of the regulation of fibrinolysis; however, there is still a great deal to learn about these topics. In section 4.6 a hypothetical model of the structure of CPN is presented. This model comes from an amalgamation of information: a crystal structure of the isolated, truncated, recombinant two-chain CPN1, molecular threading of the LRR sequence of CPN2 onto a structure of the Nogo receptor-1, *ab initio* folding models of the 13 kDa docking peptide of CPN2, and docking experiments of the 13 kDa peptide onto CPN2. As the vast majority of this model is computer-generated, it is possible that the actual structure may vary significantly from what was presented here. Therefore, ideally the x-ray crystal structure of multimeric CPN isolated from human plasma would be the next step in the structural determination of CPN. Determination of the crystal structure of plasmin-cleaved CPNc would also be an important next step, and could lead to further hypotheses with respect to the regulation of CPN by plasmin (or other serine proteases).

CPN2 is present in human plasma in excess of CPN and can alter the activity of CPN toward certain substrates. It would be beneficial to resolve the structural information regarding this free CPN2, as it could lead to new ideas on the mechanism of CPN2-mediated inhibition of CPN. For example, does the excess CPN2 circulate as monomers, dimers or higher multimers? If circulating as monomers, how does the conformation of CPN2 change to sufficiently hide its large hydrophobic region? If circulating as multimers, how many subunits are present and how do they interact? Also, what is the strength of interaction between the CPN2 subunits, and how does this compare to the

interactions within multimeric CPN, or between multimeric CPN and inhibitory CPN2 subunits?

Some of these questions could be solved by gel filtration and MALLS analyses. The multimeric state of isolated CPN2 could be determined using these methods (assuming that aggregation does not occur during the purification process). To determine the basic mechanism of interaction (hydrophobic versus electrostatic), ionic and non-ionic detergents can be used to attempt separation. Further information regarding the location of interaction of the subunits could come from truncation mutants. Although not presented in this thesis, I have created truncation mutants of the various domains of CPN2 (Figure 4.12.). The ability of these domains to interact with a full-length native CPN2 subunit could help pin-point the location of interaction of the inhibitor subunits. If free CPN2 circulates as a monomer, its structure would likely be altered to hide the large hydrophobic regions that would be exposed if the monomer circulated in the same conformation as in the predicted dimer (Figure 4.3). Major structural rearrangements such as conversion from β-sheets to α-helices could be determined by circular dichroism. The strength of interaction between the inhibitor subunits could be determined by small angle x-ray scattering and circular dichroism, or if appropriate fusion proteins can be designed, fluorescence resonance energy transfer or fluorescence anisotropy.

Other relevant structural information that remains to be determined relates to the interaction of CPN with the inhibitory CPN2 subunits. Currently, hypotheses regarding the mechanism of interaction and inhibition are purely speculative; however more information regarding the interaction of the enzyme and inhibitor could paint a clearer picture. Particularly, does the inhibitor block the access to the active site groove of CPN? Also, is the interaction through hydrophobic or electrostatic forces? Does recombinant CPN2 behave similarly with respect to structure and interactions with CPN as does CPN2 isolated from plasma? These questions could be answered using the above-mentioned techniques, such as gel filtration and MALLS, circular dichroism, and CPN2 truncation mutant studies. Additionally, a crystal structure of the inhibitor in complex with CPN would be ideal.

