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Structure and Function Investigations of Succinyl-CoA Synthetase

by

Darrin Leslie Bailey

A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Biochemistry

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"That which you are able to do may seem small compared to with what others accomplish, or compared with what you would like to do - but your little, if done well, may count for more than some ambitious task, poorly done."

Paul S. McElroy

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Structure and Function Investigations of Succinyl-CoA Synthetase* submitted by Darrin Leslie Bailey in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abstract

Succinyl-CoA synthetases (SCS) from mammalian pig heart and the bacterium, *Escherichia coli* are diverse with respect to a variety of properties. These differences have been investigated to extend our understanding of the structure and function relationships in this enzyme.

Several cDNAs encoding both the newborn and adult forms of the pig heart SCS ß subunit precursor were isolated and characterized. The deduced amino acid sequences were found to be identical. All clones used a CTG codon as a potential start site for translational initiation and contained a resultant 22 amino acid presequence for targeting into the mitochondrial matrix. The coding sequence for the mature ß subunit did not, however, contain a codon for tryptophan as had been expected from the results of previous studies by others. This was confirmed by tryptophan-specific fluorescence and amino acid compositional analyses that were carried out on highly purified samples of pig heart SCS which indicated that no tryptophan exists in this enzyme.

The functional properties of the cloned α and ß subunits of pig heart SCS were assessed through their expression in a bacterial system. Refolding isolated pig heart SCS subunits with the cloned subunits fused with protein A indicated that these complexes were able to demonstrate GTP-specific SCS activity. Coexpression of the cloned pig heart subunits resulted in the production of a GTP-specific isozyme with high catalytic competency (20 units/mg).

The tetrameric structure of *E. coli* SCS that was proposed by crystallographic studies was probed through disruption mutagenesis of the interface linking αß dimers in such a structure. The systematic removal of interactions responsible for inter-dimer associations, along with the introduction of steric and charge interference failed to perturb the quaternary structure of the enzyme from tetramer to dimer. These results suggested that an alternative arrangement of αß dimers might constitute the correct

overall tetrameric structure of the *E. coli* enzyme. This was confirmed through the successful disruption of the alternative tetramer by mutagenesis directed to the αß dimer-dimer interface in this structure. The resulting dimer mutant of *E. coli* SCS displayed full catalytic activity (55 units/mg). Like the wild type enzyme, the mutant demonstrated the ability for ATP to facilitate de-thiophosphorylation of the enzyme, an observation that had previously been attributed to a hypothetical mechanism involving alternating sites cooperativity. Instead, these results indicate that *E. coli* SCS acts as a dimer of independently active αß c

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List of Symbols and Abbreviations

αKDC	alpha-ketoglutarate dehydrogenase complex
Amp	ampere
Å	angstrom
A_{600}	absorbance at 600nm
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
ATPγS	adenosine 5'-*O*-(3-thio)-triphosphate
bp	base pair
C-	carboxy-
cDNA	complementrary DNA
CoA	coenzyme A
CNBr	cyanogen bromide
Da	daltons
DTT	dithiotheitol
EDTA	ethylenediaminetetraacetate
FPLC	fast performance liquid chromotography
g	gravity (9.81 m/s^2)
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
ITPG	isopropyl-ß-D-thiogalactopyranoside
ITP	inosine 5'-triophosphate
kbp	kilobase pairs
kDa	kilodaltons
mRNA	messenger RNA
N-	amino-
NAD or NADH	nicotinamide adenine dinucleotide
NDP	ribonucleoside 5'-diphosphate
NMR	nuclear magnetic resonance
NTP	ribonucleoside 5'-triphosphate
OD_{600}	optical density at 600nm
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
Phα57	alpha-57 subunit from pig heart SCS

Phα108	alpha-108 subunit from pig heart SCS
Phß	beta subunit from pig heart SCS
P_i	phosphate
PMSF	phenylmethylsulfonylfluoride
PRR	proton relaxation rate
$-PSO_2$	incorporated thiophosphate
PVDF	polyvinylidene difluoride
RBS	ribosome binding sequence
rpm	rotations per minute
S	Svedberg unit (10^{-13} seconds)
$_{20,w}$	sedimentation coefficient (corrected)
SCS	succinyl-CoA synthetase
SDS	sodium dodecyl sulfate
T7ø10	gene 10 promoter from phage T7
TIR	translation initiation region
Tris	tris-(hydroxymethyl)aminomethane
UTP	uridine 5'-triphosphate
v/v	volume per volume
W	watt
w/v	weight per volume

Chapter 1

AN INTRODUCTION TO SUCCINYL-CoA SYNTHETASE

I. INTRODUCTION

For over 50 years, succinyl-CoA synthetase (SCS) has been the focus of a vast array of research. From its original discovery to the recent determination of the three-dimensional crystal structure of the *Escherichia coli* enzyme [1], work on SCS (also known as succinate thiokinase) has prompted several reviews [2-5] and over 150 articles. This work has investigated the metabolic roles of SCS, its diverse biochemical characteristics and molecular reaction mechanism. Through the application of molecular biology, the isolation of various genes of SCS has resulted in a shift towards research with an emphasis on structure. In light of previous work, this has enabled a broader, more detailed understanding of the molecular aspects of this enzyme.

II. METABOLIC ROLES

A. *The citric acid cycle - the forward reaction*

Historically, SCS was first demonstrated to play a role in the citric acid cycle by studies on the oxidative decarboxylation of α-ketoglutarate to succinate. Kaufman [6] observed that succinyl-CoA was formed as an intermediate, and thereby proposed a two-step process catalyzed by two different enzymes [7]. It was suggested that α-ketoglutarate dehydrogenase produced succinyl-CoA which was subsequently cleaved by succinyl-CoA synthetase to yield succinate and CoA. Earlier studies had indicated that inorganic phosphate was esterified during these reactions [8, 9]. It was also discovered that ATP was hydrolyzed to ADP and phosphate by the actions of these enzymes when in the presence of succinate and CoA [6]. SCS was found to both esterify and liberate phosphate in these reactions [7, 10]. The combination of these observations defined the overall reversible reaction, catalyzed by SCS, to be:

$$\text{Succinyl-CoA} + \text{NDP} + \text{P}_\text{i} \overset{\text{Mg}^{++}}{\rightleftharpoons} \text{Succinate} + \text{CoA} + \text{NTP}$$

where NDP and NTP are the purine ribonucleoside 5'-diphosphate and the 5'-triphosphate respectively. Succinyl-CoA synthetase (succinate:CoA ligase, EC 6.2.1.4 [GDP-specific] and EC 6.2.1.5 [ADP-specific]) couples the conversion of succinyl-CoA to succinate with the nucleoside diphosphate dependent esterification of inorganic phosphate. Since the energy of the thioester bond of succinyl-CoA ($\Delta G^{\circ\prime}$ = -8 kcal/mol) closely approximates that of the phosphoanhydride of NTP ($\Delta G^{\circ\prime}$(ATP) = -7.3 kcal/mol), the reaction can be regarded as an interconversion of high energy intermediates.

Within the citric acid cycle (**Figure 1.1, I**), SCS catalyzes the only 'substrate-level phosphorylation' step which directly yields a high energy phosphate bond. The forward direction of the reaction shown above participates in the citric acid cycle, and aids the oxidative decarboxylation of intermediates which enter this metabolic pathway. This results in the ultimate production of ATP used to drive a myriad of energy-requiring processes within the cell. It also represents the principle function of SCS for most organisms.

The production of NTP by SCS (the forward reaction), is apparent even in organisms which lack the citric acid cycle. Trichomonad flagellates are parasites which lack mitochondria but metabolize carbohydrates through a fermentative mechanism [11]. In organelles called hydrogenosomes, acetyl-CoA is converted to acetate by the enzyme acetate:succinate CoA-transferase [12]. Succinate is regenerated by SCS in these protozoans, with the energy of the thioester linkage being converted into the phosphoanhydride of ATP.

B. *Ketone body metabolism, biosynthesis and the reverse reaction*

The reversibility of the reaction catalyzed by SCS is crucially important to ketone body metabolism. Central to this pathway is a dependence on succinyl-CoA which is produced at the expense of NTP (in the reverse direction of the above reaction). Mammals generate the ketone bodies, acetoacetate and 3(ß)-hydroxybutyrate, in the liver

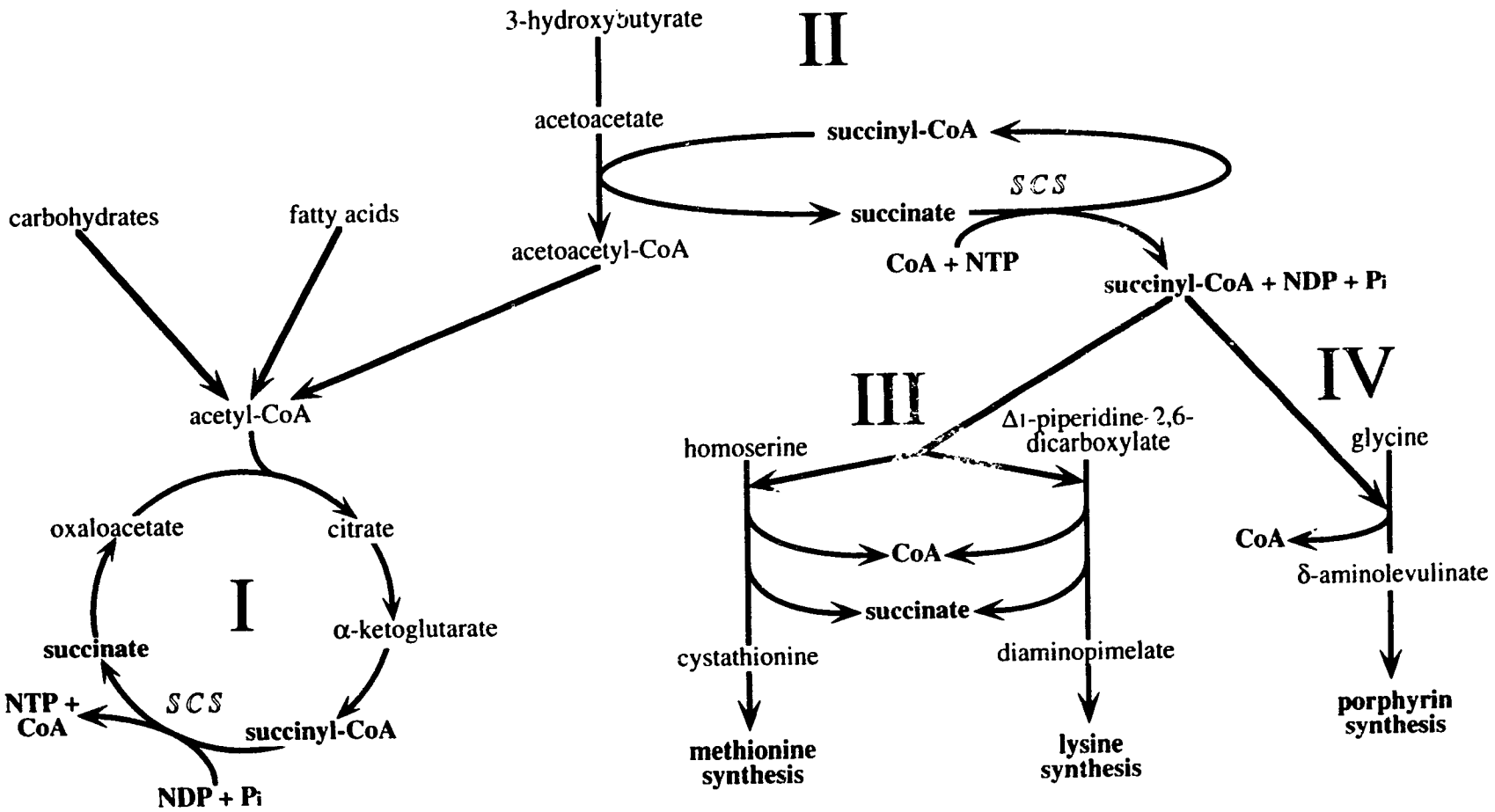

Figure 1.1 Metabolic pathways utilizing succinyl-CoA synthetase (SCS) for the production of either succinate or succinyl-CoA. The action of succinyl-CoA synthetase (indicated in outlined letters) is required for the hydrolysis of succinyl-CoA in the citric acid cycle (denoted by the roman numeral **I**). The enzyme has also been implicated for the synthesis of succinyl-CoA required during ketone body metabolism (**II**), the biosynthesis of the amino acids methionine and lysine (**III**), as well as the biosynthesis of porphyrins (**IV**). Derived from Voet and Voet [13].

when excesses of acetyl-CoA cannot be oxidatively decarboxylated by the citric acid cycle [13]. These fuels are used by the heart and renal cortex under normal conditions, or by the brain during fasting and in diabetic conditions. In order to be metabolized in these tissues, ketone bodies are converted back to acetyl-CoA. The critical step in this process is catalyzed by succinyl-CoA:3-ketoacid CoA transferase [14], which transfers CoA from succinyl-CoA to acetoacetate forming acetoacetyl-CoA (**Figure 1.1, II**). To ensure that ketone bodies are continually utilized, high levels of succinyl-CoA must necessarily be maintained [15, 16]. Since succinate results from the metabolic activation of these ketone bodies, SCS has been proposed to fulfill the role of replenishing the required succinyl-CoA levels [16].

Likewise, the production of succinyl-CoA is centrally important to the biosynthesis of methionine and lysine, and also porphyrin synthesis. In plants and bacteria, methionine synthesis is dependent upon the production of cystathionine. In turn, this essential precursor is formed by the succinylation of homoserine by succinyl-CoA, followed by the displacement of the succinate group by cysteine [13] (**Figure 1.1, III**). A similar mechanism is employed for the biosynthesis of lysine. Tetrapyrrole porphyrins including chlorophyll, cobalamin, heme and phycobilin are all derived from a single precursor, δ-aminolevulinate. This molecule is synthesized in animals and bacteria the condensation of succinyl-CoA and glycine [13] (**Figure 1.1, IV**). Although succinyl-CoA can be generated by other means including the α-ketoglutarate dehydrogenase reaction, the oxidation of odd-chain fatty acids and the degradation of some amino acids, it has been determined that SCS activity is required for the production of this anabolic precursor. Through observations made on anaerobic organisms impaired in their ability to oxidize acetate via the citric acid cycle, it was shown that SCS activity was still required for biosynthetic purposes [17].

III. BIOCHEMICAL DIVERSITY

A. *Sources of succinyl-CoA synthetase*

SCS has been investigated from a wide variety of sources including a large number of bacterial sources [18-20], *Trichomonas foetus* and *Trichomonas vaginalis* [21], *Trypanosoma brucei* [22], yeast [23], blowfly muscle [24] and many mammalian tissues [25-28] and several plant tissues [29-33]. However, the best characterized and most active preparations of SCS were derived from the bacterium *Escherichia coli* [34-39] and from pig heart tissue [40-44]. Several distinctive qualities of the enzymes from these two sources have been noted and are listed in **Table 1.1**. Subsequent to the identification of the genes encoding *E. coli* SCS [45], an overexpression system was developed [46] to both produce large quantities and aid in the future analyses of this enzyme.

B. *Substrate specificity*

A number of studies have described the substrate specificity of SCS. In both the *E. coli* [47] and pig heart [41] enzymes, the precursor of CoA, dephospho-CoA, was found to be a good substrate. Its precursor, 4-phosphopantatheine, was found to be a poor substrate for the *E. coli* enzyme [48]. The high specificity of SCS for succinate [10, 30, 36] was exemplified by studies testing a group of naturally occurring carboxylic acids. Malate at high concentrations (91 mM), the most reactive of these compounds, was only 3% as effective as succinate in net catalysis [48]. The derivative analogs, α-methylsuccinate and α-methylenesuccinate, were good substrates and are similar to succinate in reactivity [49]. Divalent cations play a role in the reaction catalyzed by SCS, with Mg^{2+}, Mn^{2+} and Co^{2+} being active in decreasing order of effectiveness [32, 33, 36].

The SCS enzymes can be sub-classified on the basis of nucleotide specificity: for all purines or for one purine. SCS from *E. coli* [50], *Rhodopseudomonas sphaeroides* [33] and a variety of other bacterial species [19, 51] comprise the first of these which

Table 1.1

Distinctive characteristics of succinyl-CoA synthetases from E. coli *and pig heart tissue*

Defining character	***E. coli* SCS**	**Pig heart SCS**
nucleotide specificity	'relaxed' ATP>GTP>ITP [50]	'restricted' ATP or GTP [25]
enzyme molecular mass	140 kDa [36, 37]	75 kDa [41]
subunit size	α = 29.5 kDa ß = 41.0 kDa [56]	α = 32 kDa ß = 42 kDa [43]
quaternary structure	$\alpha_2ß_2$ tetramer [58]	αß dimer [58]
subcellular location	cytoplasm	mitochondrial matrix
biogenesis of subunits	synthesized as mature subunits [45]	synthesized as precursor subunits [105]
coordination of subunit expression	translationally coupled [45]	unknown
folding and assembly - *in vitro* requirements	ATP, P_i (≤ 100% recovery) [64, 65]	polyethylene glycol or glycerol (≤ 60% recovery) [69]
- *in vivo* assisting factors	not chaperone mediated [63]	chaperones implicated [63]
Genetic polymorphisms	none reported	yes [25, 103]

demonstrate a 'relaxed' specificity. Its members are capable of accepting either ATP, GTP or ITP as a substrate. From a comparison of the kinetic parameters using each of these nucleotides, the *E. coli* enzyme exhibited an ordered specificity with ATP > GTP > ITP in order of effectiveness [50]. In contrast, a second group made up of some prokaryotes and all eukaryotic species of SCS studied to date, displayed a more restricted nucleotide specificity. Gram-positive bacteria [52], plants [29-33] and yeast [53] SCS were specific for ATP, whereas purified mammalian enzymes were shown to be specific for GTP or its 6-oxo precursor, ITP [54]. Pig heart SCS was found to utilize 8-aza-GTP [40] and 6-thio-GTP [41] in place of GTP. In addition, several studies have indicated the presence of distinct ATP- or GTP-specific isoenzymes of SCS in a number of diverse animal species (see **E** below).

C. *Subunit composition and quaternary structure*

From gel filtration chromatography of *E. coli* , the molecular mass of the enzyme was estimated to be 140-160 kDa [35-37]. Similar analysis of pig heart SCS determined this enzyme to have a molecular mass of 70-80 kDa, half that of the *E. coli* species [41, 43, 44]. While gram-negative bacteria contain SCS which was of a 'large' molecular size equivalent to that from *E. coli* [18, 51, 55], all gram-positive bacteria were found to contain 'small' forms of SCS which are comparable to that of the pig heart enzyme [18, 55].

Other eukaryotic enzymes including that from the yeast, *Saccharomyces cerevisiae*, are also similar in size to pig heart SCS [18, 27]. Surprisingly, SCS enzymes from the closely related Trichomonad species, *Trichomonas vaginalis* and *Trichomonas foetus* display heterogeneity with respect to their molecular size [21]. *T. vaginalis* has an apparent 'large' size of 150 kDa, whereas its protozoan cousin *T. foetus*, has a 'small' size of 70 kDa.

Denaturation of *E. coli* [56] and pig heart [43, 44] SCS revealed both enzymes were composed of two different subunits. The smaller subunit was designated the α

subunit and the larger was labelled the ß subunit. SDS polyacrylamide gel electrophoresis (SDS-PAGE) performed on *E. coli* SCS provided estimates of 29.5 kDa and 40 kDa for the α and ß subunits respectively [56]. Molecular masses of 32 kDa for the α subunit and 42 kDa for the ß subunit were reported for the pig heart SCS subunits, respectively [43]. Amino acid compositional analysis of the native *E. coli* enzyme indicated both subunits in the complex were present in equimolar amounts [4]. Considering the observed molecular weights of the enzymes, *E. coli* SCS was, therefore, a 'large' $\alpha_2\beta_2$ tetramer, while the pig heart SCS was a 'small' αß dimer. The possibility existed that the αß dimeric forms of SCS associated during catalysis to form tetrameric structures. To test this concept, gel filtration was performed on the pig heart enzyme in the presence of substrates [57]. No change was detected in the elution profile of the enzyme, suggesting the quaternary structure was unaltered. Moreover, active enzyme centrifugation further established that the *E. coli* enzyme was a non-dissociating tetramer and pig heart SCS was a non-associating dimer [58].

D. *Synthesis, folding and assembly*

The genes encoding the subunits from the bacterial forms of SCS have been cloned from *E. coli* [45], *Thermus aquaticus* B [59] and *Thermus flavus* AT-62 [60]. The organization of the ß and α subunit genes is similar in all three organisms. The termination codon of the upstream gene encoding the ß subunit overlaps the initiation codon of the downstream α subunit gene. It was suggested that this arrangement coordinates the biosynthesis of equimolar amount of ß and α subunits for subsequent assembly [45]. This is consistent with the observation that neither one of the subunits accumulated alone to any degree [61].

In *E. coli*, the two SCS genes are transcribed from the same polycistronic message as are seven other genes which contribute to three citric acid cycle enzymes [45]. Thus the bacterial cell can regulate the level of activity in this pathway directly through the expression of this transcript. Moreover, two components of the α-ketoglutarate

dehydrogenase complex are coexpressed with the SCS subunits. This may be significant since specific interactions have been observed between these two enzymes [62].

Extensive analysis *in vitro* has been directed toward the folding of the SCS subunits and the assembly of the *E. coli* enzyme. These studies have been simplified since these processes do not require the assistance of molecular chaperones [63]. Purified subunits, isolated from the denatured native enzyme, can reconstitute active tetramers following dilution or removal of the denaturant [64]. Refolding is markedly enhanced by the presence of ATP or phosphate, suggesting productive intrasubunit interactions are facilitated by occupation of the phosphoryl binding site [64, 65]. The reconstitution of *E. coli* SCS was indicated to be a two-step process [66]. The first event during renaturation occurred rapidly (within minutes) and represents the attainment of secondary structure and the association of subunits into a tetrameric structure. The following reactivation of the enzyme was a much slower step in the refolding pathway which involves small conformational rearrangements. This latter phase of recovery was determined to be the step influenced by the presence of ATP. Nearly complete levels (>90%) of original activity can be recovered from denatured *E. coli* enzyme.