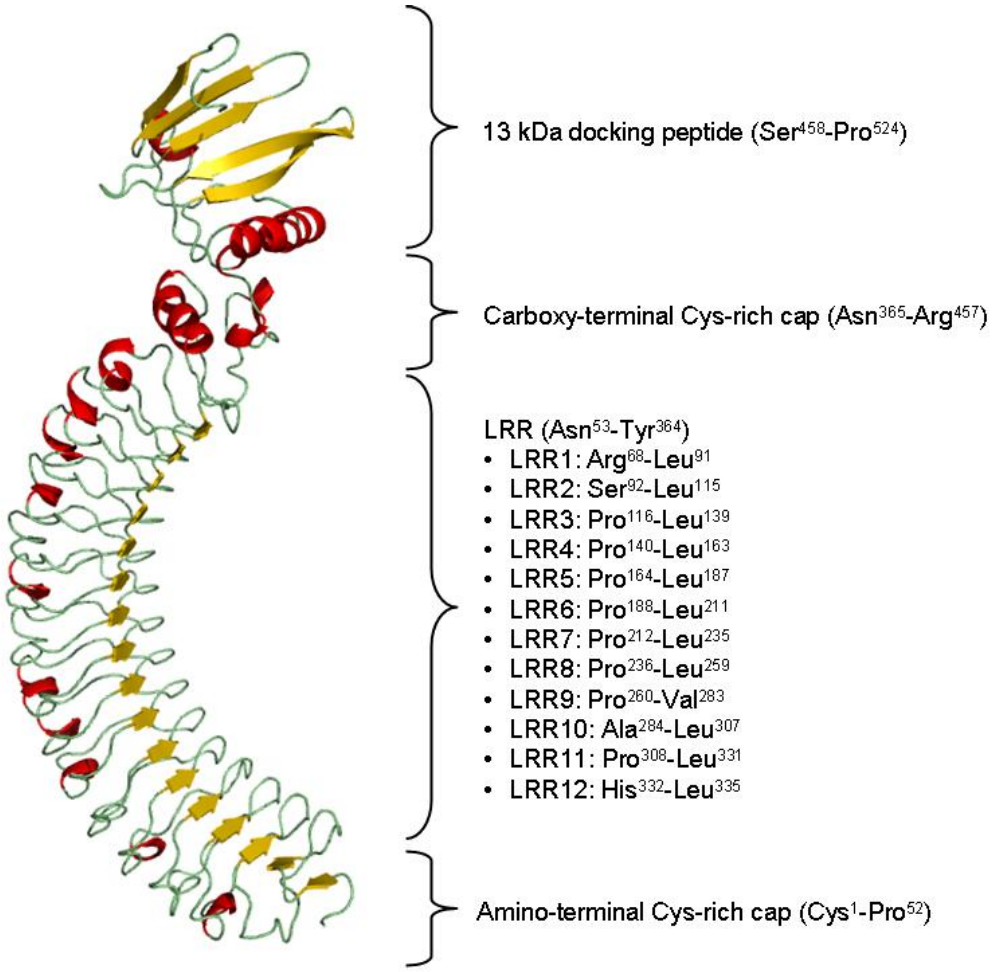

Figure 4.12: Domains of the CPN2 subunit.

The mechanism of modulation of CPN activity by free CPN2 can be further clarified by kinetic studies. Particularly, determination of the inhibitory kinetics of CPN2 toward CPN activity with all of its known substrates, determination of the inhibitory kinetics of CPN2 toward plasmin-cleaved CPNc, and determination of the inhibitory kinetics of plasmin-cleaved CPN2c toward CPN and CPNc. All of these forms (CPN, CPNc, CPN2, CPN2c) could be present in plasma at various concentrations and could therefore affect the apparent activity of CPN. Additionally, plasmin is not likely the only plasma serine protease with reactivity toward CPN. The kinetics of CPN cleavage by neutrophil elastase, kallikrein, thrombin, and other plasma proteases should be determined. Also, the cleavage kinetics should be determined for CPN2 by these enzymes, as well as determining any enhancement or reduction in the rate of cleavage caused by the

association of CPN1 and CPN2. Finally, the effect of proteolytic cleavage of CPN on its activity toward its various substrates should be determined.

Although not presented in this thesis, I have expressed (hypothetically) non-plasmin-cleavable mutant CPN subunits in mammalian cell culture. These could be purified and compared to recombinant or plasma-purified CPN with respect to activity, particularly in regards to any increase in activity during the course of fibrinolysis. Plasmin generated during fibrinolysis could cleave native CPN, forming the more active two-chain CPNc. Comparing non-plasmin-cleavable CPN to native CPN could help determine the extent of plasmin-mediated enhancement of CPN antifibrinolytic activity during the course of fibrinolysis.

Additional clinically-relevant experiments could include the development of an assay for the determination of the concentration and activity of CPN and CPN2, as described in detail in section 4.9.. This assay could be used to measure samples from a general healthy population and from patients with a variety of infectious, inflammatory, malignant and circulatory disorders. It would also be interesting to determine if other mammalian species also have a circulating free CPN2 inhibitor (and if it has similar activity to that seen in humans), or if this modulator is unique to humans.

To help explain how the mechanism of CPN2-mediated modulation of CPN activity originated, analysis of the intracellular trafficking and secretion of CPN subunits would be beneficial. It is currently unknown where the CPN subunits associate – whether this is an intracellular or extracellular process. If CPN2 is expressed in a large excess to CPN1 and is allowed to be secreted from the cells in isolation, this could explain the presence of this interesting inhibitor. The intracellular trafficking of CPN and CPN subunits could be monitored in fixed native liver cells by fluorescently-labelled antibodies raised toward CPN1, CPN2, and multimeric CPN. Additionally, although not presented here, I have cloned and expressed CPN1- and CPN2-GFP fusion proteins in mammalian cell culture, and the intracellular trafficking of these subunits could be examined in live cells via fluorescence and/or confocal microscopy. The role of glycosylation in the trafficking and multimerization of CPN could be examined similarly in cells grown in the presence of glycosylation inhibitors. Knowledge of how and where CPN is formed can lead to possible methods of CPN inhibition or activation.

4.11. Conclusions

CPN was discovered in 1962, and despite the almost five decades worth of research since then, there is still a great deal to be learned about its structure, activity, and regulation. The lack of information is compounded by what we suggest to be many erroneous conclusions that have been made over the years; many of these inaccuracies are not due to any experimental error, but rather due the tendency of scientist to be reductionists, examining individually the pieces of the puzzle. Unfortunately, due to the intricacies of CPN and its complex regulation, it must be examined as a whole, or we risk overlooking important information.

After re-evaluation of the structure and stoichiometry of CPN and its constituent subunits, it can now be concluded that CPN is a trimeric enzyme, composed of a single catalytic subunit and two regulatory subunits. Its structure is sensitive to proteolytic cleavage by plasmin, and both types of subunits (catalytic and regulatory) are digested. Plasmic cleavage enhances the activity of CPN toward certain substrates, such as large physiological compounds like (DD)E, and can therefore be considered as a form of activation of the enzyme. This activation is not required to induce CPN activity toward all substrates; in some cases, plasmic cleavage has no effect on the rate of catalysis, while in other cases, it results in a dramatic increase in the catalytic efficiency.

CPN is further regulated by a circulating inhibitor or modulator of its activity. This negative regulation is due to an interaction with a protein that is either derived from the CPN2 regulatory subunit, or exists as this subunit in its entirety. This inhibitor may circulate as a monomer or a multimer and may be prone to proteolysis by plasmin. Plasmic cleavage of the inhibitor could constitute a further mechanism of regulation; however, these details have not yet been determined. Interaction of CPN with its inhibitor affects only certain activities of CPN; for example, the activity of CPN toward FA-Ala-Lys is unchanged, while the activity toward (DD)E is significantly reduced.

It is likely due to the presence of this inhibitor in plasma that the antifibrinolytic activity of CPN was not previously identified. The experiments that demonstrated a lack of antifibrinolysis by CPN were performed in plasma or whole blood, which contains the CPN inhibitor. Following immunodepletion of the inhibitor, the role of CPN in fibrinolysis of reconstituted plasma clots becomes evident. CPN can now be defined as an

antifibrinolytic enzyme that is positively regulated by plasmic activation, and negatively regulated by a circulating inhibitor.

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