In part due to the complexity of eukaryotic systems, much less is known about the folding and assembly of the dimeric mitochondrial SCS. Mammalian subunits destined for the mitochondrion are synthesized as higher molecular weight precursor proteins [67]. An amino (N-)terminally located polypeptide sequence serves to direct the translocation of precursor subunits through the mitochondrial membranes and into the matrix [68]. These 'signal sequences' are removed from the precursors likely by a matrix processing protease, resulting in mature subunits [68]. Although no information is available on the subsequent assembly of the dimeric enzyme *in vivo*, it has been suggested that molecular chaperones may participate in this and perhaps other translocation events [63]. Nevertheless, *in vitro* studies have been conducted on the refolding of denatured subunits isolated from pig heart SCS [69]. These renaturation experiments clearly demonstrated

that the folding and assembly of this dimeric enzyme was different from that of the *E. coli* tetramer. First, the presence of the nucleotide substrate, GTP, had no effect on the refolding and assembly of the mammalian enzyme. Second, the reconstitution process required the presence of 20-25% (v/v) polyethylene glycol or glycerol. It was believed that this is reflective of the high protein concentrations found within the mitochondrial matrix. Third, a maximum of only 60% of the original activity could be recovered. This inefficient recovery of active enzyme implicated that other factors, possibly chaperones, may be necessary in the assembly process.

E. *Genetic polymorphism and functional roles of dimeric SCS*

More than one distinct species of SCS has been observed in a variety of animal species. Those occurrences were consistent with a genetic polymorphism of the dimeric enzyme in eukaryotes. For example, two discrete GTP-specific enzymes were isolated from mouse liver mitochondria [70]. These two forms were distinguishable on the basis of a number of criteria including pH stability, heat inactivation and their electrophoretic behavior. Furthermore, levels of one of these enzymes was elevated 300% under conditions of stimulated porphyrin synthesis. This indicated a role for GTP-specific SCS in the biosynthesis of these compounds. Although SCS from mammalian tissues had been generally regarded as GTP-specific [54], the initial characterization of the enzyme from muscle tissue supported ATP-linked activity [6, 7, 10]. Investigation of a wide variety of animal species including mammals [25, 71, 72] observed both ATP- and GTP-specific SCS activities. Furthermore, the different tissue sources examined displayed varying ratios of these two specificities [25, 72]. These specificities were shown to be attributed to two distinct and separable proteins, establishing that more than one species of SCS functions in mammalian tissues [25].

To rationalize the existence of distinct ATP- and GTP-dependent forms of SCS, it was proposed that these isozymes were designed to fulfill the different metabolic roles that SCS played in animal tissues [16]. For efficient ketone body breakdown, SCS must

maintain high levels of succinyl-CoA required (by CoA transferase) for the continual utilization of this pathway [15, 16]. Estimates of the GTP/GDP concentration ratio of approximately 100 appeared to be much greater than that of the ATP/ADP concentration of 1 in mammalian mitochondria [71]. As such, the higher chemical potential available to a GTP-specific isozyme in this environment was hypothesized to drive the replenishment of succinyl-CoA necessary for ketone body metabolism [16]. Support of this concept was demonstrated by increases in the level of the GTP-specific activity in diabetic animals, while alternatively the ATP-specific levels decreased [26].

The ATP-specific SCS appears suited to a role in the citric acid cycle. Since ATP/ADP concentration ratios were near unity in the mitochondria, an ATP-dependent enzyme would be more responsive to succinyl-CoA levels. Its operation, in the direction of succinate formation, would have been driven by the continual provision of succinyl-CoA by the preceeding oxidative steps. Evidence to support this theory was provided from studies with *Trypanosoma brucei* [22]. The life cycle of these parasites has two stages: a bloodstream form which has repressed citric acid cycle activity, and a procyclic form found in the insect gut which displays a fully functional cycle. When entering the procyclic stage, these parasites demonstrated increases in ATP-specific SCS alone [22]. This is consistent with a role for the ATP-specific enzyme in the citric acid cycle.

Regardless, together these reports have suggested that genetic polymorphism existed for SCS. Proof of this supposition was provided by indications of multiple genes for the α subunit in the protozoan parasite, *Trichomonas vaginalis* [73], and in the yeast, *S. cerevisiae* [23]. Knock out mutations of a known yeast α subunit gene failed to produce the expected phenotypic defect in respiration. This result was likely due to the presence of a duplicated and complementing α subunit gene since heterologous sequences in genomic DNA were detected under hybridization conditions of low stringency. Further to this point, two alternatively spliced cDNAs encoding the α subunit

of pig heart SCS were isolated from an expression library constructed from newborn pig heart tissue (see **V.A** below).

IV. THE HYPOTHESIZED CATALYTIC MECHANISM

A. *Exchange reactions and the overall mechanism*

The hypothesized reaction mechanism of SCS was defined from observations of enzyme catalyzed isotope exchange reactions. These occurred between [ß-^{32}P]-ADP and ATP (Mg^{2+} required) [30, 74], [^{18}O]-phosphate and succinate (ATP and CoA or succinyl-CoA required) [75] and [^{14}C]-succinate and succinyl-CoA (phosphate and Mg^{2+} required) [30, 74]. These experiments suggested that reaction intermediates were involved in overall catalysis. Furthermore, each exchange reaction was interpreted to represent a partial reaction in the complete mechanism.

Phosphorylated enzyme intermediates were predicted on the basis of exchange between ATP and ADP in the presence of Mg^{2+} [74]. This was confirmed in both the pig heart and *E. coli* enzymes by the covalent incorporation of [^{32}P] label from reaction with [γ-^{32}P]-NTP [76, 77]. The formation of the phosphoenzyme is favorable as exemplified by the equilbrium constants (K*eq*) of 2.1 and 31 for the mammalian [43] and *E. coli* [37] enzymes, respectively. The phosphorylated residue was identified to be a histidine [78, 79], and later to be a 3-phosphohistidyl isomer [80]. The phosphoenzyme was determined to be an obligatory intermediate in the reaction mechanism through steady state kinetic measurements [81].

Exchange reactions catalyzed by SCS between [^{18}O]-phosphate and succinate implicated a second reaction intermediate, succinyl phosphate [75, 82]. It was proposed that the oxygen atoms of phosphate could be directly interchanged with those of the carboxyl oxygens of succinate via the formation of the mixed anhydride, succinyl phosphate. The existance of this high energy intermediate was confirmed by Nishimura

and Meister [83] when they synthesized succinyl [^{32}P]-phosphate using succinate, [γ-^{32}P]-ATP and substrate quantities of SCS. The legitimacy of succinyl phosphate as a reaction intermediate was proven by its ability to drive the phosphorylation of SCS and the subsequent formation of ATP from ADP [83]. In the opposite direction, succinyl phosphate can, in the presence of CoA, drive the enzymatic synthesis of succinyl-CoA. However, the latter reaction can occur nonenzymatically [49], and its rate approximated that measured for the enzyme catalyzed partial reaction [48]. It is apparent from the measured rate constants that succinyl phosphate reacts faster with CoA than it dissociates from the enzyme [48]. This is consistent with the fact that the intermediate cannot be detected during net catalysis. These results suggest that the final partial reaction producing succinyl-CoA from succinyl phosphate and CoA likely occurs by an uncatalyzed mechanism on the enzyme.

Considering these results has led the enzymatic mechanism of SCS to be described by three sequential partial reactions:

$$E + NTP \overset{Mg^{2+}}{\rightleftharpoons} E\text{-}P + NDP$$

$$E\text{-}P + \text{succinate} \rightleftharpoons E\cdot\text{succinyl-}P$$

$$E\cdot\text{succinyl-}P + CoA \rightleftharpoons E + \text{succinyl-CoA} + P_i$$

where E-P is the phosphoenzyme intermediate and E·succinyl-P is the enzyme bound form of the intermediate, succinyl phosphate. (The direction as listed is opposite to that occurring in the citric acid cycle.) It is worth noting that each partial reaction contains a high energy form of a product and reactant. In this manner, no energy is lost in the overall transition from the phosphoanhydride of NTP to the thioester of succinyl-CoA.

B. ***Binding constants and kinetic pathways***

Through steady state kinetic analysis of SCS, the Michaelis constants for the variety of substrates have been determined for the enzymes from *E. coli* [84, 85], pig

heart [40] and artichoke [32]. These are listed in **Table 1.2**. Comparison of these Km values reveals that those for succinyl-CoA, CoA, ATP and ADP are one to two orders of magnitude smaller than those for succinate and phosphate. From the kinetic sequence defined for these enzymes [40, 84, 85], these differences are generally reflected in the order of substrate addition and product release. A lower Km value and therefore a higher enzyme affinity is observed for those substrates and products which contain a nucleotide moiety.

In the mechanism proposed for *E. coli* SCS [84, 85], ATP is the first substrate to bind to the enzyme while ADP is the last product to leave. This pathway is described as partially random, fully sequential and is outlined in Panel A, **Figure 1.2**. Succinate and CoA associate with the enzyme in random fashion forming a ternary complex with ATP before the reacted products, succinyl-CoA and phosphate leave the pathway.

The reaction scheme for the pig heart enzyme, determined from initial velocity and product inhibition studies, is ambiguous [40]. In random order, GTP and CoA add first to the enzyme while the products succinyl-CoA and GDP are last to dissociate randomly (Panel B, **Figure 1.2**). The kinetic analysis indicated that succinate, the third substrate, enters the reaction pathway either prior or subsequent to the release of the product, phosphate. However, net catalysis is dependent upon the formation of the reaction intermediate, succinyl phosphate. If phosphate left the pathway before succinate associated with the enzyme, abortive complexes would result preventing succinyl-CoA synthesis. Therefore the reaction pathway for the pig heart SCS outlined in dashes on Panel B, **Figure 1.2**, is highly unfavorable.

C. *Structural modification through catalysis*

A number of observations have concluded that the binding of substrates and the formation of reaction intermediates induce conformational changes in SCS. Structural alterations occurred upon phosphorylation of the enzyme. Rates of inactivation of *E. coli* SCS were higher for the dephosphorylated form as compared to the the phosphoenzyme.

Table 1.2

Michaelis kinetic constants of succinyl-CoA synthtase from E.coli, *pig heart and artichoke*

Enzyme source	Substrate					
	CoA	succinyl-CoA	ADP/GDP[1]	ATP/GTP[1]	succinate	phosphate (P_i)
E. coli [84, 85]	1.5×10^{-6} M	7.7×10^{-6} M	1.2×10^{-5} M	2.0×10^{-5} M	1.0×10^{-4} M	2.6×10^{-3} M
Pig heart [40]	2.0×10^{-5} M	3.0×10^{-5} M	8.0×10^{-6} M	1.0×10^{-5} M	8.0×10^{-4} M	6.0×10^{-4} M
Artichoke [32]	2.2×10^{-6} M	5.6×10^{-5} M	1.2×10^{-4} M	1.4×10^{-4} M	2.0×10^{-3} M	1.4×10^{-3} M

[1] The nucleotide used in kinetic anaylsis was dependent on the source tested. ADP and ATP were used as substrates for the *E. coli* and artichoke enzymes, while GDP and GTP were used for the pig heart enzyme.

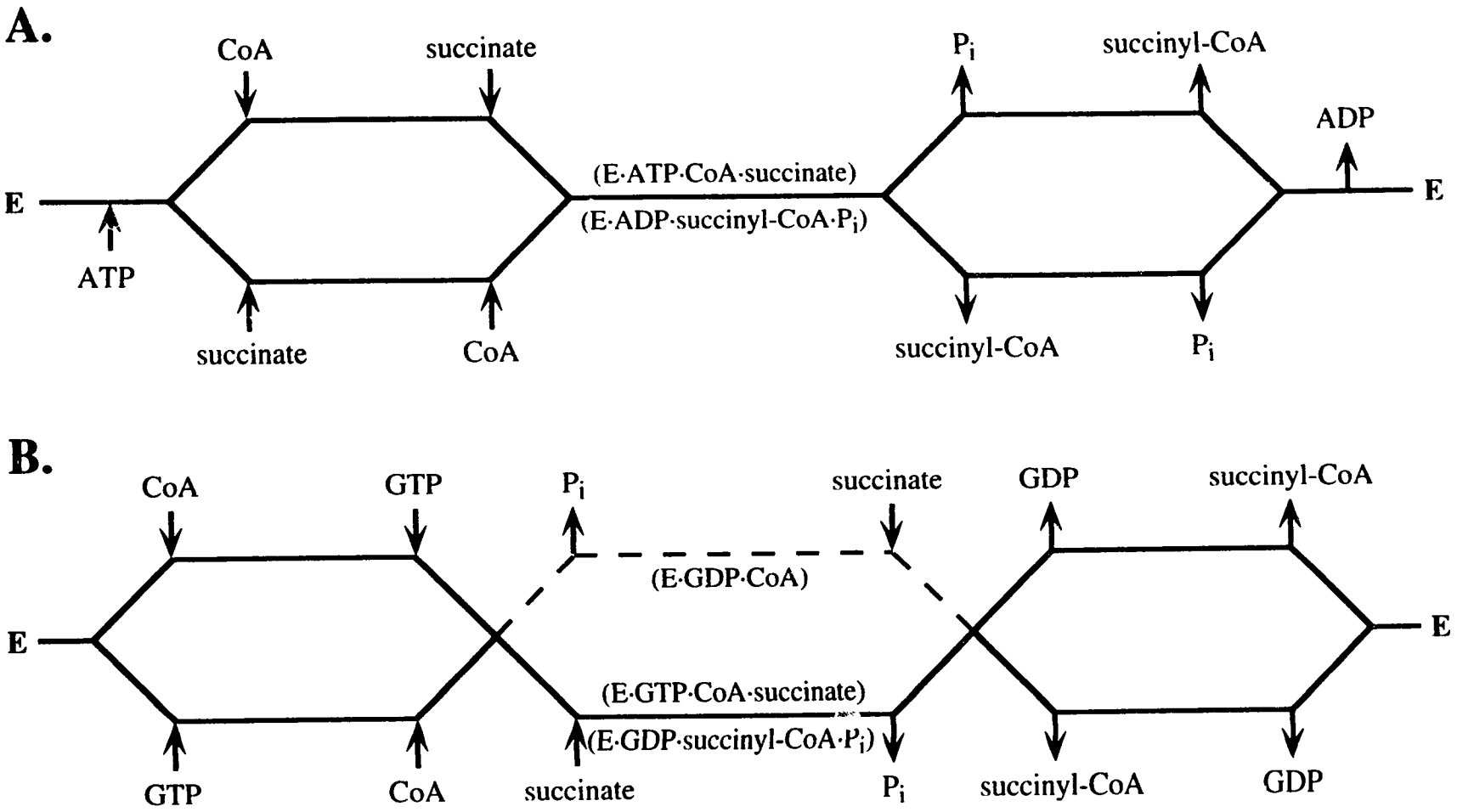

Figure 1.2 Reaction schemes for SCS from *E. coli* and pig heart. Steady state kinetic measurements [40, 84, 85] were used to deduce the order of substrate addition and product release from *E. coli* SCS (**A**) and pig heart SCS (**B**). The reaction scheme proceeds from left to right. Substrates associating with the enzyme (E) are indicated by arrows feeding into the pathway, whereas dissociating products are shown with arrows directed away from the pathway. The formation of the ternary complexes, E·NTP·CoA·succinate and E·NDP·succinyl-CoA·Pi, leads to productive enzyme catalysis. The dashed pathway for pig heart SCS allows phosphate (Pi) to dissociate before succinate binds, resulting in the formation of abortive complexes.

Treatment with trypsin has shown the level of digestion to have been two orders of magnitude greater for the dephosphorylated enzyme [86]. Since the phosphoenzyme was almost completely protected from proteolysis, phosphorylation of SCS appeared to shift the conformation of the enzyme into a more compact, protease resistant form.

The binding of CoA to *E. coli* SCS was observed to cause changes in the flexibility of the regions labeled with dansyl-chloride [87]. Subsequent studies have shown a decreased level of quenching of tryptophan fluorescence by acrylamide when either ATP or CoA was bound to the bacterial enzyme [88]. These fluorescence changes were consistent with modifications in the conformation of SCS.

NMR studies have also detected significant structural changes occurring at the active site of the enzyme. Using Mn(II) as a probe, measurements of the proton relaxation rate (PRR) were modulated by the binding of substrates [89]. CoA or succinyl-CoA increased the PRR enhancement, whereas succinate and CoA together produced a decrease. Thus, the conformation of the active site seemed to respond differently to the binding of various substrates. Other evidence suggesting that significant structural modifications occur during catalysis was provided by [^{31}P]-NMR studies on the phosphoenzyme of *E. coli* [90]. The binding of CoA to this form effected a broadening of the phosphohistidine resonance. This result was interpreted as a manifestation of two exchanging conformations; one competent to transfer the high energy phosphate to ADP, the other capable of phosphoryl transfer to succinate (in the direction of succinyl-CoA synthesis). In the presence of CoA and the competitive inhibitor, 2,2'-difluorosuccinate, the resonance narrowed suggesting the phosphoryl group was locked into an orientation permissive to succinyl-CoA formation.

These fluorescence and NMR studies detected little conformational change in the enzyme when succinate or its inhibitor analog were added alone [88-90]. This implied that the major structural transitions can be attributed to CoA binding and phosphorylation by ATP. Therefore, the enzyme appeared to be 'poised' for catalysis when

phosphorylated and bound with CoA. The final binding of 'low affinity' succinate to this poised enzyme may serve to couple the three partial steps during the reaction.

D. *Substrate synergism*

Ramaley *et al.* [37] noted that the exhange reaction between ATP and ADP occurred at a rate which was significantly slower than that of the overall reaction. However, the exchange in this partial reaction was stimulated by the addition of succinyl-CoA [37, 81]. This phenomenon is termed 'substrate synergism' and referred to the rate acceleration of a partial reaction by a substrate not directly involved. Enzymes demonstrating this effect are only fully active when all the binding sites are occupied by substrates. Substrate analogs, such as desulfo-CoA, were also able to elicit synergistic effects. This unreactive analog could dramatically stimulate the formation of ATP from ADP and succinyl phosphate [49] and also the synthesis of succinyl phosphate from ATP and succinate [91]. In addition, the production of succinyl phosphate from succinate and the phosphoenzyme was enhanced by the addition of ATP [90]. It has been suggested that the observed synergism is a result of conformational modifications which occur following the binding of all the substrates.

E. *Half-of-the-sites phosphorylation and alternative sites cooperativity in* E. coli *SCS*

The subunit composition of *E. coli* SCS implied this $\alpha_2\beta_2$ tetrameric structure contained two active sites, and, therefore, two sites of phosphorylation. However, a number of early studies observed only a single mole of phosphate incorporated per mole of enzyme if phosphorylation was attempted with ATP [35, 37, 86]. The apparent inability of SCS to become bis-phosphorylated was consistent with a concept of 'half-of the-sites' reactivity with respect to phosphorylation [86]. This observation implied that some asymmetry existed between the two αß dimer halves of the tetramer. Multisubunit enzymes displaying these characterisics may also demonstrate catalytic cooperativity. Bild *et al.* [57] tried to rationalize the tetrameric structure of *E. coli* SCS in terms of its

catalytic properties. They examined the steady state oxygen exchange between medium [^{18}O]-phosphate and succinate per mole of ATP cleaved. It was observed that while the dimeric pig heart enzyme exhibited no change in oxygen exchange as ATP concentrations were lowered, an increase was detected for *E. coli* SCS. They suggested, based on the idea of 'half-of-the-sites' phosphorylation, that the binding of ATP at one active site facilitates the formation and release of succinyl-CoA at the other catalytic site. In this alternating sites cooperative mechanism, subsequent phosphorylation at the first site, through reciprocal conformational change, would prepare this site for the next catalytic turnover. By using a hybrid species of *E. coli* SCS, Wolodko *et al.* [92] noted that [^{35}S]-labeled unphosphorylated α subunits became phosphorylated upon brief exposure to substrates. This result evinced the capacity for alternating activity between dimers. As well, it established that 'half-of-the-sites' reactivity was not a consequence of permanent asymmetry. Vogel and Bridger have provided corroborating evidence through the use of [^{31}P]-NMR [90, 93]. They observed a signal corresponding to succinyl phosphate only when ATP was added to a reaction solution containing phosphorylated enzyme and succinate. A schematic representation of this hypothetical model is presented in **Figure 1.3**.

A number of studies employed the slowly reacting analog, ATPγS, as a probe for subunit interactions in SCS. In *E. coli* SCS, the rate of discharge of the thiophosphate from 'monothiophosphorylated' enzyme (approximately one mole thiophosphate incorporated per $\alpha_2\beta_2$ tetramer) in the presence of succinate and CoA was stimulated by ATP [94]. Nonhydrolyzable analogs of ATP had no stimulatory effect on the discharge. This result implied that the phosphorylation event precipitated the reciprocating conformational changes necessary for catalysis at the second site. In the reverse direction, the interaction of thiophosphorylated enzyme with ADP to form ATPγS was stimulated by succinyl-CoA [95]. Thiophosphate discharge by this means, however, was not accompanied by phosphorylation at the second site. Positional isotope exchange

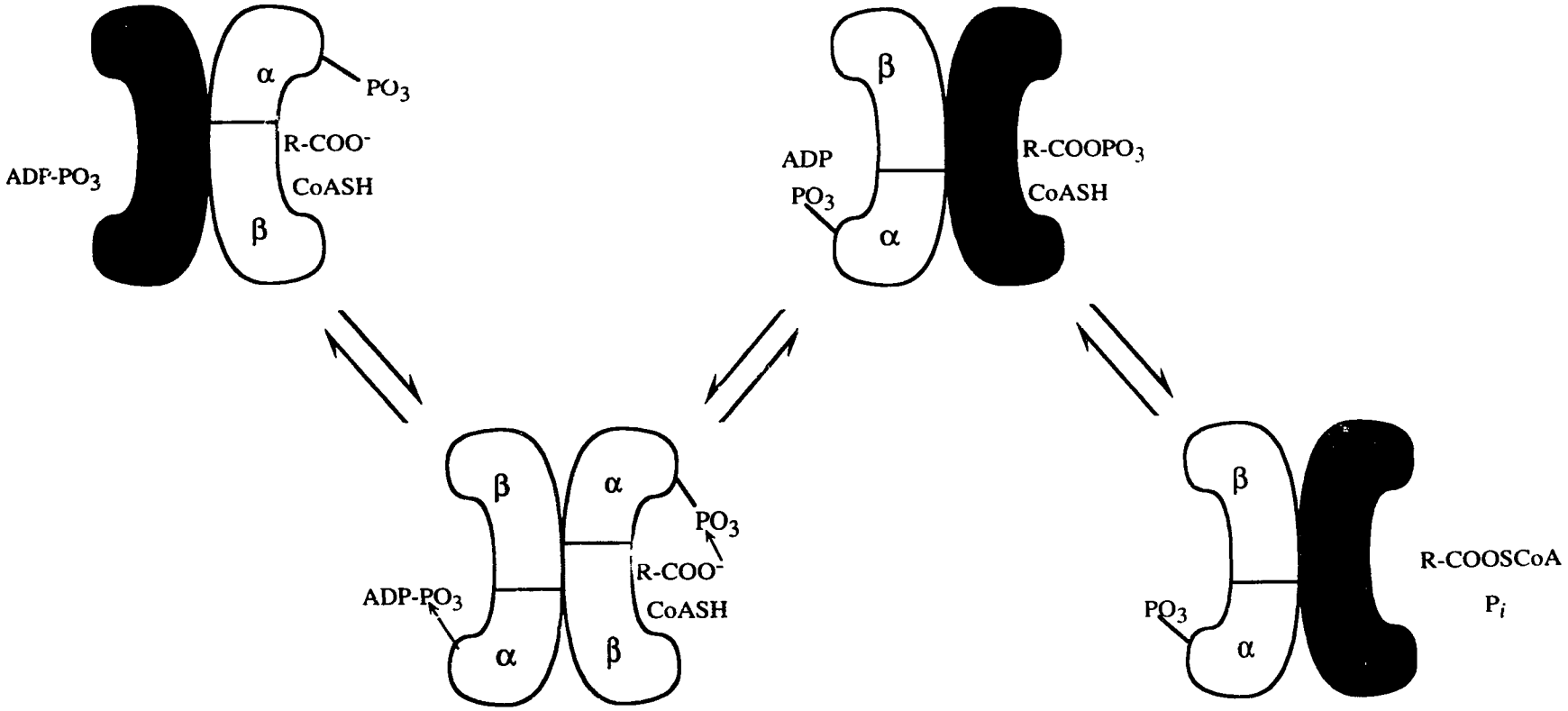

Figure 1.3 Alternating cooperative sites model for catalysis by *E. coli* SCS. The unshaded and darkly shaded shapes represent αß halves of the tetrameric enzyme with different conformations, which are proposed to alternate during the course of catalysis. The lightly shaded tetramer represents subunits with intermediate or transitory conformations. In the proposed catalytic route, phosphoryl transfer from ATP to the left-site is shown to be concerted with the phosphoryl transfer from the right-site to succinate ($R\text{-}COO^-$). Further intermediate steps between the formation of succinyl-phosphate and that of succinyl-CoA are omitted for clarity. Derived from Williams and Bridger [96].

experiments were carried out on 'monothiophosphorylated' *E. coli* SCS using ATP labeled with [^{18}O] at all oxygen positions on the terminal γ phosphate ([ß,γ-^{18}O]-ATP) [96]. No scrambling of isotope into the non-bridge position was detected except in the case where all three substrates were present. This indicated that no bis-phosphorylation occurred and scrambling was a result of discharge of thiophosphate by 'synergistically' acting substrates.

Although this preceeding evidence was highly suggestive, a number of confounding observations argued against an alternating sites cooperative mechanism. First, various reports of the phosphorylation levels achieved for *E. coli* SCS have indicated stoichiometries which approach two (instead of one) moles of phosphate per mole of enzyme [37, 38, 97-100]. Several of these reports have been the result of enzyme dephosphorylation prior to phosphorylation with ATP, or phosphorylation with succinyl-CoA and phosphate. This degree of phosphate incorporation was inconsistent with the 'half-of-the-sites' phosphorylation (one of the tenets of the alternating cooperativity proposal). Second, the interpretation of the results obtained from the [^{18}O]-exchange experiments, upon which the model was originally suggested, may be obscured due to technical limitations [95]. High concentrations of pig heart SCS enabled this enzyme to demonstrate exchange properties similar to the *E. coli* enzyme [93]. Third, de-thiophosphorylation experiments on pig heart SCS revealed that thiophosphate discharge in this dimeric enzyme was stimulated by GTP [101]. Since this de-thiophosphorylation effect was facilitated by NTP in both dimeric and tetrameric forms of SCS, it was suggested that the nucleotide (ATP or GTP) acts through a single site to mechanistically displace the covalently bound phosphate [5, 101]. Fourth, hybrid tetramers of *E. coli* SCS were created in which one of the catalytic histidine residues was inactivated through mutagenesis [99]. These mutant enzymes were catalytically active and exhibited properties of substrate synergism. From these studies it was concluded that the *E. coli*

tetramer is actually composed of two independent active dimers which associate to form a 'dimer of dimers'.

V. STRUCTURAL ASPECTS AND FUNCTIONAL CORRELATIONS

A. *Primary structure*

SCS has been characterized genetically from seven different sources [23, 45, 59, 60, 67, 73, 102]. The genes encoding the *E. coli* enzyme were the first to be identified [45]. They are located at the end of a gene cluster which encodes several other citric acid cycle enzymes. The *sucC* gene encodes the 388 amino acid ß subunit of SCS with a calculated molecular mass of 41.4 kDa. The *sucD* gene encodes the 288 amino acid α subunit of SCS which is 29.6 kDa. The molecular masses are consistent with values previously estimated from sedimentation and chromatographic studies [36, 37, 56].

Two different complementary DNA sequences (cDNAs) encoding the α subunit from pig heart SCS have been isolated [103] demonstrating the polymorphic nature of SCS within mammalian systems. The deduced proteins from these two messages are distinguished from one another by an internally replaced stretch of polypeptide sequence (see **Figure 3.1**, page 60). The two forms arise from an alternative splicing event of distinct exons during mRNA synthesis. One, labeled Phα57, contained a cassette of 57 nucleotides coding for a protein sequence which is identical with that from a cDNA encoding the rat liver α subunit. The calculated molecular mass of Phα57, at 32.1 kDa, was in agreement with that derived from chromatographic analysis of the denatured enzyme [43]. The generation of the alternative message, Phα108, was found to involve atypical mRNA splicing donor and acceptor sites [103], correlating with the decreased frequency of this message [104]. The spliced exon of 108 nucleotides coded for a novel polypeptide sequence that bears no similarity to any of the other cloned α subunits. It has

been suggested that the Phα108 protein is differentially expressed in fetal or newborn myocardial tissue, from whence it has been observed [104].

The presently available amino acid sequences for the α subunit of SCS were deduced from genes cloned from: *E. coli* [45], *T. vaginalis* [73], *T. flavus* [60], rat liver [67], pig heart [103, 104] and yeast [23]. An alignment of these highly conserved subunit sequences is found in **Figure 1.4**. Comparison of the amino acid sequence of the *E. coli* α subunit with those from dimeric mammalian sources (rat liver, pig heart) revealed that the amino acid sequences were about 70% identical and 90% similar when conservative replacements are included [67] (see **Figure 3.1**, page 60).

Of the seven sources from which α subunit genes have been isolated, in only three have the genes encoding the ß subunit been identified. These included those from *E. coli* [45], *T. flavus* [60] and *T. vaginalis* [102]. SCS from each of these sources has previously been reported to be tetrameric in subunit structure. Thus, no ß subunit sequence representative of a dimeric form had thus far been cloned. An alignment of ß subunit protein sequences is presented in **Figure 3.4**, page 70. In constrast to the α subunit proteins, the primary sequence of the ß subunits were only 45% identical and 65% similar when conservative changes are considered. The residues in the carboxy-(C)terminal region of the ß subunits displayed more conservation than in the amino-(N)terminal region, implying a greater functional significance for the C-terminal section of the subunit. As apparent from the three dimensional crystal structure of *E. coli* SCS (see **B.** below), this region, along with the highly conserved α subunit, were found to make contributions to the active site.

It is worth mentioning that three codons for tryptophan were found in the gene sequence encoding the ß subunit of *E. coli* SCS [45]. This was in agreement with that determined from amino acid compositional analysis on the whole enzyme and isolated ß subunit [88]. The low Trp content of the enzyme accounted for the observed A_{280} (10 mg/ml) of 5.11 [37] and 4.9 [105]. On the basis of substrate-protected, quenching of

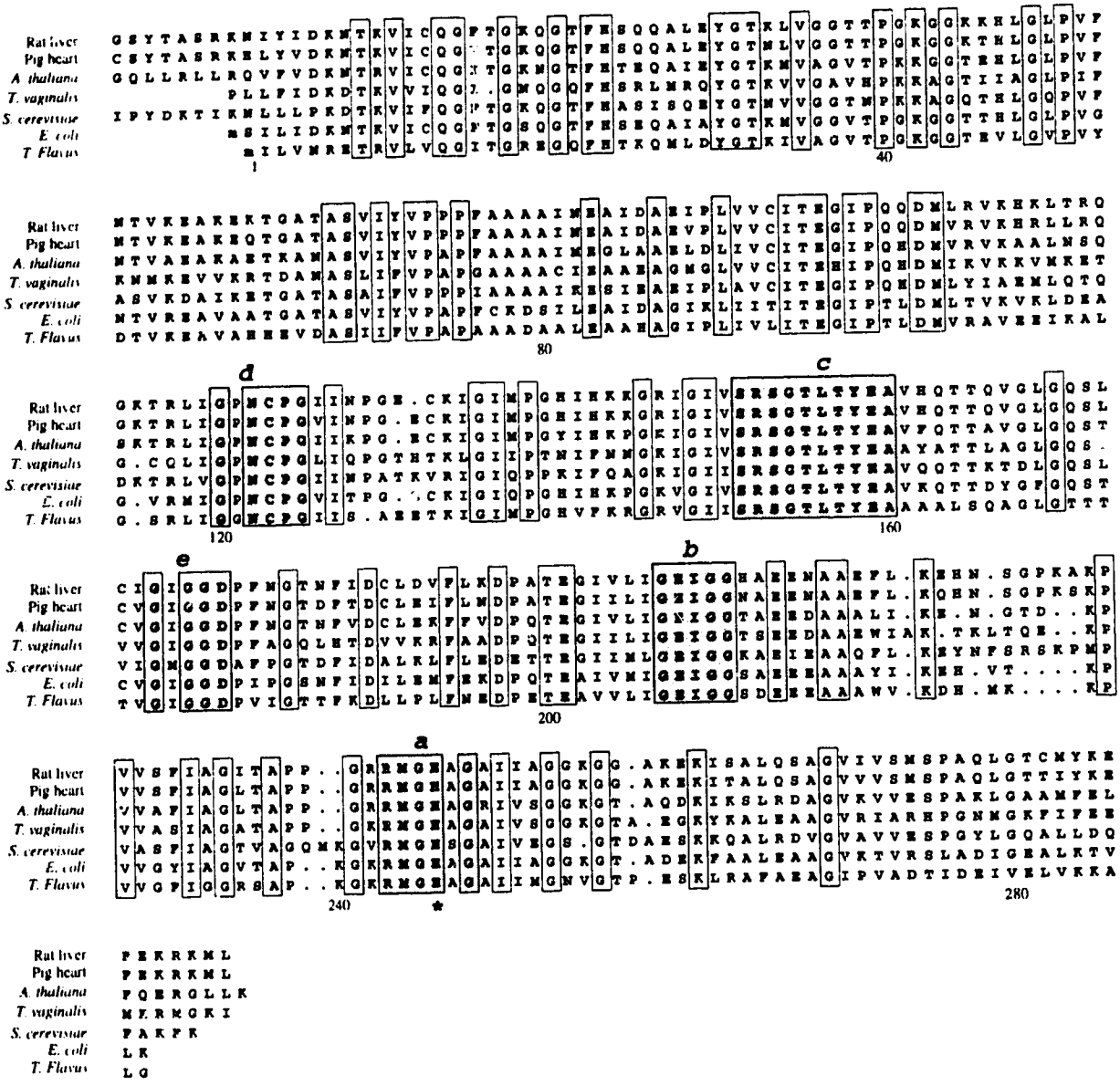

Figure 1.4 Comparison of the amino acid sequence of the α subunits of SCS. The primary structures of cloned α subunits were taken from the following sources: Rat liver [67], Pig heart [103], *Arabidopsis thaliana* [Swiss Protein Data Base], *Trichomonas vaginalis* [114], *Saccharomyces cerevisiae* [23], *Escherichia coli* [45] and *Thermus flavus* [60]. Boxed regions indicate identity among all seven α subunit sequences. Shaded boxes highlight stretches of conserved α subunit sequence and are identified by a letter (*a-e*). The numbering refers to that of the *E. coli* sequence. The asterix denotes the position of the catalytic His246α residue. Taken from Ryan [104].

tryptophan fluorescence by acrylamide [88], it has been concluded that at least one of the three Trp residues can act as a reporter group for events occurring at the active site. This claim was corroborated by chemical modification of one Trp by N-bromosuccinimide [106]; the resulting enzyme was inactive with respect to the overall reaction, but capable of being phosphorylated. The pig heart enzyme has been characterized as well, with respect to tryptophan content and possible function of as a marker group [69, 107]. Amino acid analysis has indicated one Trp residue is located in the ß subunit [69]. This was consistent with a similarly low A_{280} (10 mg/ml) value of 3.5 [44]. Tryptophan fluorescence studies on pig heart SCS demonstrated that fluorescence quenching by acrylamide was also decreased by CoA and GTP. Unlike the *E. coli* enzyme, succinate protected against acrylamide quenching. These data suggested, as in *E. coli* SCS, that the single Trp residue of the pig heart enzyme was close to the active site. Moreover, it was also suggested that this Trp residue has been conserved due to important contributions it makes to the catalytic activity of the enzyme [5].

The α subunit was determined to contain the active site histidine which is phosphorylated during catalysis [56]. Through trypsin digestion of [^{32}P]-phosphorylated *E. coli* enzyme and amino acid sequencing of the resulting labeled peptide, this residue was determined to be His246 [108]. It is, along with some surrounding sequence, highly conserved in all of the α subunits (see **Figure 1.4*a***).

Eukaryotic forms of the α and ß subunits were synthesized as precursors containing N-terminal presequences which direct the protein into the mitochondria (rat liver [67], pig heart [103] and yeast [23]) or hydrogenosome (*T. vaginalis* [73, 102]). These signal sequences shared typical properties common to mitochondrial matrix-directed proteins. They included: an Arg residue at the -2 position relative to the signal sequence cleavage site [109] and more importantly, the potential to adopt an amphiphilic α-helical conformation with polar or charged residues on one side and nonpolar residues on the other [110]. The pig heart α subunit precursors were noticeably less stable than

their mature counterparts [104]. It has been suggested that the signal sequence prevents the complete protein folding of these precursors, allowing the attainment of a translocation competent state.

B. *The crystal structure of* E. coli *SCS*

The three dimensional structure of SCS from *E. coli* was determined to 2.5 Å resolution by x-ray crystallographic methods [1]. Due to the ambiguous choice associated with the symmetrical packing of tetramers within the crystal, two alternatives existed for the quaternary structure. The proposed model [1] was chosen based on its consistency with available experimental data. In the schematic representation of the structure in **Figures 1.5 and 1.6**, the α subunits lie at opposite poles of the tetramer and do not contact one another directly. Rather, each α subunit interacted primarily with one of the ß subunits, thereby comprising each αß dimer. All associations between the αß dimers within the tetramer were, therefore, entirely mediated by the ß subunit (see **Figure 1.5**). Previous cross-linking studies on the *E. coli* enzyme (using dimethylsuberimidate and *o*-phenylenedimaleimide) had confirmed the $\alpha_2ß_2$ structure and indicated the close contact between the α and ß subunits [111]. The ß subunits associated through several electrostatic salt bridge and hydrogen bonding interactions (see **Figure 7.2**, page 145). This inter-ß subunit association was symmetrical and, therefore, two interactions existed for every bonding partnership.

The polypeptide chains of the α and ß subunits were observed to fold into distinct N- and C-terminal domains (see **Figures 1.7 and 1.8**). The structural elements which comprise most of the active site were made by contributions from the C-terminal domain of the ß subunit and both domains of the α subunits. Each of these was characterized by a doubly wound parallel ß-sheet or nucleotide binding motif ('Rossmann fold', [112]). The N-terminal domain of the ß subunit provided several of the dimer-dimer contacts. This domain can be further subdivided into large (N_L) and small (N_S) subdomains (**Figure 1.8**). A short helix from the N_S subdomain created part of the CoA binding site

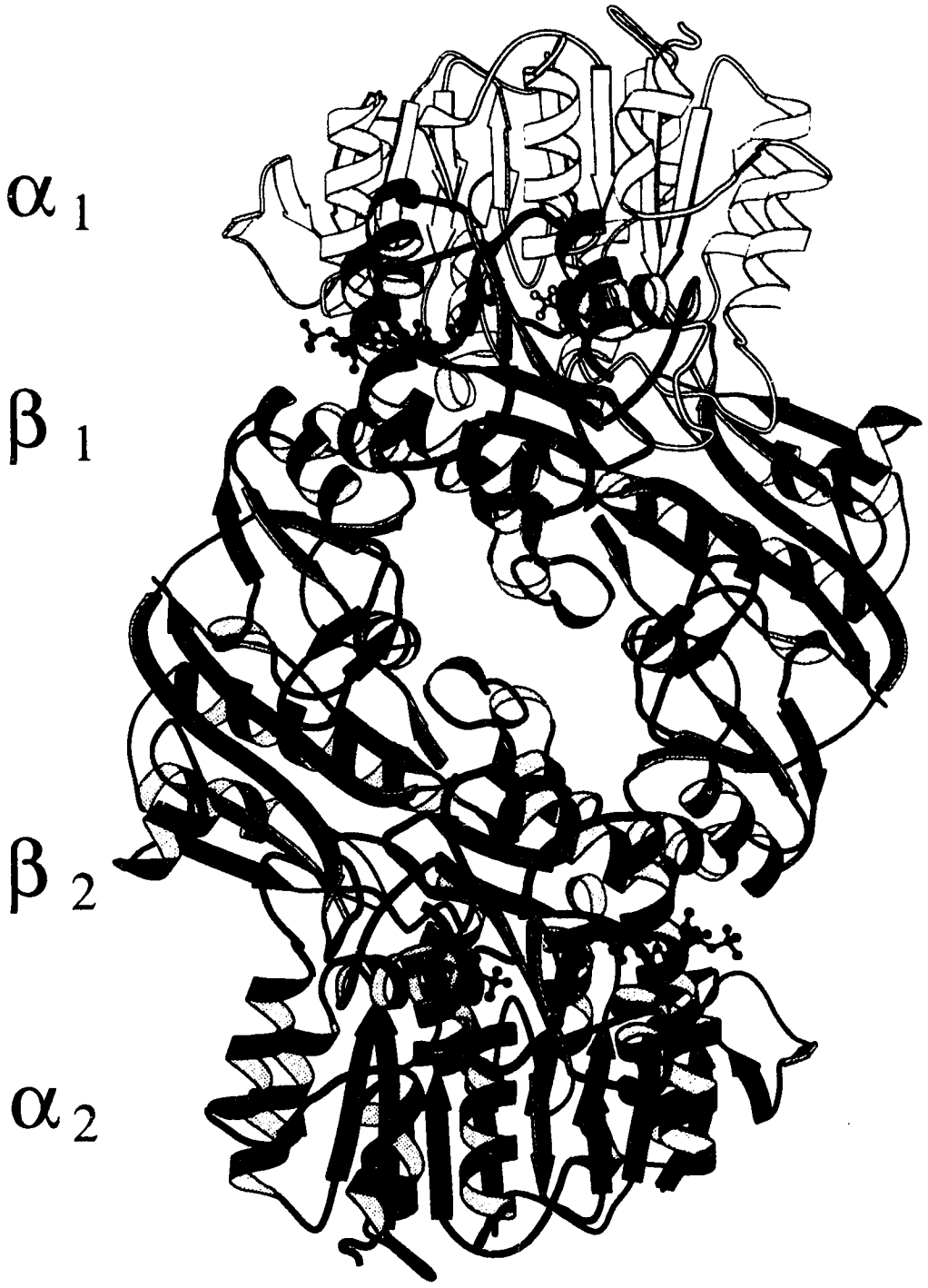

Figure 1.5 Schematic representation of the tetramer of SCS from *E. coli* (A). The two α subunits are colored in yellow and blue, and the ß subunits in green and magenta. The phosphorylated catalytic residues, His246α, are represented as ball-and-stick models in each α subunit. The two molecules of CoA are colored red and are also shown as ball-and-stick models. The non-crystallographic two-fold axis of symmetry relating the α1ß1-dimer to the α2ß2-dimer lies perpendicular to the page. Taken from Wolodko *et al.* [1].

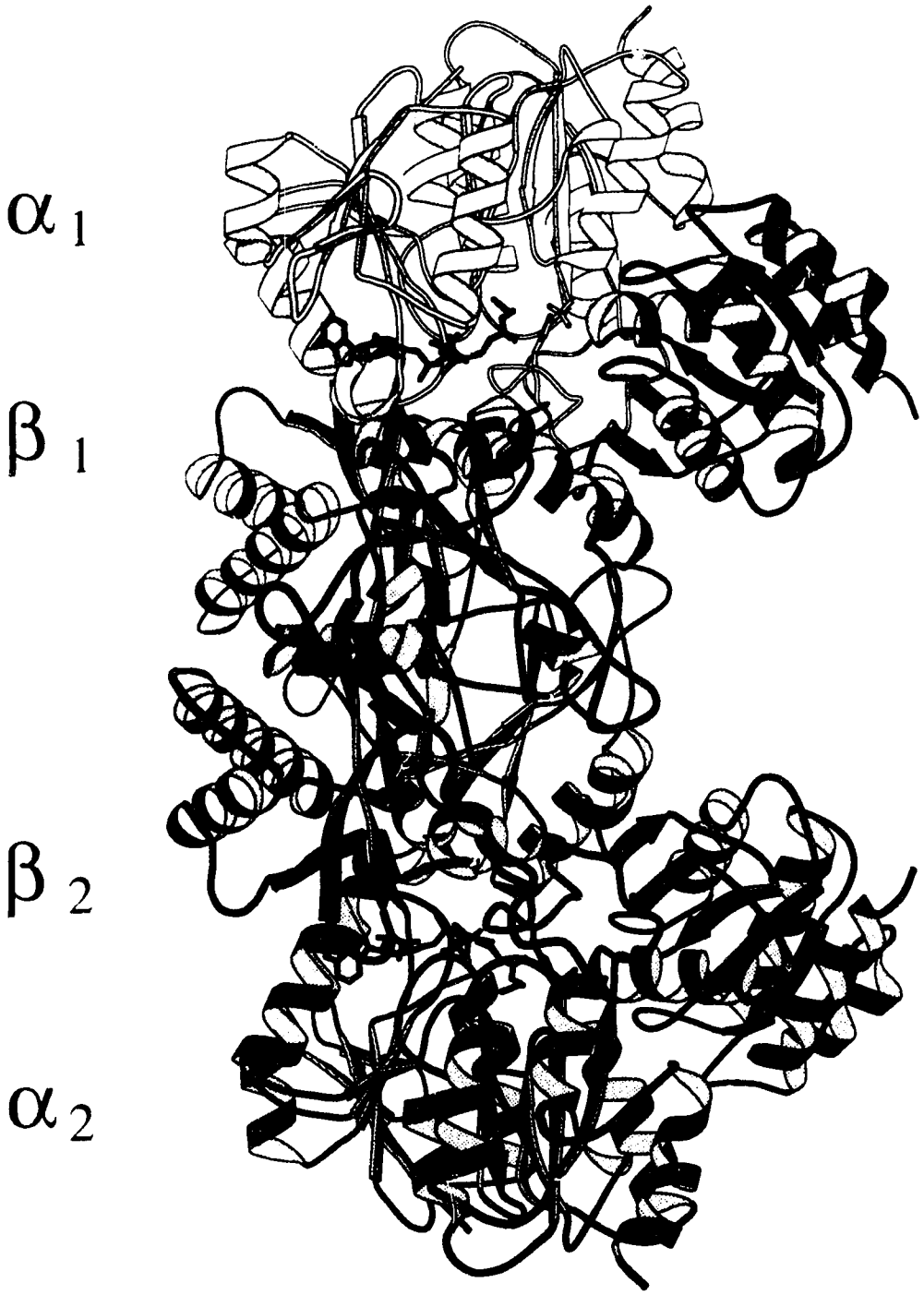

Figure 1.6 Schematic representation of the tetramer of SCS from *E. coli* (B). The tetramer represented here is related to that shown in **Figure 1.5** by a 90° rotation to the right along a vertical axis in the page. The non-crystallographic two-fold axis of symmetry relating the two dimers within the tetramer therefore lies horizontal to the page. Interactions between a helix of ß2 (magenta) and CoA bound to the α1ß1-dimer (yellow/green) are apparent. Taken from Wolodko *et al.* [1].

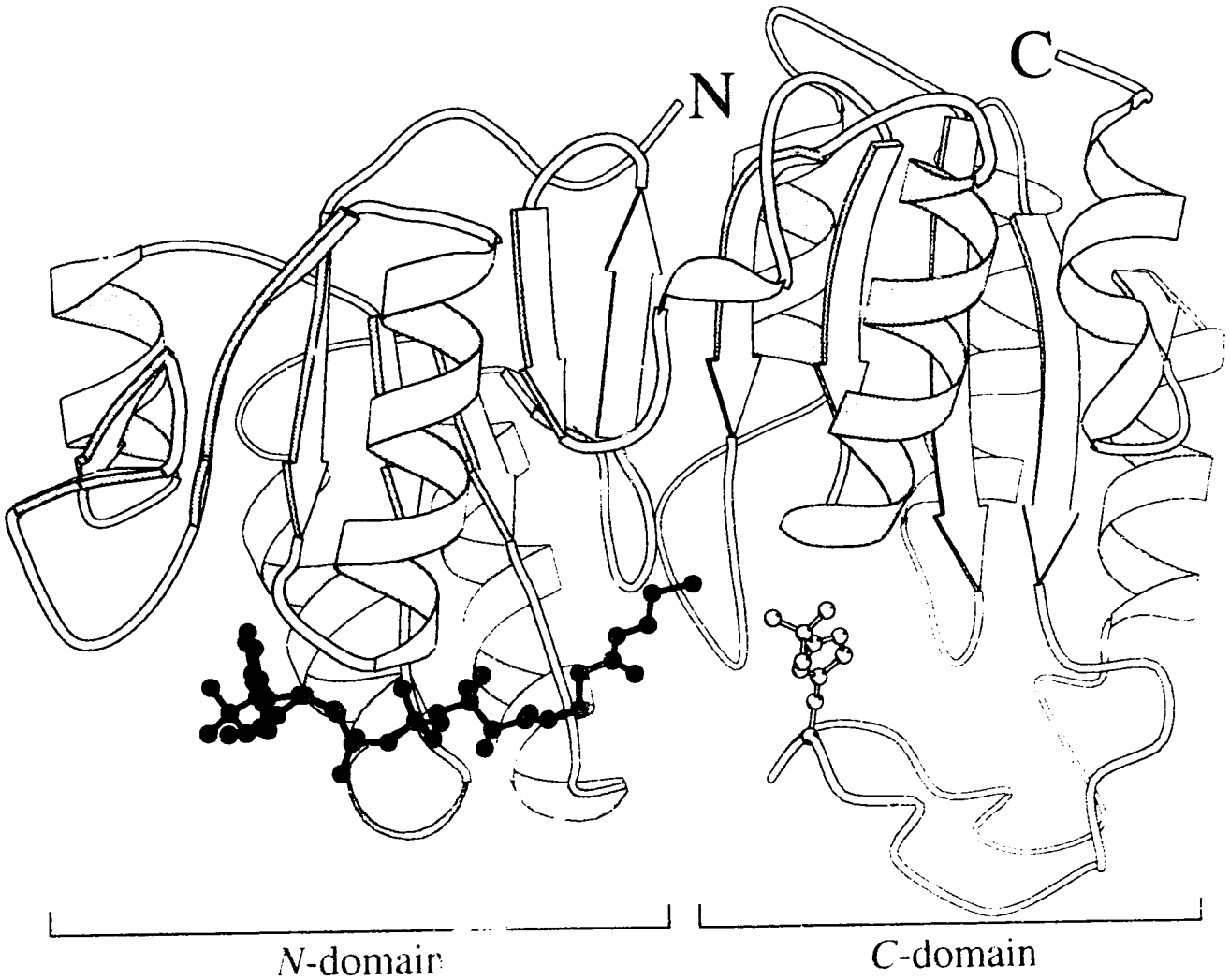

Figure 1.7 Schematic representation of the α subu[illegible] from *E. coli* SCS. The labels, N and C, [illegible]e the N and C termini of the polypeptide, respectively. The subunit is comprised [illegible] domains which are indicated by the horizontal [illegible] and are designated by *N* and *C* (corresponding to the termini which are contained with[illegible]e phosphorylated residue, His246α, is depic[illegible] ball-and-stick model as is CoA (red), which is bound in the *N*-domain. The power helix of the *C*-domain is represented in orange color. Residues at the end of this α-helix are close to an oxygen atom of the catalytic phosphohistidine re[illegible]due. Taken from Wolodko *et al.* [1].

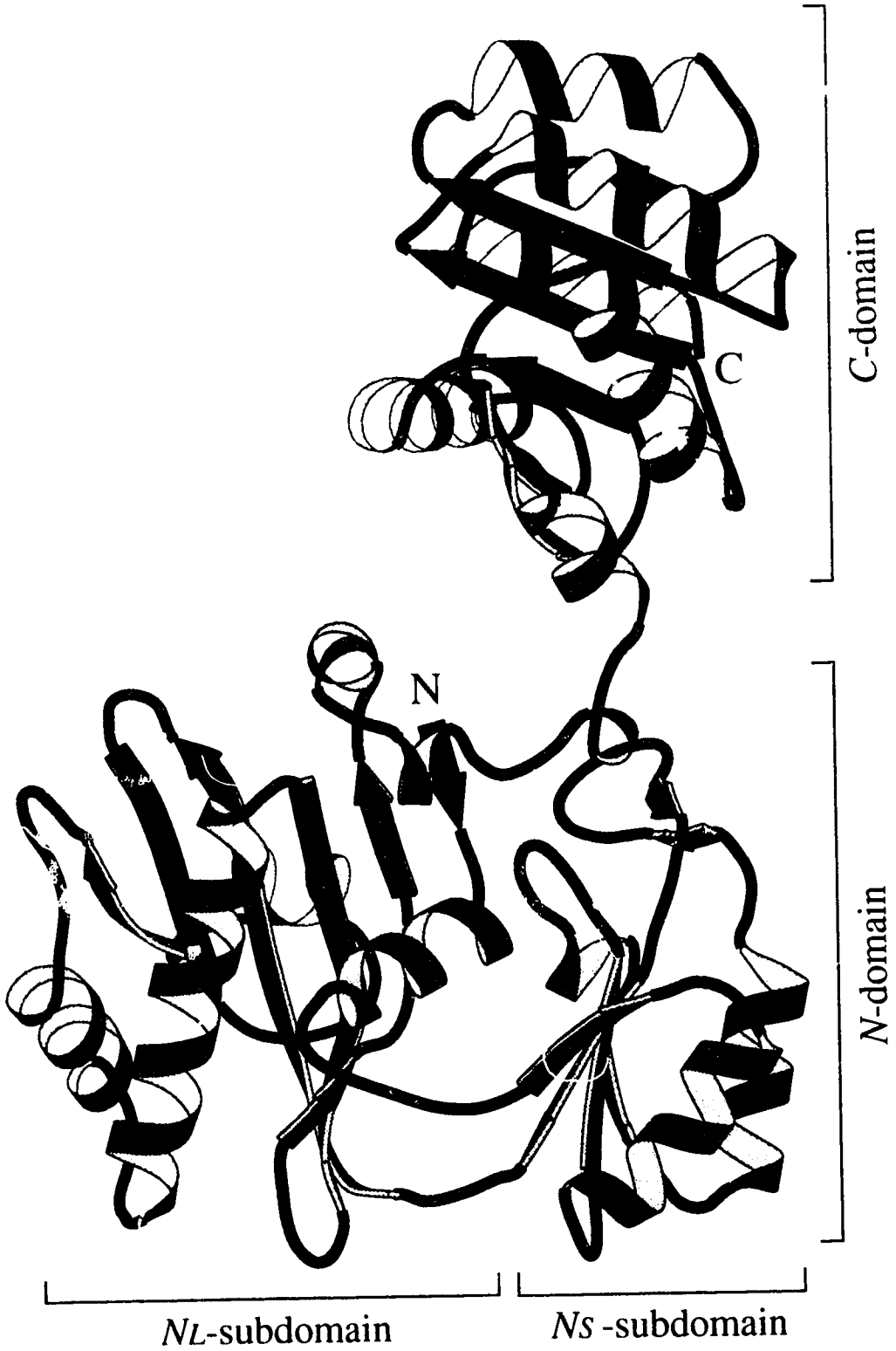

Figure 1.8 Schematic representation of the ß subunit from *E. coli* SCS. N and C labels denote the N and C termini of the protein. The ß subunit contains two distinct domains which are separated by a sequence of hydrophilic residues. The *N*-domain can be further subdivided into a large subdomain (*NL*) and a small subdomain (*NS*). The *C*-domain interacts primarily with the α subunit to form an active site. The 'power' helix in the ß subunit is shown in black for emphasis. Taken from Wolodko *et al.* [1].

in the adjacent dimer. The participation of the ß subunit in the formation of the active site in the neighboring dimer furnished a structural rationale for the tetrameric structure of *E. coli* SCS.

The crystal structure revealed that the two αß dimers of the tetramer were nearly symmetrical both structurally and functionally. Two molecules of CoA were bound in the structure; one in each CoA binding site (see **Figure 1.6**). Perhaps more importantly, the catalytic histidine residues, Hisα246, were phosphorylated in both α subunits. This stoichiometry agreed with several reports [37, 38, 97-100] and was at variance with those which led to the proposal of 'half-of-the-sites' phosphorylation [86]. Together, these observations were inconsistent with a hypothetical mechanism of alternating sites cooperativity [57] in *E. coli* SCS.

Two of the phosphoryl oxygens of the catalytic phosphohistidine were within hydrogen bonding distance of the N-termini of two α-helices, one from each subunit of the αß dimer (see **Figure 1.9**). The amino acid sequence of both these 'power' helices was highly conserved. In the α subunit, the sequence of this helix comprised the longest conserved sequence in the protein (see **Figure 1.4 *c***). At the N-terminal end of this α subunit helix, the side chain hydroxyl groups of Serα153 and Thrα155 and the amide nitrogen of Glyα154 interacted with one phosphoryl oxygen atom. The backbone amide nitrogen atoms of Alaß266 and Glyß267 at the N-terminus of the ß subunit helix served to coordinate the second oxygen. The longitudinal axes of both helices were observed to be aligned with these phosphoryl oxygens (see **Figure 1.9**). It was suggested that the partial positive charges developed through the helical dipoles, enables these 'power' helices to stabilize the two negative charges of the phosphoryl group [1] (see **Figure 1.10**). This structural feature, in part, explained the high stability of the phosphoenzyme.

The imidazole group of the phosphohistidine interacts at the Nγl position with the carboxylate group of Gluα208. This residue and surrounding sequence were believed to play a role in the phosphorylation event [1]. The functional significance of this region

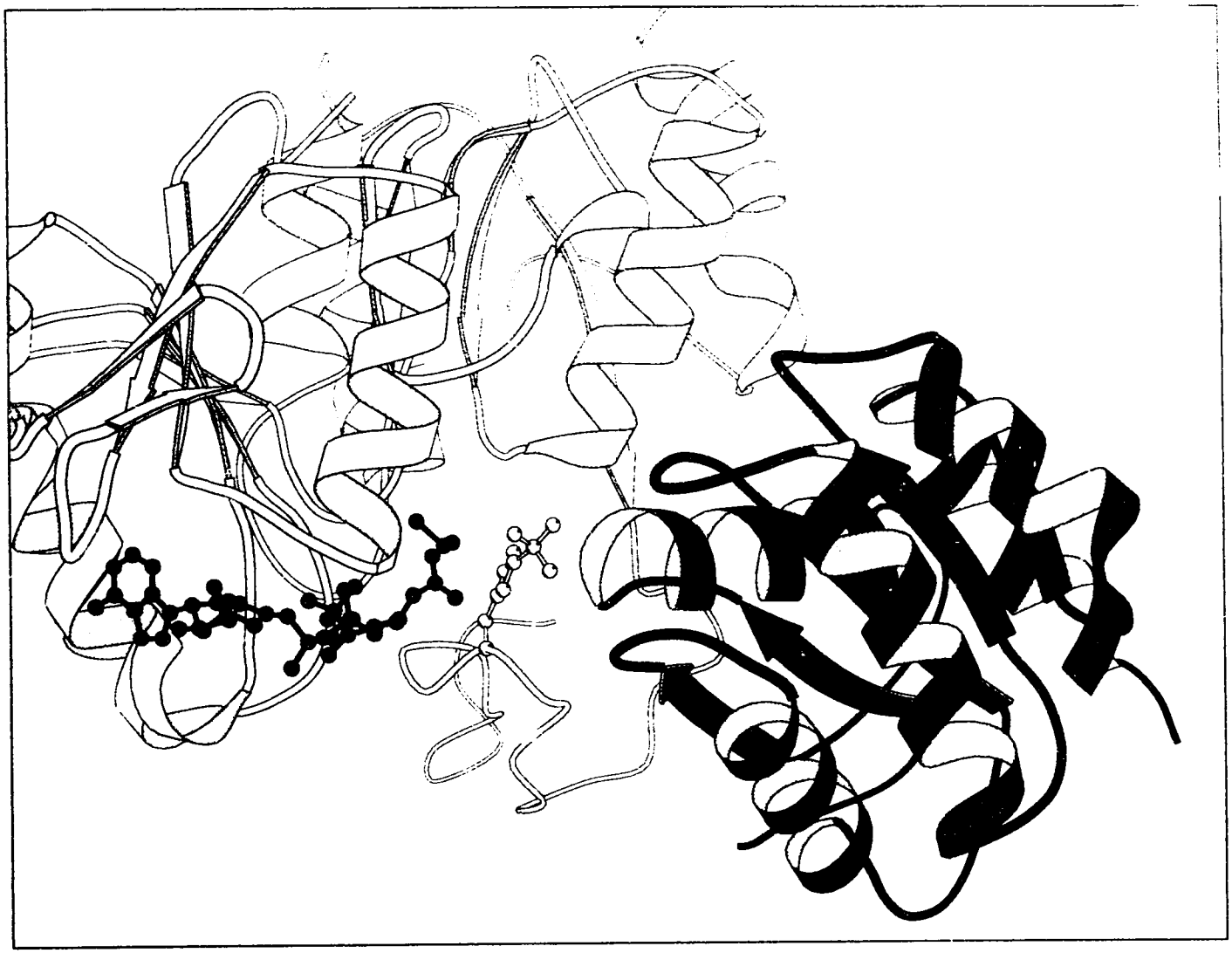

Figure 1.9 Arrangement of the 'power' helices at one active site in *E. coli* SCS. Represented in this figure is the active site pocket formed between the α1 subunit (yellow) and the ß1 subunit (green). This view is the same as that shown in **Figure 1.6**. The 'power' helices of the α and ß subunits are colored orange and black, respectively, to show their orientation within the structure relative to the phosphohistidine residue (shown as a yellow ball-and-stick model). The sulfhydryl end of CoA, depicted as a red ball-and-stick model, is also oriented toward the phosphohistidine. Taken from Wolodko *et al.* [1].

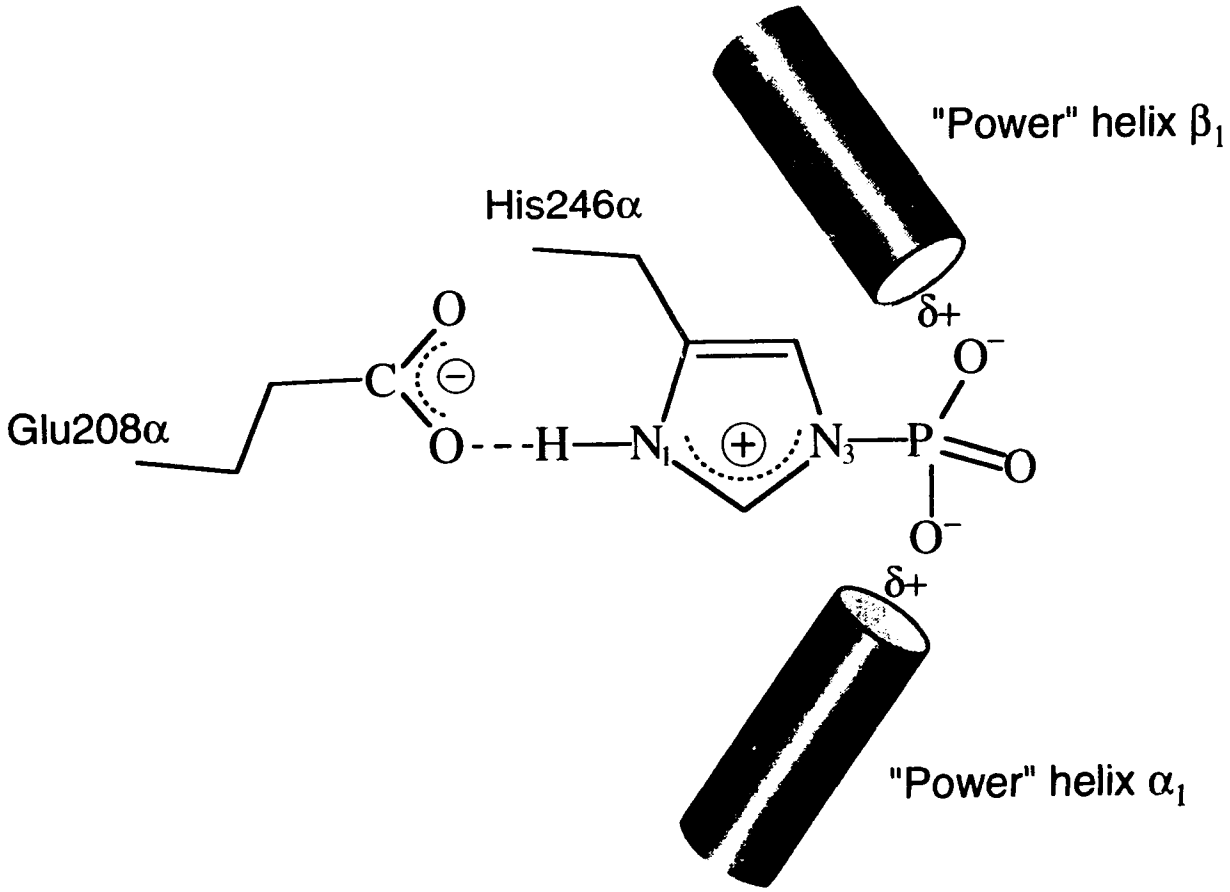

***Figure 1.10 Schematic representation of the stabilizing environment near the active site phosphohistidine in E. coli* SCS.** Illustrated are the active site determinants which stabilize the 3-phosphohistidyl isomer of the catalytic residue, His246α. Partial positive charges developed from the helical dipoles of the 'power' helices (α_1 and β_1) stabilize the negative charges of two phosphoryl oxygens. Glu208α forms a hydrogen bond with the N-1 of the imidazole ring, thereby stabilizing the tautomer with a lone pair of electrons at N-3 for nucleophilic attack on the phosphoryl group of ATP or succinyl-phosphate. Taken from Wolodko *et al.* [1].

was implied by its extremely well conserved nature among α subunits (**Figure 1.4 *b***). The phosphohistidine residue was confirmed to be a N-3 isomer [80]. Therefore, the phosphorylation event was believed to be a nucleophilic attack by the N-3 atom of Hisα246 on either the terminal phosphate of ATP or the phosphoryl group of succinyl phosphate [93]. However, the favourable tautomeric form of the imidazole ring was one which contained the lone pair of electrons at the N-1 position. In order to satisfy chemical requirements for phosphorylation, Vogel and Bridger [90] have suggested an anionic group stabilizes the tautomer with a lone pair of electrons at the N-3 position. Structurally, Gluα208 was suitably positioned to fulfill this role (**Figure 1.10**).

Whereas the binding sites for CoA have been determined from the structure, those for succinate and ADP/ATP have not been identified. CoA was bound primarily by the 'Rossmann fold' in the N-terminal domain of the α subunit and by interactions from the neighboring ß subunit in the proposed quaternary structure (see **Figures 1.6, 1.7 and 1.8**). The thiol group of CoA extended to within 7 Å of the phosphoryl group of the phosphohistidine (**Figure 1.9**). The third oxygen of the phosphoryl group, not interacting with the 'power' helices, was observed to be oriented directly toward the thiol group of CoA. Conceivably, the intervening area between these reactive groups could accommodate succinate. With regard to the potential ADP/ATP binding sites, an unoccupied nucleotide-binding motif occurred in each of the C-domains of the α and ß subunits (see **Figures 1.7 and 1.8**). Both were at an appropriate distance and relation to the phosphohistidine to provide a binding domain for ADP/ATP. The isolated α subunit has the ability to be phosphorylated by ATP [64], suggesting the nucleotide binding site was contained entirely within this subunit. The binding of affinity-label analogs of ADP/ATP has provided mixed results. Treatment with ADP-dialdehyde led to cross-linking of the α and ß subunits suggesting the ADP/ATP binding site is shared between the two subunits [113]. However, the use of 8-azido-ATP resulted in exclusive labeling

of the *E. coli* ß subunit [100]. These data agreed with the possibility that at least part of the binding site of the nucleotide moiety of ADP/ATP is found on the ß subunit.

VI. RESEARCH OBJECTIVES

A. *Cloning, characterization and expression of a mammalian isoform of SCS*

As presented earlier, a number of structural and functional differences exist between the SCS enzymes isolated from *E. coli* and pig heart tissue. To reiterate, the bacterial enzyme is tetrameric in structure with broad nucleotide specificity, whereas the mammalian enzyme has a dimeric quaternary structure with restricted specificity for either ATP or GTP. To further characterize these distinctive properties and to provide a rationalization for them, additional structural evidence is required. A remarkable degree of conservation exists between the amino acid sequences of the α subunits from these enzymes. It is plausible, therefore, that several of the observed differences between these two forms may be attributable to variations in the amino acid sequences of the ß subunit.

In contrast to the amino acid sequences of the ß subunit known from several tetrameric enzymes, none have been reported from a dimeric source. The isolation of a cDNA encoding the mammalian SCS ß subunit will constitute an initial step towards identifying differences found among these forms of SCS. The value of such primary structure information becomes apparent when considering that αß dimer-dimer interactions within the proposed quaternary structure of *E. coli* SCS are mediated entirely by the ß subunits [1]. If mammalian ß subunit residues are found to not be conserved with the equivalent *E. coli* SCS residues responsible for inter-dimer associations in the tetramer, these residues may have been changed to prevent oligomerization. Such sites in the *E. coli* enzyme will be employed for mutagenesis to disrupt this tetrameric structure (see **B.** below).

The isolation of a cDNA encoding the mammalian ß subunit will also permit its coexpression with a cloned mammalian SCS α subunit. The significance of this will be manifold. Since mammalian tissues contain many isoforms of SCS, this polymorphic nature has precluded the isolation of a single species of enzyme. The expression of the cloned mammalian subunits will enable the functional assessment of a homogeneous isozyme of SCS. As a consequence, a direct link may be made between the primary structure of component α and ß subunits and the resulting nucleotide specificity. High yields of these proteins could be obtained through bacterial expression. Hence, the over-production of a cloned dimeric isozyme by this methodology may aid its purification, since enzyme isolation from mammalian tissues is difficult and yields of final product are low.

The polymorphic heterogeneity of mammalian SCS in preparations from tissues has prevented the growing of crystals suitable for x-ray diffraction studies of this enzymatic form. 'Homogeneous' preparations of expressed proteins will remove the problems of 'contaminating' isoforms. In addition, the genetic identification and characterization of the isoforms will extend our understanding of the relationship between polymorphism and metabolism.

B. *Investigation of the quaternary structure assignments for* E. coli *SCS and rationalization of this tetrameric structure*

Due to the symmetrical relations of SCS molecules within the crystal, two possibilities existed for the choice of the tetrameric structure of the *E. coli* enzyme. On the basis of available experimental data, one of these two viable quaternary structure arrangements was proposed [1]. To determine whether this prediction was correct and corresponds with the tetrameric structure of the enzyme in solution, the interactions between αß dimers will be probed through site-directed mutagenesis. Residues observed to be responsible for close contacts at the dimer-dimer interface will be changed to disrupt the native quaternary structure of *E. coli* SCS. If such attempts, based on the

published structure, were unsuccessful in altering the quaternary structure of the enzyme in solution, the alternative arrangement of dimers would be implicated as the proper configuration for the tetramer. Similar disruption mutagenesis directed to the αß dimer-dimer interface of the alternative quaternary structure would be carried out to confirm this conclusion.

An important question can be posed with regards to the quaternary structure and function of *E. coli* SCS. What advantage is provided by a tetrameric structure when dimeric forms are as catalytically effective? An alternating sites cooperative mechanism has been proposed as a rationale for the subunit structure of this enzyme. Yet many lines of evidence suggest otherwise and indicate that tetrameric *E. coli* SCS is truly composed of independently-acting dimers. The mutagenic studies, outlined above, will yield important information as to the purpose of this 'larger' oligomeric form. If a dimeric form of *E. coli* SCS can be obtained by the disruption of a dimer-dimer interface, its functional properties would be assessed. In the case where such a mutant was found to be catalytically active, a mechanism of alternating sites cooperativity may not be relevant to the native *E. coli* SCS; other rationales for a tetrameric enzyme constructed of independent dimeric enzymes should be sought.

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Chapter 2

MATERIALS AND GENERAL METHODS

I. MATERIALS

Buffer, growth media and other reagent grade chemicals were supplied by Aldrich, Bio-Rad, BDH, BRL, Kodak, ICN, New Jersey Biolabs or Sigma.

DNA modifying and restriction enzymes were purchased from BRL, Boehringer Mannheim or New England Biolabs and were used as recommended by the manufacturers. Sequenase™ and Sequenase™ Version 2.0 were from United States Biochemical Coorporation. *Thermus aquaticus* and Vent™ DNA polymerases were purchased from Perkin Elmer-Cetus and Amersham. Deoxynucleotides were from Pharmacia.

Several cDNA libraries were obtained from the following sources: a rat liver λgt11 library was kindly provided by M. Mueckler (Massachusetts Institute of Technology, Cambridge, Ma.); adult pig heart and rat heart cDNA libraries (in λgt11) were purchased from Clontech; and an infant pig heart λgt11 library was created by T.W. Lin in this laboratory [1].

Nitrocellulose membranes were obtained from Schleicher & Schuell, Amersham and Bio-Rad; Hybond N nylon membranes were bought from Amersham; GeneScreen™ hybridization membrane was from Du Pont; Immobilon™ transfer membranes and general filtering membranes were from Millipore.

Radioisotopes were obtained from Amersham, Du Pont or ICN. Chromatographic resins were supplied by Bio-Rad, Pharmacia.

II. GENERAL METHODS

Unless otherwise stated, all methods employing molecular biology are essentially those described in Sambrook *et al.* [2].

A. Screening of the λgt11 cDNA libraries

1. *Screening libraries using antibodies*

Methods described herein are derived from Sambrook *et al.* [2]. Stocks of *E. coli* plating bacteria were prepared by inoculating a single Y1090 colony into 25 ml of Luria Broth medium supplemented with 50 µg/ml Ampicillin and 0.2% (w/v) maltose. These cultures were grown overnight at 37 °C with shaking. Bacteria in stationary phase were then harvested and resuspended in 10 ml of 10 mM $MgSO_4$.

Aliquots (0.2 ml) of the plating bacteria suspension were transfected with $3x10^4$ pfu of λgt11 bacteriophage and incubated at 37 °C for 20 minutes. These bacteria infected with phage were then mixed with 3 ml of liquified 0.7% (w/v) agarose prepared in LB medium and then spread onto a LB/Ampicillin plate (85 mm in diameter). The phages were allowed to replicate at 42 °C for about 3.5 hours before each plate was overlaid with a circular nitrocellulose filter previously saturated with 10 mM IPTG. After an additional 4 hours of incubation at 37 °C, the position of the filter was marked. The filter was then removed and incubated briefly in 50 mM Tris-HCl (pH 7.5) and 150 mM KCl (TK buffer buffer) at room temperature before being washed a second time for 30 minutes in the same buffer. The filters were then transferred to a solution of 5% (w/v) skim milk powder with 0.1% (v/v) Antifoam A for 30 minutes at room temperature. This blocking solution was removed and the filters were washed briefly in water before being incubated with antibodies specific to the ß-subunit of pig heart SCS. Antibody adsorption was allowed to occur overnight at room temperature with constant agitation. The filter discs were then washed at room temperature in the following manner: first in a large volume of TK buffer; then through a second wash of TK buffer with 0.05% (v/v) of the anionic detergent Tergitol NP-40 (TKN buffer); and thirdly in TK buffer alone. Washed filters were then incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (from Cappel, diluted 3 x 10^3 times in TK buffer with 3% (w/v) BSA) for 3

hours at 37 °C. The nitrocellulose discs were then washed as before (1- TK buffer; 2- TKN buffer; and 3- TK buffer) before colour development in a solution of 0.33 mg/ml nitro blue tetrazolium, 0.165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl and 50 mM $MgCl_2$. The chromogenic reaction was stopped by rinsing the filters in water.

Duplicate filter lifts were done on initial screening of the λgt11 libraries. When doing so, the first filter was removed from the plate after 4 hours of incubation at 37 °C and then a second filter was overlaid and incubated for an additional 4 to 6 hours at 37 °C. The position of both filters coincided since the position of the second disc was made using the same asymmetric markings of the first filter.

2. *Screening libraries using DNA probes*

Following protocols outlined above for screening libraries with antibodies, λgt11 bacteriophage libraries were plated out onto LB/Ampicillin plates employing Y1090 *E. coli* as the lytic host. The plates were incubated overnight at 37 °C, followed by chilling for at least 1 hour at 4 °C.

The bacteriophage plaques were transferred to nylon membranes by placing untreated filter discs on the cooled plates for 1 minute [3]. (A second membrane was left on each plate for 3 minutes as a duplicate filter lift.) After adsorption, filters were carefully lifted and soaked for 5 minutes on Whatman filter paper saturated with 0.5 M NaOH and 1.5 M NaCl to denature the filter-bound phage DNA [3]. The filters were then neutralized by placement onto filter paper saturated with 1M Tris-HCl (pH 8.0) and 1.5 M NaCl for 5 minutes. Filters were briefly rinsed in 0.45 M NaCl and 45 mM sodium citrate·$2H_2O$ (pH 7.0) for 5 minutes and then air dried. Phage DNA was cross-linked to the nylon filter by ultraviolet radiation using a Stratagene Strata-linker™.

Prior to hybridization, filters were prehybridized at 68 °C for about 2 hours, with shaking, in 0.75 M NaCl, 75 mM sodium citrate·$2H_2O$ (pH 7.0), 0.1% (w/v) N-lauroylsarcosine (sodium-salt), 0.02% (w/v) SDS and 1% (w/v) Boehringer Mannheim

Blocking Reagent™ [4]. The prehybridization solution was removed and replaced with a similar hybridization solution containing approximately 5 ng/ml freshly denatured DNA probe (containing digoxigenin-11-dUTP). The filters were hybridized at 68 °C overnight with shaking.

Steps to remove unbound and nonspecifically bound probe were as follows [4]. Filters were washed twice for 10 minutes at room temperature with 0.3 M NaCl, 30 mM sodium citrate·$2H_2O$ (pH 7.0), 0.1% (w/v) SDS, and twice for 20 minutes at 68 °C with 15 mM NaCl, 1.5 mM sodium citrate·$2H_2O$ (pH 7.0), 0.1% (w/v) SDS.

Probe-target DNA hybrids were detected using the following protocol [4]. Filters were washed briefly in 100 mM Tris-HCl (pH 7.5) and 150 mM NaCl (BTK buffer), then incubated with this solution containing 2% Blocking Reagent™ for 30 minutes, followed by a washing step in BTK buffer. An anti-digoxigenin antibody conjugated to alkaline phosphatase was diluted 5 x 10^3 times in BTK buffer and incubated with filters for 30 minutes. Unbound antibody-conjugate was removed by washing twice for 15 minutes in BTK buffer. Color development of the specifically bound antibody-hybrid complexes was similar to that previously described.

B. DNA preparation and purification

1. *Small scale plasmid DNA preparations by the alkaline lysis method*

Routinely, plasmid DNA was prepared for cloning and restriction enzyme analysis without modification to the protocol as described in Sambrook et al. [2].

Double-stranded plasmid DNA used for sequencing was prepared in a similar manner except as follows. Cellular lysates were treated with 20 µg/ml RNase A (DNase free) for 20 minutes at 37 °C, before extraction with phenol and chloroform. After resuspension of precipitated and washed DNA, a polyethylene glycol (PEG) precipitation step was included to further purify the plasmid DNA. Here, NaCl and PEG (average

8000 g/mol) were added to 0.4 M and 6.5% (w/v) respectively to precipitate the DNA cooled to 0 °C. The nucleic acids were collected by centrifugation at 4 °C, washed in 70% (v/v) ethanol, dried under vacuum and resuspended in deionized water.

2. *Isolation of bacteriophage λ DNA*

In contrast to the plate lysate method of λ DNA purification [2], the following culture method was used [5, 6]. Approximately 1 x 10^5 pfu of bacteriophage suspension were incubated with 0.2 ml of freshly prepared Y1090 plating bacteria for 20 minutes at 37 °C. Added to the infected cells was a 10 ml solution of LB, with 10 mM $MgCl_2$, 0.1% (w/v) glucose and 50 µg/ml ampicillin. This culture was grown overnight at 37 °C with shaking. Cells were removed by centrifugation, and the high titre supernatant was then centrifuged at 30 x 10^3 rpm for 30 minutes using a SW41 rotor (Beckman). Pelleted bacteriophage particles were then resuspended in 200 µl of SM buffer and treated with 200 µl of freshly made 1 mg/ml proteinase K (in SM buffer) at 37 °C for 2 hours. The suspension was extracted once with phenol, and once with chloroform before the nucleic acids were precipitated at 0 °C with 100 µl of 7.5 M ammonium acetate and 1 ml of 95% (v/v) ethanol. After centrifugation, the λ DNA was washed with 70% (v/v) ethanol before drying and resuspension in TE buffer. DNA purified by this protocol typically yielded approximately 20 to 30 µg and was useful in further analysis and cloning steps.

3. *Purification of single stranded DNA from M13 bacteriophage*

Ten millilitres of early log phase JM103 cells were inoculated with approximately 1 x 10^{10} pfu of M13 bacteriophage and grown for 8 hours at 37 °C with shaking. The cultures were centrifuged at 3 x 10^3 rpm for 30 minutes and the supernatant containing the single stranded DNA viral particles was collected. M13 phage were precipitated overnight at 4 °C by the addition of 2.5 ml of 20% (w/v) PEG(8000) in 2.5 M NaCl. The virus precipitated with PEG was collected by centrifugation at 3 x 10^3 rpm for 30 minutes, and then resuspended in 750 µl of water. The viral suspension was extracted

twice with an equal volume of phenol equilibrated with Tris-HCl (pH 8.0) and once with chloroform. M13 DNA was then precipitated with 50 µl 3 M sodium acetate (pH 4.5) and 1 ml of 95% (v/v) ethanol at -20 °C overnight. Single stranded template DNA was pelleted by centrifugation, washed with 70% (v/v) ethanol, dried and resuspended in TE buffer. A yield of 5 to 10 µg of DNA was obtained by this method and was suitable for DNA sequencing and oligonucleotide-directed mutagenesis.

4. *DNA isolation from agarose gels via glass powder*

DNA retrieval from agarose gels using glass powder elution was routinely used as a preparative step in the purification and ultimate cloning of DNA. The glass powder suspension and sodium iodide solution were prepared by established methods [7, 8].

TAE buffered agarose gels were used to electrophoretically separate DNA samples. A gel slice containing a target DNA fragment was removed and melted in 2.5 volumes of saturated sodium iodide solution at 50 °C. DNA was then allowed to bind to the glass powder slurry at 0 °C for 5-10 minutes. Bound DNA was pelleted with the glass powder by brief centrifugation. After removing the liquid phase, the glass fines were resuspended and pelleted three times by washing in 250-500 µl of 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1 M NaCl and 50% ethanol (v/v) chilled to -20 °C. The DNA fragment was then eluted from the glass powder by resuspending twice in TE buffer, heating at 50 °C for 3 minutes and collecting the supernatant after centrifugation.

C. Gel electrophoresis

1. *SDS Polyacrylamide gel electrophoresis (SDS-PAGE)*

To separate and characterize protein samples based on their molecular size, SDS PAGE, a method originally established by Laemmli [9], was employed using the following conditions.

Discontinuous denaturing slab gels were routinely cast with the separating gel containing final component concentrations. Acrylamide/N,N'-methylenebis-acrylamide concentrations were at 8% (w/v) / 0.21% (w/v), or 10% (w/v) / 0.27% (w/v), or 12% (w/v) / 0.32% (w/v). The final concentration of Tris-HCl buffer (pH 8.8) was 0.4 M, SDS 0.1% (w/v), TEMED 0.04% (v/v), and ammonium persulfate, the catalyst of polymerization, 0.07% (w/v).

The final concentration of components in the stacking gel solution was: acrylamide / bis-acrylamide, 5% (w/v) / 0.13% (w/v), Tris-HCl buffer (pH 6.8), 60 mM, SDS, 0.1% (w/v), TEMED, 0.045% (v/v), and ammonium persulfate, 0.2% (w/v).

For increased resolution and sharpened protein bands, gradient SDS-PAGE was also used [2]. Using a gradient former, separating gels of this type were cast having a 7-15% (w/v) gradient of acrylamide concentration in the direction of protein migration.

A Tris-glycine electrophoresis buffer for the system was composed of 25 mM Tris-HCl, 192 mM glycine (electrophoresis grade) and 0.1% (w/v) SDS. The pH was adjusted to 8.3.

Protein samples were prepared in buffer consisting of 50 mM Tris-HCl (pH 6.8), 2.5% (w/v) SDS, 1% (v/v) ß-mercaptoethanol, 6% (v/v) glycerol and 0.1% (w/v) bromophenol blue and boiled for 10 minutes before applying to the gel. The electrophoresis was continued until the dye front reached the bottom of the gel. Proteins were visualized by staining the gel with 0.5% (w/v) Coomassie blue, 50% (v/v) methanol and 10% (v/v) acetic acid, and then destained in 45% methanol and 10% acetic acid to remove excess dye from the background.

2. *Agarose gel electrophoresis*

DNA characterization was achieved using non-denaturing agarose gel electrophoresis with Tris-acetate-EDTA (TAE) as the buffering components. Gels were prepared and used in electrophoresis according to established methods [2]. DNA samples were loaded onto agarose gels using a 6X gel-loading buffer consisting of 0.25% (w/v)

bromophenol blue, 0.25% (w/v) xylene cyanol, and 30% (v/v) glycerol. Agarose gels were stained with 0.5 µg/ml ethidium bromide and visualized under ultraviolet radiation.

3. *DNA sequencing gel electrophoresis*

Nucleotide sequence of templates was determined using the dideoxy-chain termination method [10] and denaturing gel electrophoresis. Sequencing gels were cast to be either 0.4 mm or ultra-thin spaced with field gradients [11-13]. The latter system was employed to improve resolution and extend the limit of useful sequencing information that could be obtained. Customarily, sequencing gel solutions consisted of 6% (w/v) acrylamide, 7.0 M urea, 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA) pH~8.0, and were polymerized with 0.05% (w/v) ammonium persulfate and 0.05% (v/v) TEMED. Gel electrophoresis occurred at a constant power output of 60 W. When completed, sequencing gels were fixed with 10% (v/v) methanol and 10% (v/v) acetic acid for 20 minutes before being dried and exposed to x-ray film.

D. Protein and DNA transfer onto membranes

1. *Western blots*

Subsequent to SDS-PAGE, gels containing separated protein samples were equilibrated in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% (w/v) SDS and 20% (v/v) methanol) [2]. The soaked gel was assembled into the blotting apparatus (Bio-Rad) with an equilibrated nitrocellulose membrane closest to the anode. Electrophoretic transfer of proteins onto the solid support was carried out 12-16 hours at 25 volts followed by 65 volts for one hour.

Following transfer, all steps were performed according to the protocol outlined above (see section "screening with antibodies"). These steps included the blocking of the membrane, the binding of primary antibodies (SCS specific) and secondary antibodies, all the washing steps, and the colorimetric detection.

2. *Electrophoretic transfer of DNA*

DNA separated on agarose gels buffered with TAE, was electrophoretically transferred onto a nylon-based transfer membrane [14] in a similar manner to that described for Western blotting.

The DNA was first denatured in 0.2 M NaOH and 0.6 M NaCl for 30 minutes. The gel was then washed and equilibrated in transfer buffer (25 mM Na_2HPO_4 (pH 6.5)) for 1 hour with three changes of buffer. After assembly and immersion of the cassette in the electrophoresis tank, transfer was carried out initially at 0.25 Amps and 9 volts for 1-2 hours at 4 °C, followed by 1 Amps and 26 volts for 1-2 hours (to complete the transfer of large fragments). DNA that had been transfered was then irreversibly cross-linked to the membrane.

E. Sequencing of DNA templates by the chain termination method

1. *Sing' stranded templates*

Single stranded DNA templates derived from M13 [15] were used routinely for most of the sequencing throughout this project. Prepared as described above, both the quality and the quantity of these templates were suitable for their subsequent sequencing by the dideoxy-chain termination method [10]. Sequenase™ and Sequenase™ Version 2.0 (USB) were utilized as the DNA polymerases in all sequencing reactions.

General protocols for single stranded sequencing followed those given for use with the Sequenase polymerases [16]. Briefly, primer was annealed to the M13 template (approximately 1 µg), by heating the mixture at 65 °C for 2 minutes and cooling slowly over a period of 30 minutes. (A 1:1 molar ratio of primer to template DNA was maintained.) Labeling and termination reactions were carried out as described by United States Biochemical [16].

In order to obtain sequence information very close to, or far away from the primer, variations were made in both the concentration of labeling and termination mixes, and in the time of labeling and termination. Specifically, for reading sequences close to the primer, the labeling mixture was diluted 1:10 with water, the amounts of the primer and the template were doubled, and the reaction times were kept to under 3 minutes each. When extending sequences farther from the primer, labeling mixes were used undiluted, additional labeled dATP was used, the labeling reactions were lengthened to 5 minutes, and the termination reactions were extended by diluting the dideoxy-termination nucleotides 1:2 with sequence extending mix [16].

Regions of secondary structure in the product DNA resulting in gel artifacts or compressions were usually eliminated by the use of dITP instead of dGTP in the labeling mix [16].

2. *Double stranded templates*

Double stranded plasmid DNA was prepared for sequencing according to the alkaline denaturation method [17-21]. The DNA was denatured by adding 0.1 DNA solution volumes of 2 M NaOH, 2 mM EDTA and incubating 30 minutes at 37 °C. The mixture was neutralized by adding 0.1 reaction volumes of 3 M sodium acetate (pH 4.5), and the DNA was precipitated with 2-4 reaction volumes of ethanol at -70 °C for 30 minutes. After washing the pelleted DNA with 70% (v/v) ethanol, it was redissolved in water and sequenced directly using similar conditions applied to single stranded templates.

F. Oligonucleotide directed mutagenesis

All oligonucleotide directed mutagenesis carried out during the course of this work followed essentially the method of Kunkel *et al.* [22]. Modifications to the protocol are as follows. Recombinant M13 bacteriophage containing the genes encoding the

subunits of *E. coli* SCS (M13mp18.202), were propagated in the *E. coli* strain CJ236, deficient for dUTPase and uracil N-glycosylase. The progeny viruses that were generated were isolated in order to purify uracil-enriched, single stranded template DNA used for mutagenesis (see above). A tenfold molar excess of phosphorylated mutant oligonucleotide was annealed to the template in 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl and 1 mM dithiothreitol at 65 °C for 5 minutes and then cooled to room temperature. Extension and ligation of primed mutant hybrids were completed using 2.5 units of T4 DNA polymerase and 2 units of T4 DNA ligase in the presence of 0.5 mM of each dNTP, 1 mM ATP, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 25 mM NaCl and 5.5 mM dithiothreitol. This mutagenesis reaction was initiated for 10 min.at 0 °C, followed by 10 minutes at room temperature, and continued for 3-4 hours at 37 °C.

The parental strand of the filled in duplex hybrid which contained uracil was degraded selectively by transfection into an *E. coli* strain with wild type uracil N-glycosylase activity. Specifically, 5.0 µl of the mutagenesis reaction mixture was transfected directly into transformation competent JM103 *E. coli* for 1 hour at 0 °C. Cells were heated at 42 °C for 90 seconds, then chilled at 0 °C for 2 minutes. Afterwards, 150 µl of freshly grown JM103 along with 3.5 ml of liquified top agarose were added and poured onto LB plates. Plates were cooled to solidify the top agarose, and the bacteria and phage were grown overnight at 37 °C.

Plaques were picked the following day, high titre stocks of the potential mutants were developed, and single stranded templates were prepared. Due to the highly efficient production of mutants by this technique [22] (typically, > 50% of the plaques analyzed were mutated), mutants were screened for by DNA sequencing analysis using an primer upstream of the mutagenic site.

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Chapter 3

CLONING AND SEQUENCING OF THE ß-SUBUNIT PRECURSOR cDNA OF NEWBORN PIG HEART SUCCINYL-CoA SYNTHETASE

I. INTRODUCTION

Fundamental differences exist among succinyl-CoA synthetases from various sources. The enzyme from *E. coli* [1-3] and a variety of other gram-negative species [4-6] have a quaternary structure composed of an $\alpha_2\beta_2$ tetramer. In contrast, SCS from all mammalian sources investigated to date has an $\alpha\beta$ dimeric structure [7-9]. When considering the nucleotide specificity of SCS, the enzyme from *E. coli* [10] and a number of bacterial species [4, 11, 12] has been found to be broadly specific, accepting either ATP, GTP or ITP. In comparison, the eukaryotic counterparts are much more discriminating, with a restricted specificity to either ATP or GTP [13-18]. Indeed, from various lines of evidence it has been surmised that genetic polymorphism is responsible for distinct ATP- and GTP-specific enzymes in mammalian tissues, each having a discrete metabolic role [19-23]. Taken together, these differences in quaternary structure and nucleotide specificity serve to classify these enzymes into one of two groups. The first group is represented by the *E. coli* enzyme, and is typified by a tetrameric ($\alpha_2\beta_2$) structure exhibiting broad nucleotide specificity. The second class, represented by the pig heart enzyme, is manifested by dimers ($\alpha\beta$) with strict ATP or GTP specificity.

In order to fully characterize the structural differences between the two classes of SCS enzymes, the knowledge of protein sequence information was required in addition to the other biochemical data. Buck *et al.* [24] have sequenced the SucC and SucD genes of *E. coli* encoding the ß and α subunits, respectively. The α subunits from rat liver [25] and pig heart [26] SCS have also been cloned and sequenced. Especially in light of the above noted differences, it is surprising that the amino acid sequences of the α subunits from *E. coli* and the mammalian sources are about 70% identical and over 90% similar when conservative replacements are included **Figure 3.1** [25]. While this remarkable degree of conservation of sequence provides little clue for the differences in the properties between the two forms of the enzyme, it does make an important suggestion. Perhaps to a greater degree, the majority of class differences could be attributed to

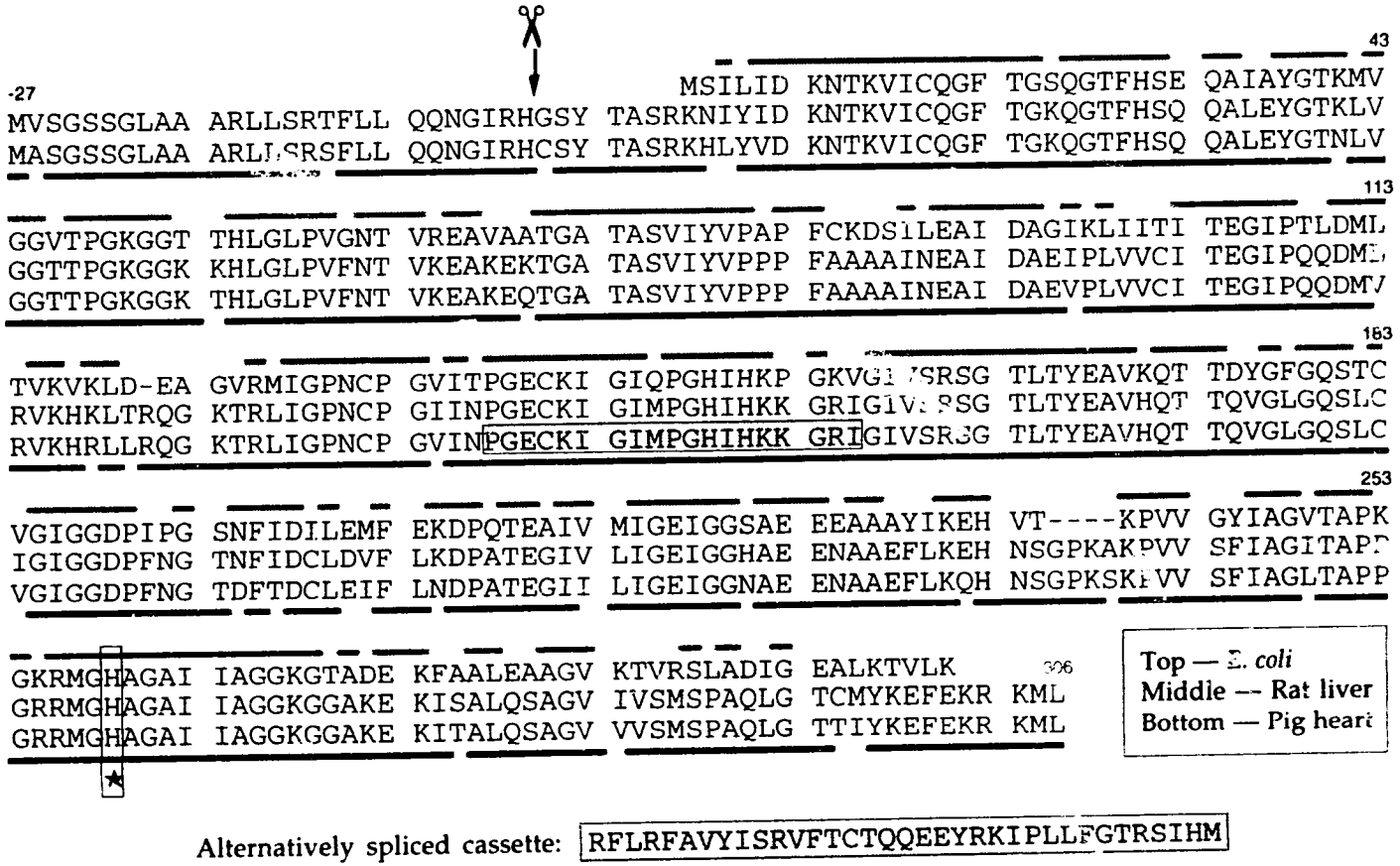

Figure 3.1 Comparison of the predicted amino acid sequences of cloned α subunits from *E. coli*, rat liver and pig heart. The sequences of the subunits are taken from the following: *E. coli* [31]; rat liver [32]; and pig heart [33, 34]. The numbering is based on the rat liver sequence. Homology between sequences is indicated with bars above or below compared pairs. The upper bar indicates identity between *E. coli* and rat liver sequences, while the lower bar indicates identity between rat liver and pig heart sequences. The shaded blocks represent the sequence of alternatively spliced cassettes in pig heart α57 (in alignment) and α108 (shown below). Scissors indicate the point of proteolytic cleavage to remove the signal sequence for the eukaryotic proteins. The box with star indicates the position of the conserved active site histidine residue.

differences in the ß subunits and not the α subunits. Since we wished to study the relationship between structure and function of SCS, and no protein sequence for a ß subunit from a dimeric enzyme had yet been determined, we sought to isolate and sequence a cDNA clone for the ß subunit from pig heart SCS. This is the subject of this Chapter.

II. MATERIALS AND METHODS

A. Bacterial strains, vectors and cDNA library

The following *E. coli* strains were used throughout this work: Y1090 was used as a lytic host for λ bacteriophage propagation during the screening of the λgt11 cDNA libraries; JM103 and JM109 were used for the construction and propagation of M13 derivatives. The genotypes of these bacterial strains can be found in **APPENDIX I.** M13mp18 was used as a sequencing vector for all sequencing. A cDNA λgt11 expression library, created using messenger RNA isolated from the heart tissue of a 2 day old piglet [27], was used in these experiments.

B. N-terminal protein sequence determination

N-terminal amino acid sequence analysis of the ß subunit of pig heart SCS was obtained after separation of the purified enzyme into its subunits by SDS-PAGE followed by electrophoretic transfer of the protein onto polyvinylidine difluoride membrane [28]. These immobilized protein samples were directly subjected to automated Edman degradation using an Applied Biosystems Model 470A instrument.

C. Internal protein sequence determination by cyanogen bromide cleavage

Cyanogen bromide (CNBr) cleavage of purified pig heart ß subunit was used to create protein fragments for internal amino acid sequence determination [29]. Approximately 4 nmol of purified pig heart ß subunit was extensively dialyzed against

4% (v/v) acetic acid and then dried by vacuum. The protein sample was resuspended in 50 µl of CNBr in 70% (v/v) formic acid. For effective cleavage, the CNBr concentration was a 300 mol excess over the estimated methionine content of the protein sample. The digestion was carried out for 24 hours at room temperature (22 °C), before being diluted with 500 µl of water and dried down by vacuum overnight. The fragmented protein sample was resuspended in acetonitrile and 0.05% (w/v) trifluoroacetic acid, and the resultant solution was applied onto a Reverse Phase Hydrophobicity HPLC column. Fractions containing protein were collected and dried before being sequenced by automated Edman degradation (see above).

D. Pseudo-screening of polyclonal antibodies to the ß subunit of pig heart SCS

Polyclonal antibodies directed against the ß subunit of pig heart SCS were developed in rabbits following established protocols [30]. The antigen was prepared from the electro-eluate of an SDS-PAGE gel slice containing the ß subunit of GTP-specific, pig heart SCS. It was mixed with Freund's adjuvant and was injected intramuscularly. The resultant antiserum was diluted and subjected to the following pseudo-screening protocol.

Non-recombinant λgt11 bacteriophage was plated out on 10 LB/agar plates, using Y1090 as a lytic host, to obtain a semi-confluent lysis of the bacterial lawn (approximately 10^5 plaques/plate; see **Chapter 2**). Nitrocellulose plaque lifts of the lysed lawns were taken [30], and these saturated filters were incubated for 6 hours with polyclonal antibodies directed against the ß subunit of pig heart SCS. Purified antibodies were collected for further use in immunological screening of expression libraries.

E. Antibody screening, isolation and sequencing of clones from a newborn pig heart cDNA library

A cDNA library used in these studies was constructed using heart tissue from a newborn piglet and generated in λgt11 as reported elsewhere [27]. (This cDNA library

will be referred to henceforth as a 'newborn pig heart cDNA library'.) Pseudo-screened polyclonal antibodies directed against the ß subunit of pig heart SCS were used to screen nylon plaque lifts according to a modified method described by Huynh *et al.* [31] (see **Chapter 2**).

Positive plaques were selected and eluted in lambda dilution buffer (35 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$, 0.1M NaCl and 0.1% (w/v) gelatin). These phage mixtures were repeatedly re-screened with antibodies until the positive clone was pure, after which high titre stocks of the this phage were developed [30]. Using these high titre stocks, recombinant λ phage DNA for each potential positive was prepared from 50 ml liquid cultures. The DNA was digested with EcoRI, and the fragments were separated by preparative agarose (0.7% (w/v)) gel electrophoresis. Bands containing inserts were excised from the gel, the DNA was purified by glass powder [32, 33] and then subcloned into similarly digested M13mp18. These sequencing vector constructs were propagated in *E. coli* JM103 or JM109 to generate single stranded template, suitable for subsequent sequencing [30].

F. Construction of digoxigenin-labeled DNA probes

Digoxigenin-11-dUTP (Boehringer Mannheim) labeled DNA probes used in re-screening the newborn pig heart cDNA library were created by random priming [34, 35] a clone encoding the ß subunit of SCS from newborn pig heart tissue (see **RESULTS AND DISCUSSION**).

Approximately 0.5-1 µg of linear DNA purified from an agarose gel (see **Chapter 2**) was denatured by boiling for 15 minutes followed by quickly cooling in ice for 5 minutes. The random priming reaction mixture consisted of 6.25 OD/ml of random hexanucleotide, 100 µM each of dCTP, dGTP and dATP, 65 µM dTTP, 35 µM digoxigenin-11-dUTP, 50 mM Tris-HCl (pH 7.2), 10 mM $MgCl_2$, 0.1 mM DTT, 0.2 mg/ml bovine serum albumin and 2-3 units of Klenow (large fragment) DNA polymerase [36]. Extension of the randomly annealed hexanucleotides was allowed to occur for

approximately 20 hours at 37 °C. The reaction was terminated with the addition of 4 mM EDTA (final concentration), and the labeled probes were precipitated overnight at -70 °C with 100 mM LiCl and 3.5 reaction volumes of 95% (v/v) ethanol. The nucleic acids were collected by centrifugation at 14 x 10^3 g for 15 minutes. The pellet was washed with 40 µl of 70% (v/v) ethanol and then dried under vacuum before being redissolved in 50 µl TE buffer (37 °C, 30 minutes). The randomly primed probe prepared in this manner was stable at -20 °C for 1-2 years.

G. Screening, isolation and sequencing of clones from a newborn pig heart cDNA library using DNA probes

Digoxigenin-containing DNA probes created from randomly priming a clone of the ß subunit of SCS from newborn pig heart tissue (see above) were used to re-screen the newborn pig heart λgt11 cDNA library [36] (see **Chapter 2**).

Positive clones were re-screened repeatedly until isolated to homogeneity. Insert DNA was amplified directly from the viral stocks by the polymerase chain reaction (see below). The amplification products were digested with restriction endonucleases, separated by agarose gel electrophoresis and further purified by glass powder elution. These isolated DNA fragments were then subcloned into the M13mp18 sequencing vector. Recombinants were isolated, and single stranded templates were then prepared and sequenced.

H. Amplification of DNA by the Polymerase Chain Reaction (PCR)

PCR amplification of DNA was carried out using the methods developed by Saiki *et al.* [37]. The various component and temperature parameters used to create an acceptable level of amplification were determined empirically. Routinely, 10-100 ng of each primer was used in the amplification reaction. The oligonucleotide sequence of the primers used can be found in **APPENDIX I** (see **RESULTS AND DISCUSSION** below for details). When λ phage was used as a template in the PCR, typically 10^5 pfu were

denatured at 94 °C for 3 minutes before adding Taq DNA polymerase and initiating thermal cycling.

III. RESULTS AND DISCUSSION

A. Protein sequence determination

In order to verify the identity of potential positive clones encoding the ß subunit of pig heart SCS, protein sequence information was required from the purified pig heart ß subunit. This was obtained by sequential Edman degradation [38] of two different preparations of isolated ß subunit. First, intact ß subunit was used in two separate determinations to provide the following amino acid sequences at the N-terminus:

a) L-N-L-Q-E-Y-Q-S-K-K-L,

b) L-N-L-Q-E-Y-Q-S-K-K-L-M-S-D-N-G-V-K-V.

Second, fragments produced by cyanogen bromide treatment of the ß subunit were used in order to obtain internal polypeptide sequence. Only one purified cleavage product submitted for analysis was unambiguous with respect to the amino acid sequence derived. Its sequence was P-E-V-V-G-Q-L-A.

B. Isolation and sequencing of cDNA clones encoding the ß subunit of pig heart SCS

Before use in the immunological screening of expression libraries, polyclonal antibodies directed against the ß subunit of pig heart SCS were purified by a 'pseudo-screening' procedure [39] to remove antibodies which recognized other *E. coli* proteins or those of the non-recombinant λgt11 bacteriophage. By decreasing the level of the background detected, this simple procedure served to increase the sensitivity of the antibody probes, thus aiding in the detection of weak positive signals.

Approximately 4.2 x 10^5 plaques from a newborn pig heart cDNA library in the expression vector λgt11 [27] were screened with the 'purified' antibodies specific for the ß subunit. Fourteen potential positive clones were isolated and purified to homogeneity

through repetition of the screening protocol. The λ DNA from each clone was isolated and the insert DNA was excised by endonuclease digestion with EcoRI. The insert DNA fragments were then subcloned into the sequencing vector M13mp18. Sequencing analysis subsequently confirmed that two of these (apparently identical) clones (2C and 2D) encoded the ß subunit of SCS for the reasons given below.

C. Sequencing strategy for ß subunit clones from newborn pig heart

Insert DNA from the positive clones 2C and 2D was cleaved into 800 base pair (bp) and 600 bp fragments when digested with EcoRI. It was confirmed through PCR amplification of the purified λ phage stock, using λ specific primers (λF and λR, see **APPENDIX I**) on either side of the insert site, that these fragments alone made up the 1.4 kilobase pair (kbp) DNA insert. Each of the two smaller fragments were repeatedly sequenced in both orientations. To aid in the sequence determination of remote regions downstream from the primers, deletions of the M13mp18 recombinants were created using restriction sites internal to both the 800 bp (SalI) and the 600 bp (SphI) fragments. One unique, continuous open reading frame assisted in assigning the orientation of the fragments with respect to one another. As well, a polyA tail at the 3' end of the 600 bp fragment served to place it downstream of the 800 bp 5' fragment. Sequence through the internal EcoRI site was obtained from a sequencing construct created by a λ DNA HindIII-SphI fragment in M13mp18. The scheme for all sequences completed on the SCS ß subunit clone from newborn pig heart is presented in **Figure 3.2**, and the complete sequence is shown on **Figure 3.3**.

D. SCS ß subunit sequence from newborn pig heart

The clones 2C and 2D were confirmed to be that of the ß subunit of pig heart SCS for the following reasons. First, the amino acid sequence derived from the DNA of these two clones matched the N-terminal protein sequence obtained for the mature pig heart ß subunit (see **Figure 3.3**). As well, internal amino acid sequence determined from

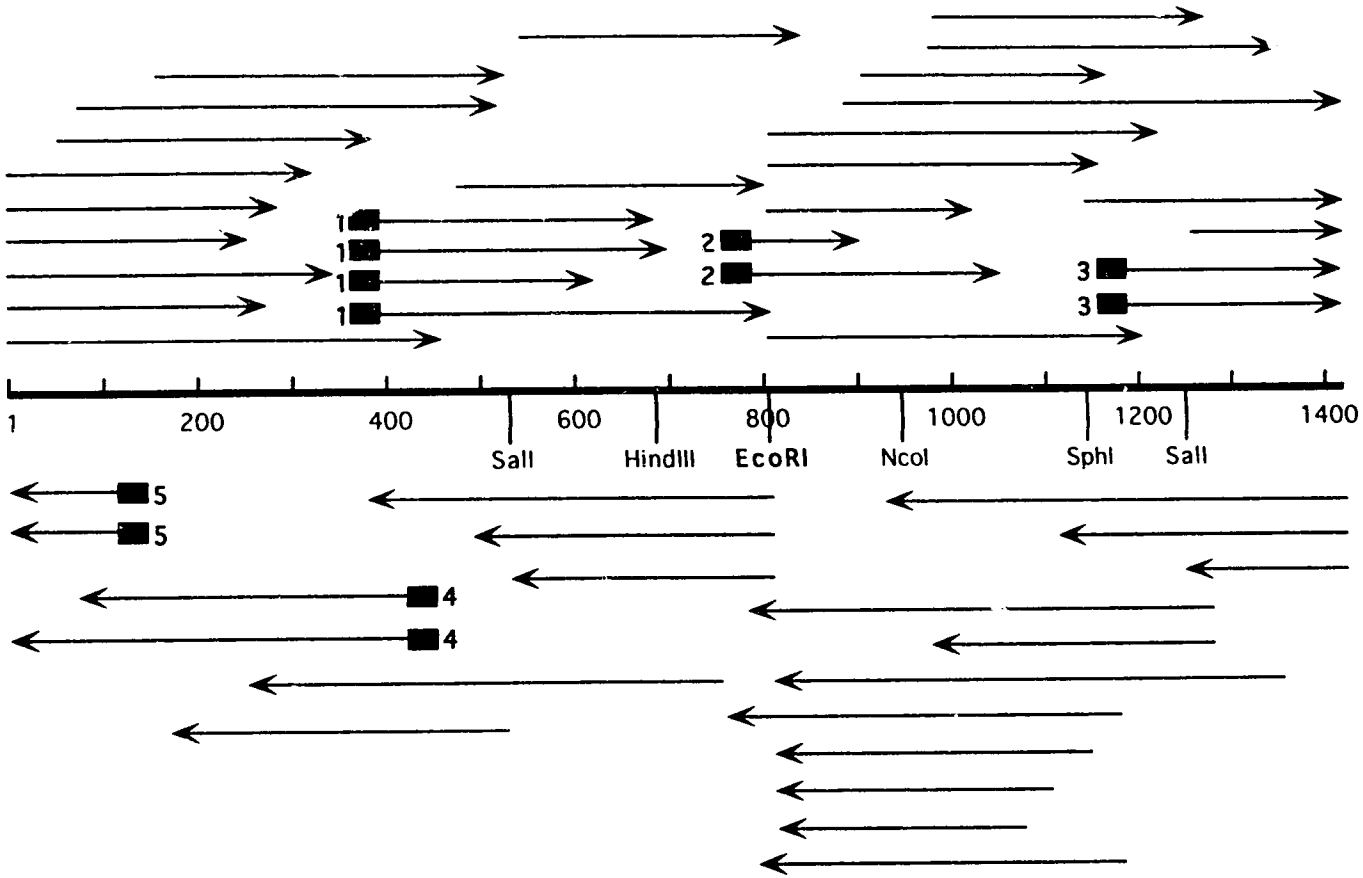

Figure 3.2 Schematic map of sequencing trials completed on the clones of the newborn pig heart ß subunit. Sequences were determined from 2C, 2D, PH5 and PH6 clones. Represented is the 2C/2D clone with numbering in base pairs. The numbered bars labeled from 1 to 5 represent sites where synthetic oligonucleotides were used as primers.

GAATTCCGGCGGCCCCCGTGGCAGCTCAGGCCCGGAAACTTCTGAGAGAC *50*

CTGGCGTTCCGGCCCCCGCTCTTGGCGGCTAGGTCCCAGGTGGTTCAATTAACCCCCAGAAGATGGCTGAAGCTG
-22 *M A F R P P L L A A R S Q V V Q L T P R R W* L N L

CAGGAATACCAGAGCAAGAAACTGATGTCTGACAATGGAGTGAAAGTTCAAAGATTTTTTGTAGCAGACACTGCC *200*
4 Q E Y Q S K K L M S D N G V K V Q R F F V A D T A

AATGAAGCTCTCGAGGCTGCTAAGAGACTAAATGCAAAAGAAATTGTTTTAAAAGCCCAGATCTTAGCTGGAGGA
29 N E A L E A A K R L N A K E I V L K A Q I L A G G

AGAGGAAAAGGTGTCTTCAGTAGCGGTTTGAAAGGAGGTGTTCATTTAACAAAAGACCCTGAAGTTGTGGGTCAG *350*
54 R G K G V F S S G L K G G V H L T K D P E V V G Q

TTGGCTAAACAGATGATTGGGTATAATCTAGCAACAAAACAAACTCCAAAAGAAGGTGTGAAAGTTAACAAGGTG
79 L A K Q M I G Y N L A T K Q T P K E G V K V N K V

ATGGTAGCTGAAGCATTGGACATTTCCAGGGAAACCTACCTGGCCATTCTTATGGACCGGTCTTGCAATGGTCCC *500*
104 M V A E A L D I S R E T Y L A I L M D R S C N G P

GTATTGGTGGGCAGCCCTCAGCAAGGTGTCGACATTGAAGAAGTGGCAGCTTCAAACCCAGAACTTATTTTTAAG
129 V L V G S P Q G G V D I E E V A A S N P E L I F K

GAGCAAATTGACATTATTGAAGGAATAAAGGACAGCCAGGCTCAGCGGATGGCAGAAAATCTAGGCTTCCTCGGG *650*
154 E Q I D I I E G I K D S Q A Q R M A E N L G F L G

CCTTTGCAAAACCAGGCTGCAGATCAAATTAAGAAGCTTTATAATCTCTTCCTGAAAATCGATGCTACTCAGGTG
179 P L Q N Q A A D Q I K K L Y N L F L K I D A T Q V

GAAGTGAATCCCTTTGGTGAAACTCCAGAAGGACAAGTTGTCTGTTTTGATGCCAAGATAAACTTTGATGACAAT *800*
204 E V N P F G E T P E G Q V V C F D A K I N F D D N

GCAGAATTCCGACAGAAGGATATATTTGCTATGGACGACAAATCAGAGAATGAGCCCATTGAAAATGAAGCTGCG
229 A E F R Q K D I F A M D D K S E N E P I E N E A A

AAGTATGATCTAAAATACATAGGACTGGATGGGAACATCGCCTGCTTTGTAAATGGGGCTGGACTTGCCATGGCT *950*
254 K Y D L K Y I G L D G N I A C F V N G A G L A M A

ACCTGTGACATCATTTTCTTGAATGGTGGGAAGCCAGCCAACTTCTTGGATCTTGGAGGTGGTGTAAAGGAATCT
279 T C D I I F L N G G K P A N F L D L G G G V K E S

CAGGTTTATCAAGCATTCAAACTGCTCACAGCCGATCCTAAGGTTGAAGCCATCCTAGTGAATATTTTTGGTGGA *1100*
304 Q V Y Q A F K L L T A D P K V E A I L V N I F G G

ATCGTCAACTGTGCCATCATTGCCAATGG**G**ATCACCAAAGCATGCCGGGAGCTGGAACTCAAGGTGCCTCTGGTG
329 I V N C A I I A N G I T K A C R E L E L K V P L V

GTCCGGCTTGAAGGAACCAATGTCCACGAAGCCCAGAACATTCTCACTAACAGCGGACTCCCCATTACTTCGGCT *1250*
354 V R L E G T N V H E A Q N I L T N S G L P I T S A

GTCGACCTGGAGGATGCAGCCAAGAAGGCTGTGGCCAGCGTGACTAAGAAGTGATGCCTTTGTCCTGATCCCTCG
379 V D L E D A A K K A V A S V T K K •

GAGGAAGAAGTTTTCCCATAAGAAG**A**GATTGTTCTCTCATCACTGTGAAAGAAATGGTTATCCCAATGAGCCGAA *1400*

AAAAAAGGAATTC *1413*

Figure 3.3 Nucleotide sequence of the cDNA (2C,2D) encoding the ß subunit of succinyl-CoA synthetase from newborn pig heart, with deduced amino acid sequence. Numbers in plain text correspond to protein sequence, numbers in bold italics refer to nucleotide sequence. Amino acid sequence is shown to begin at the AGACCTGGC consensus sequence for non-AUG translation initiation, resulting in a 22-residue mitochondrial signal sequence (italics) and a mature polypeptide of 395 amino acids. N-terminal and internal protein sequences obtained by sequential Edman degradation agree with the deduced protein sequence and are underlined. The cDNA clones obtained from a mature pig heart library (see **Chapter 4**) were nearly identical in sequence except for two silent nucleotide substitutions at positions 1,130 and 1,351, noted in bold.

fragments obtained through cyanogen bromide cleavage coincided with residues 72-79 in the translated amino acid sequences. It should be noted that this internal sequence does not arise from CNBr-specific cleavage since Asp, not Met, is proximal to this Pro in the deduced amino acid sequence from the clone. Rather, this fragment likely arose due to the sensitive nature of Asp-Pro bonds under the acidic conditions used during CNBr fragmentation. Second, there was a high degree of sequence similarity (approximately 45% identity, see **Figure 3.4**) when the deduced amino acid sequences were aligned to the ß subunits of other SCS sources. Third, there was good agreement between the amino acid composition determined for the purified pig heart ß subunit [40, 41] and that corresponding to the amino acid sequence derived from a region of the cDNA encoding the mature form of the pig heart ß subunit (see **Table 5.1**, page 101).

The cDNA clone was 1,413 bp in length. A single continuous open reading frame from nucleotide 117 to a stop codon at nucleotide 1302 encodes the mature form of the pig heart ß subunit. The translation of this coding sequence would produce a protein consisting of 395 amino acids with a calculated mass of 42,502 Da, in good agreement with that estimated previously by SDS-PAGE [8].

E. The potential problem of the absence of tryptophan and of an ATG start codon

The pig heart ß clones have superfluous nucleotide sequence upstream of the start site corresponding to the N-terminus of the mature protein. It is obvious, therefore, that these clones have the potential to code for a precursor form of the ß subunit containing a mitochondrial targeting sequence. However, repeated sequencing of the clones in this region failed to reveal an ATG start codon which could be the precursor start site. In addition, the absence of a tryptophan codon in the coding sequence of the mature protein was unexpected, considering that others have reported that based on both protein fluorescence and amino acid analysis that, purified pig heart SCS contains one Trp residue located in the ß subunit [41] (neither the α subunit from either pig heart or rat

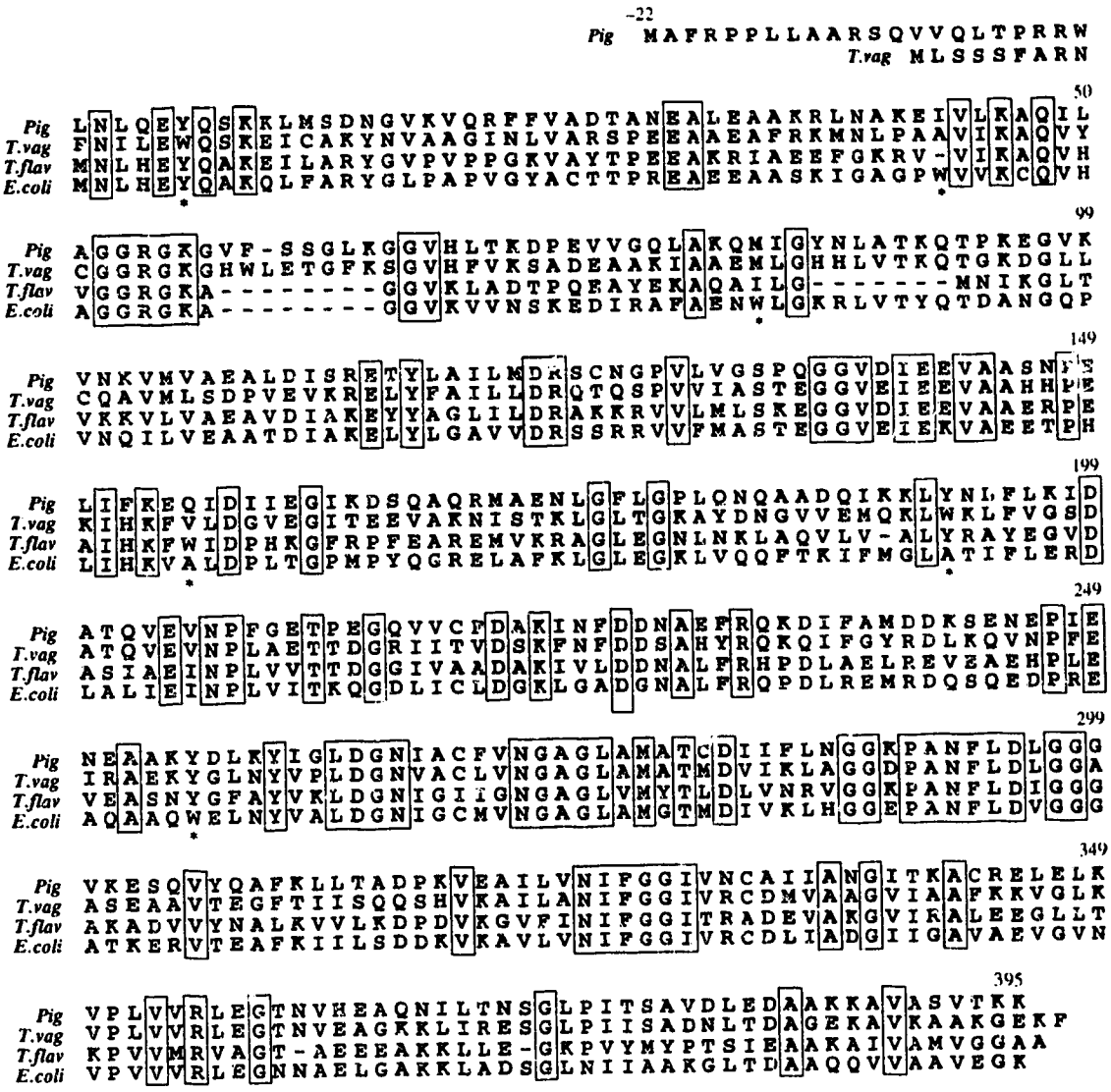

Figure 3.4 Comparison of the amino acid sequences of the ß subunit of SCS from various sources. Numbering is based on that of the pig heart sequence. Sequences include those obtained from: newborn pig heart (this work), *Trichomonas vaginalis* (*T. vag*) [51], *Thermus flavus* (*T. flav*) [52], and *Escherichia coli* (*E. coli*) [24]. Putative N-terminal signal sequences are shown for the pig heart sequence (22-residue leader for mitochondrial targeting), and *T. vaginalis* (9-residue hydrogenosomal signal sequence). Regions of sequence identity for all four species are boxed. Sites with tryptophan residues in any of the sequences are marked with asterisks. Numbering is based on the pig heart ß subunit sequence.

liver contains any Trp residues).[1] These two potential problems prompted re-screening the pig heart cDNA library to search for independent ß subunit clones that may have had 5' extensions with ATG start codons and coded for a mature amino acid sequence that contains a Trp residue. Instead of using antibodies raised against the ß subunit as probes for additional clones, the cDNA clone of the pig heart ß subunit was randomly primed to create DNA probes labeled with digoxigenin (see **MATERIALS AND METHODS**).

Under stringent hybridization conditions, 8 positive clones were isolated from screening approximately 3 x 10^5 pfu of the newborn pig heart cDNA library. Insert DNA was amplified from these positive λ phage stocks by PCR using λgt11 primers (λF and λR) and subsequently subcloned into M13mp18 (see **MATERIALS AND METHODS**). Restriction enzyme analysis indicated that 6 of the 8 clones had 800 bp and 600 bp EcoRI fragments, which were later confirmed through sequence analyses to be identical to the original pig heart ß subunit clones, 2C and 2D. The two remaining clones, PH5 and PH6, appeared identical to one another in terms of restriction endonuclease analysis, yet different from the original ß subunit clones (see **Figure 4.2**, page 87). The insert DNAs of PH5 and PH6 were comprised of 3 EcoRI fragments, 1,200 bp, 800 bp and 350 bp in length. Surprisingly, when these insert fragments were sequenced, they were discovered to contain exactly the same coding sequence for the mature pig heart ß subunit as did the original clones. The larger fragment was simply a 3'-extension of the 600 bp fragment of the original clones. Through PCR methods, the small 350 bp fragment was found to be related distally, downstream of the 1,200 bp fragment, and therefore contained only untranslated sequence. The 800 bp fragments coded for the same N-terminal portion of the ß protein with the exception that the PH5 and PH6 fragments were truncated 12 nucleotides at the 5' end compared to the 2C and 2D clones. No ATG start codon was

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Since the PH5 and PH6 clones were basically identical to the original cDNA of the clones 2C and 2D, the latter remained as the prototype clones for the newborn pig heart ß subunit, and any further discussion will be in reference to them.

F. Atypical initiation codon and signal sequence of the newborn ß subunit clone

The lack of an ATG codon in the region upstream of the mature protein coding sequence implied that the initiation of translation was atypical. In a few cases, initiation has been noted to occur at codons other than AUG: most frequently at CUG, but also at ACG and less frequently at GUG [42]. From a search for potential start sites, only the CTG codon beginning at nucleotide 51 met the requirements for the efficient initiation of translation from a non-AUG codon [43]. With its surrounding sequence, AGACCTG**GC**G, this site clearly fulfilled the contextual sequence preferences for non-AUG initiation: if the C of the start codon was designated +1, the -3 position was occupied by a purine, the +4 position by guanine, and the +5 position was favourably filled with cytosine [42-44]. Moreover, it had been shown that secondary structures 12 to 15 nucleotides downstream of non-AUG codons facilitated the initiation of translation [45], and nearly all mRNAs naturally supporting non-AUG initiation have GC-rich and, hence, highly structured leader sequences [43]. It is possible that the leader sequence of the ß subunit clone had the potential to form secondary structure 12 nucleotides downstream of the initiation site. Although secondary structure predictions in RNA are extremely complex and dependent on several assumptions, one possible structure is presented in **Figure 3.5**. This structure may have assisted in stalling the 40S ribosomal subunit momentarily with its initiation site recognition center in close proximity to the CTG codon, thereby facilitating initiation.

By selecting the CTG initiation codon as the start site for translation, the one continuous open reading frame of the ß clone extended from nucleotides 51 to 1,302 and

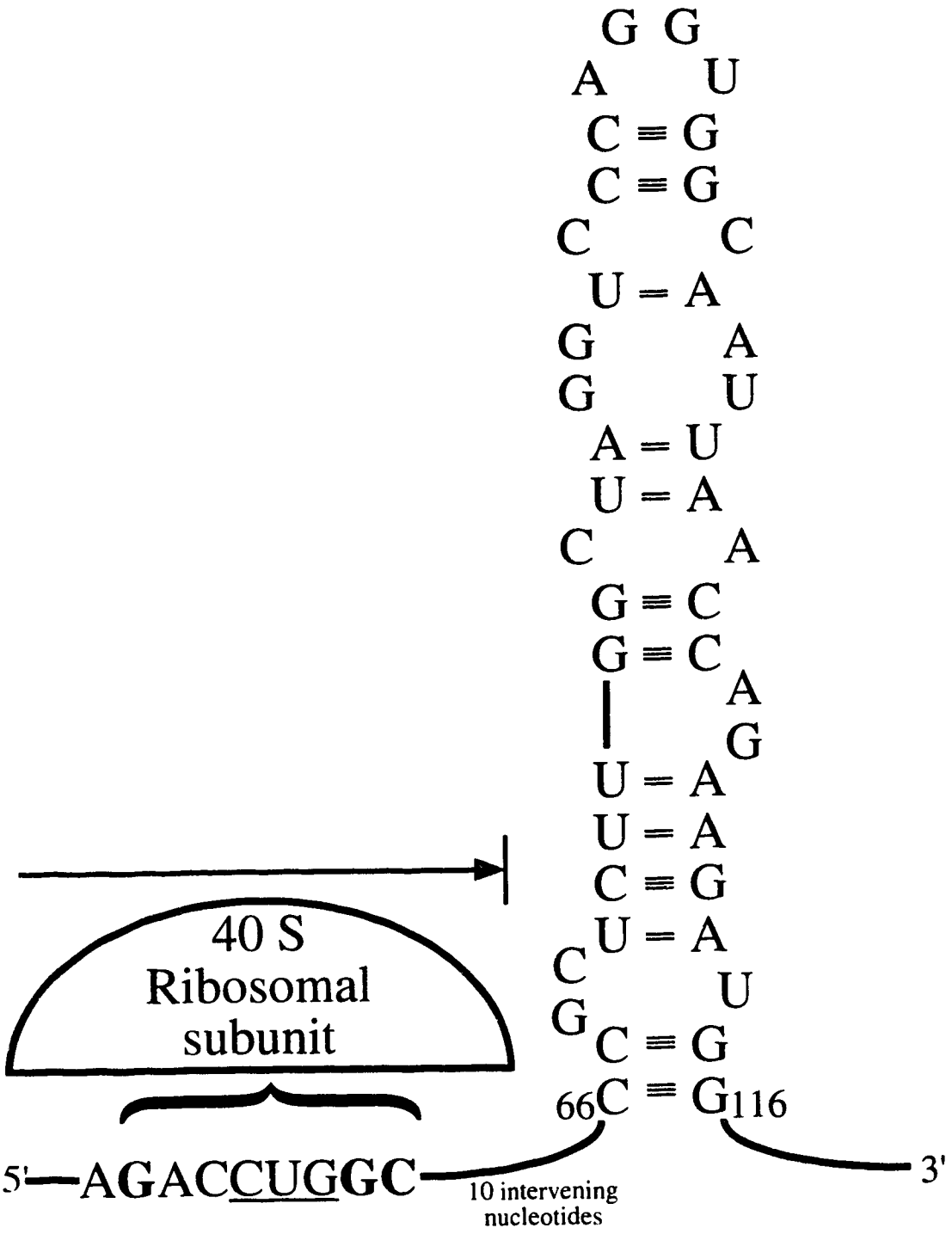

Figure 3.5 Secondary structure prediction of the mRNA for the infant pig heart ß subunit. This putative hairpin structure lies 12 nucleotides downstream of the non-AUG start codon and may facilitate initiation by stalling the scanning 40 S ribosomal subunit, and properly positioning its AUG-recognition site over the CUG codon (underlined). Nucleotides which fill contextual requirements for non-AUG initiation sites are shown in bold.

would encode a 417 amino acid protein corresponding to the cytoplasmic precursor of the ß subunit. The first 22 amino acids before the mature N-terminus would constitute the mitochondrial matrix targeting sequence (see **Figure 3.3**). Although the signal sequences for the ß subunit and the α subunit [25] have typical attributes of mitochondrial signal sequences [46], it is noteworthy that they have little amino acid similarity. Due to the electrochemical potential which exists across the inner mitochondrial membrane, the preponderance of basic residues and the noted lack of acidic residues would serve to drive the insertion of the matrix-targeting signal into the mitochondrial membrane [47]. One of these basic residues, Arg at the -2 position (relative to the mature sequence start site), meets the consensus preferences for mitochondrial targeting sequences [48, 49] and likely directs cleavage of the signal sequence by a matrix endoprotease [50].

G. Primary structure comparisons between the ß subunits of *E. coli* and pig heart SCS

The principle objectives of this work were to determine the sequence for the ß subunit of pig heart SCS and to compare it to that of the *E. coli* enzyme. It was anticipated that such a comparison might provide a rationale for the diversity of the SCS enzymes, as well as insight into the structure and function relationship. As shown in **Figure 3.4**, the sequence alignment reveals there are substantial stretches of sequence identity or similarity throughout the subunits. Identical sequences that are conserved among all known ß subunit sequences may act structurally or play a catalytic role common to all forms of the enzyme. An intriguing stretch of polypeptide (residues 58-64 of the pig heart sequence) that is absent in both the bacterial ß subunits raises suspicion as a sequence likely to be involved in class differences. Other stretches of significant sequence difference between the pig heart and *E. coli* enzymes are seen at residues 20-29, 162-173, and 364-371 (pig heart subunit numbering), although many isolated nonconservative changes occur throughout much of the sequence. Some of these differences might be important in the evolution from an $\alpha_2ß_2$ tetramer observed in *E. coli*

to an αß dimer in pig heart. This point of the interaction of the αß dimers in the quaternary structure of the *E. coli* enzyme became the focus of later work (see **Chapters 7 and 8**). As well, the cloning of the ß subunit from pig heart SCS was crucial in developing the successful expression of the entire pig heart enzyme (see **Chapter 6**) necessary for future studies.

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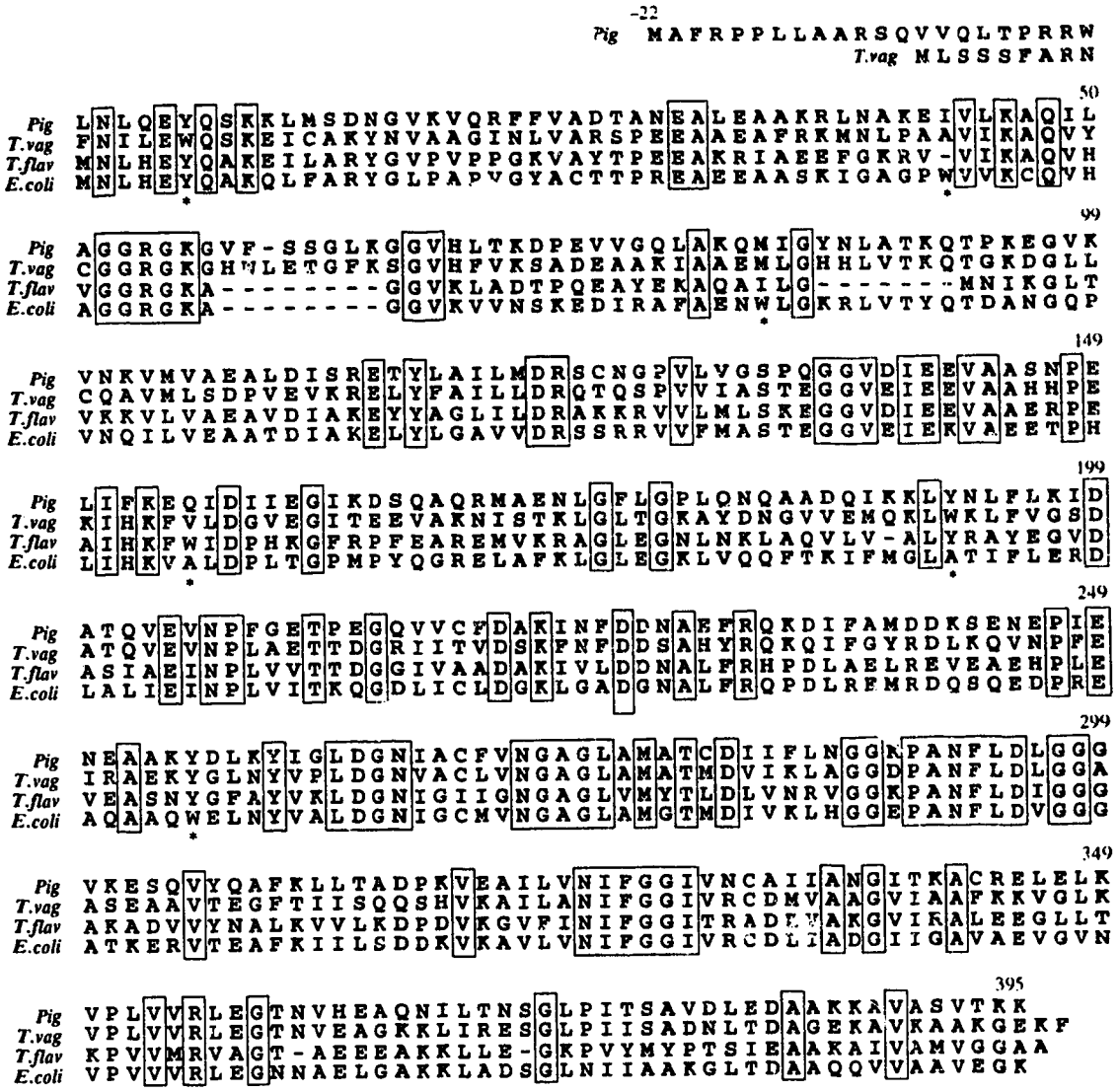

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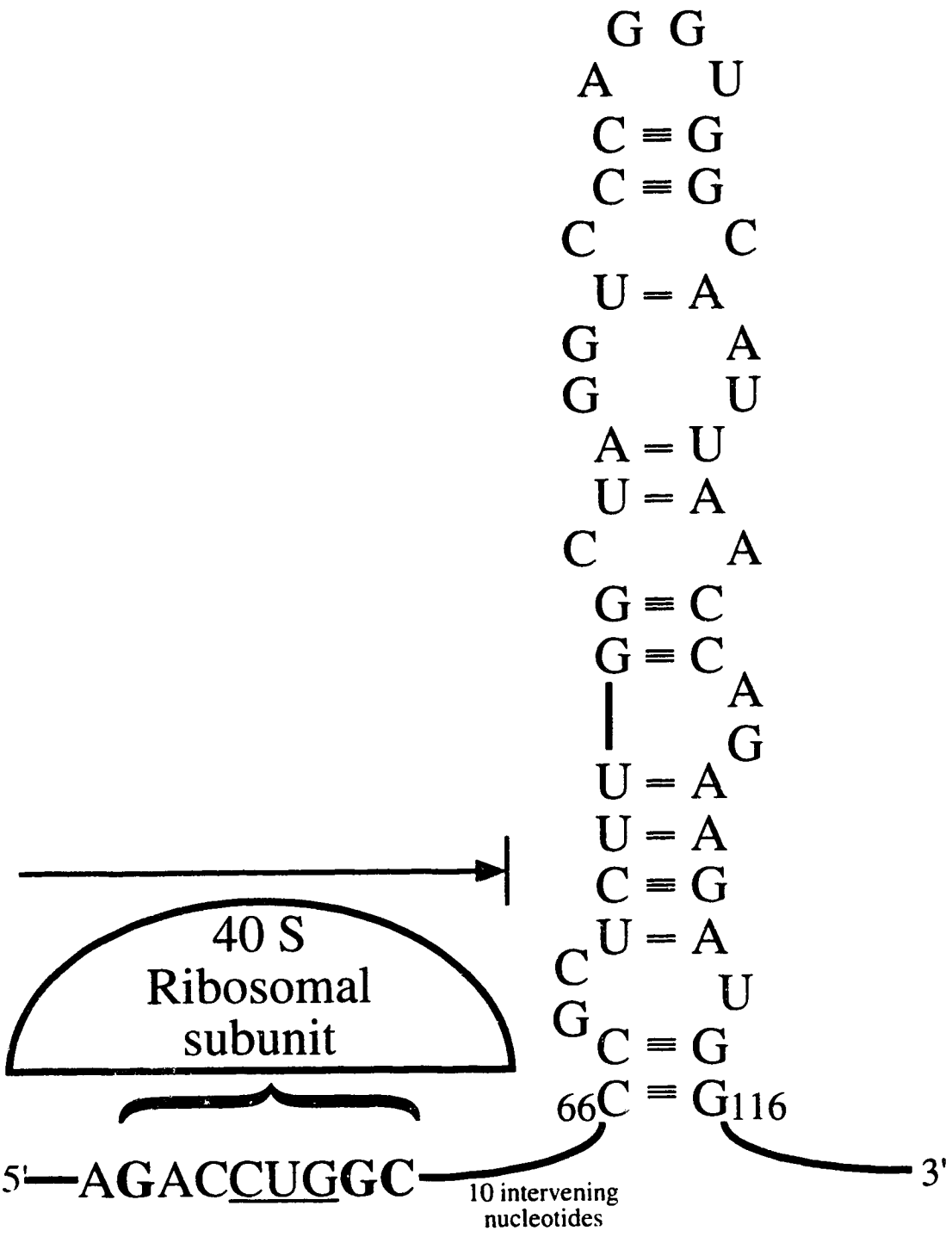

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Chapter 4

ISOLATION AND CHARACTERIZATION OF cDNAs ENCODING AN **ADULT** ISOFORM OF THE ß SUBUNIT FROM PIG HEART SUCCINYL-CoA SYNTHETASE

I. INTRODUCTION

Nishimura and colleagues [1] had previously reported that pig heart SCS contains one Trp residue and that it was located in the ß subunit. The notable absence of a single tryptophan residue encoded anywhere in the mature sequence of the SCS ß subunit clone isolated from the newborn pig heart cDNA expression library remained enigmatic. Furthermore, it was both surprising and perplexing to discover that a Trp residue was encoded in the open reading frame as the last residue of the signal sequence (see **Figure 3.3**, page 68). It would be anticipated, however, that during the targeting of this precursor from the cytoplasm to the mitochondrial matrix, this Trp residue would be cleaved on its C-terminal side by the mitochondrial signal peptidase to liberate the mature pig heart ß (Phß) subunit. Nevertheless, the proximity of this Trp residue to the mature sequence of the protein aroused suspicion: could the post translocational processing somehow leave the Trp residue with the mature form? N-terminal protein sequence of the mature subunit was determined repeatedly and unambiguously by dansyl-Edman sequencing (see **Chapter 3 - Protein sequence determination**). The results confirmed that the site of post translational processing occurs between the Trp residue in the targeting signal and the Leu residue in the mature sequence. This Trp residue, therefore, is not part of the mature sequence.

Nishimura's work demonstrating that one Trp residue was present in the Phß subunit was done using purified adult pig heart SCS. The Phß clone had, however, been isolated from a newborn pig heart cDNA library and found to contain no Trp codons in the mature coding sequence and a non-AUG codon for translational initiation. The possibility was therefore considered that this Phß subunit clone was an isoform that is differentially expressed in newborn pig heart tissue. It is well established that eukaryotic systems employ structurally distinct protein isoforms which are produced according to the state of cell differentiation and physiological functions [2]. For example, several isoforms of hemoglobin have been observed to suit the differing physiological conditions

of embryonic, fetal and adult stages of development. It is known that multiple species of SCS exist in eukaryotes as well. Mouse liver mitochondria [3], trypanosomes [4], yeast [5], and a wide variety of animal species including mammals [6-8] exhibit more than one distinct species of SCS enzyme based on nucleotide specificity or metabolic role. These reports point to the possibility that in eukaryotes, genetic polymorphism of different SCS subunit genes are responsible for the variety of distinct enzymes detected. This polymorphism has been observed in the pig heart system as well [9, 10]. Two different cDNA copies of the pig heart α subunit were isolated from the newborn pig heart cDNA library [9]. One, labeled Phα57, encoded a protein which shares a high degree of similarity (70% identity) with all regions of the previous cloned α subunits from *E. coli* and rat liver. The other, Phα108, coded for an internal replacement of a different stretch of 36 amino acid residues from 19 in Phα57 (see **Figure 3.1**, page 60). This new sequence in Phα108 was not observed in any other α subunit clones that have been isolated to date (*E. coli*, rat liver, yeast, *A. thaliana*, *T. vaginalis* and *T. flavus*) [5, 11-14]. It is therefore possible that pig heart SCS may undergo an isoform change from fetus to infant and that the Phα108 message could be a fetal remnant found in the newborn pig heart cDNA library. Since polymorphism is observed in the pig heart α subunits, it might similarly extend to the ß subunit of the same enzyme. To examine this possibility, an adult pig heart cDNA library was screened to isolate clones encoding the mature isoform of the Phß subunit. This chapter deals with the search for a cDNA of an adult isoform of the Phß subunit which contained a Trp residue in its coding seqeunce for the mature protein.

II. MATERIALS AND METHODS

A. Plasmids, bacterial strains and cDNA library

The plasmid, Bluescript SK(-) (Stratagene, Inc.), was employed as a general vector for the cloning and sequencing of cDNA inserts. The *E. coli* strains used throughout this work were as follows: Y1090, a lytic host for λ bacteriophage propagation during the screening of the λgt11 cDNA library; DH5α, for the construction and propagation of Bluescript derivatives; and JM103, a F' episome containing bacteria, for single stranded DNA template rescue from Bluescript derivatives. The genotypes of these bacterial strains can be found in **APPENDIX I**. An adult porcine heart cDNA library constructed in the cloning vector λgt11 was obtained from Clontech, Inc.

B. Construction of digoxigenin-labeled DNA probes

Digoxigenin-11-dUTP (Boehringer Mannheim) labeled DNA probes used in re-screening the newborn pig heart cDNA library were created by random priming [15, 16] a clone (2C) encoding the ß subunit of newborn pig heart SCS (see **Chapter 3 - RESULTS AND DISCUSSION**).

Methods followed are exactly as found in **Section F, MATERIALS AND METHODS, Chapter 3.**

C. Screening, isolation and sequencing of clones from an adult pig heart cDNA library using DNA probes

Digoxigenin-containing DNA probes created from randomly priming a clone of the ß subunit of newborn pig heart SCS (see above) were used to screen an adult pig heart λgt11 cDNA library (see **GENERAL METHODS**).

Positive clones were re-screened repeatedly until purified to homogeneity. Insert DNA was amplified directly from the viral stocks by the polymerase chain reaction (see below). The amplification products were digested with EcoRI and applied onto agarose gels. Upon completion of electrophoresis, DNA bands were excised from the gel and

purified by glass powder elution [17]. These isolated DNA fragments were then subcloned into Bluescript SK(-) plasmid. Recombinants were isolated, and the orientations of the DNA insert fragments were determined. Sequencing of the cloned inserts was carried out either utilizing single or double stranded template. When using single stranded templates, recombinant Bluescript plasmids were propagated in JM103 and the helper phage, M13K07, was used to rescue the single stranded DNA according to protocols established by Stratagene™ [18]. These templates were sequenced by dideoxy chain termination methods using universal (M13) forward and reverse primers (see **APPENDIX I**). Double stranded templates of recombinant plasmid DNA were prepared by agarose gel purification. These templates were sequenced by the Taq Dye-deoxy terminator method and analyzed on an automated DNA sequencer (Applied Biosystems, Inc.).

D. Amplification of λ insert DNA by the Polymerase Chain Reaction

Following previously developed methods for PCR [19], insert DNA was amplified directly from purified λ viral stocks using the following protocol. The amplification reaction mixture contained: 32 ng each of λgt11 forward (λF) and reverse (λR) oligonucleotide primers (see **APPENDIX I** for sequences), approximately 10^5 pfu of the recombinant clone viral stock, 200 μM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% (w/v) gelatin. The virus capsid proteins were denatured first at 94 °C for 4 minutes before adding 2.5 units of *Thermus aquaticus* DNA polymerase. The reaction mixture was cycled 40 times, each cycle with a denaturation step of 30 seconds at 94 °C, an annealing step at 60 °C for 1 minute and an extension step of 2 minutes at 72 °C. When the cycling was completed, the reaction was extended 15 minutes longer at 72 °C before being cooled to 10 °C.

III. RESULTS

A. Screening and sequence of cDNA clones encoding the ß subunit of adult pig heart SCS

The successful application of antibodies as probes for screening expression libraries is strictly dependent on a number of variables [20]. The cDNA insert of the target clone must reside in the proper orientation and reading frame for its expression to be immunodetected. Additionally, λgt11 insert cDNAs are expressed as fusion proteins with β-galactosidase, conferring on the target protein the potential problems of misfolding and epitope masking. Considering this rather fortuitous nature of library screening using antibodies, a more specific and direct method of identifying adult ß subunit clones could be achieved by the use of DNA probes constructed from the isolated newborn Phß clone. In the use of this more reliable methodology, a pool of digoxigenin-labeled DNA probes was synthesized using the the newborn Phß subunit clone as template. This probe population was synthesized in a random fashion by priming the newborn Phß clone with degenerate hexanucleotides at various positions along the cDNA sequence and then incorporating the digoxigenin label through extension of this primed complex (see **MATERIALS AND METHODS**). The probes produced in this manner represented all regions of the coding sequence and were, therefore, potentially suitable to identify related adult ß subunit clones.

Nylon plaque lifts of approximately 3 x 10^5 phage from an adult pig heart cDNA library (Clonetech™) were screened using the digoxigenin probe pool described [21]. Using standard hybridization and washing conditions, two positive clones (labeled Aß1 and Aß2) were identified and isolated. These clones were purified to homogeneity and the insert DNA was amplified directly from the virus using PCR with λ vector based oligonucleotide primers (λF, λR - see **APPENDIX I** for sequences). Amplified insert was digested with EcoRI and the resulting DNA fragments were analyzed by agarose gel electrophoresis. It was ascertained that the two clones were dissimilar in that Aß1 was

composed of 800 and 600 bp fragments (much like the newborn clones 2C and 2D), whereas the Aß2 clone consisted of 500 and 625 bp EcoRI fragments. These DNAs were subcloned into the EcoRI site of pBluescript SK(-) and the resulting recombinants were sequenced according to the strategy outlined in **Figure 4.1**.

B. Adult SCS pig heart ß subunit sequence

The nucleotide sequences obtained from the two positive adult clones were confirmed to be that of the ß subunit of PDH. In fact, these adult Phß subunit cDNAs were essentially identical to the newborn clones except for the following differences (see **Figure 4.2**). First, both adult clones were truncated at the 5' end of their cDNAs: the Aß1 clone was 45 nucleotides shorter than the complete sequence of the newborn Phß clones 2C and 2D, whereas the nucleotide sequence of Aß2 began at nucleotide 255 of the newborn clones. The truncation observed in the adult clones likely arose from incomplete extension of the mRNAs by reverse transcriptase during the first strand synthesis of cDNA construction. Nevertheless, the differences between the Aß1 and Aß2 cDNAs establish them as independent clones of the adult Phß subunit. The other difference between the adult and newborn pig heart ß clones was the substitution of two nucleotides occuring at positions 1,130 and 1,351 (with respect to the numbering of the newborn sequence, see **Figure 3.3**, page 68). The first of these changes at 1,130 was A instead of G, and although it occurs within the coding sequence the effect is silent: a GGG triplet is changed to GGA, both of which code for a glycine residue. The second substitution at 1,351 was found to be a G instead of A, and again had no effect on the ß subunit protein sequence since the transition occurred in the 3' noncoding region of the message. Due to the near identity of the infant and adult nucleotide sequences, the two substitutions may likely be attributable to the porcine subspecies rather than developmental variation. Therefore, it was concluded that the amino acid sequences of the ß subunit from adult and newborn pig heart are the same.

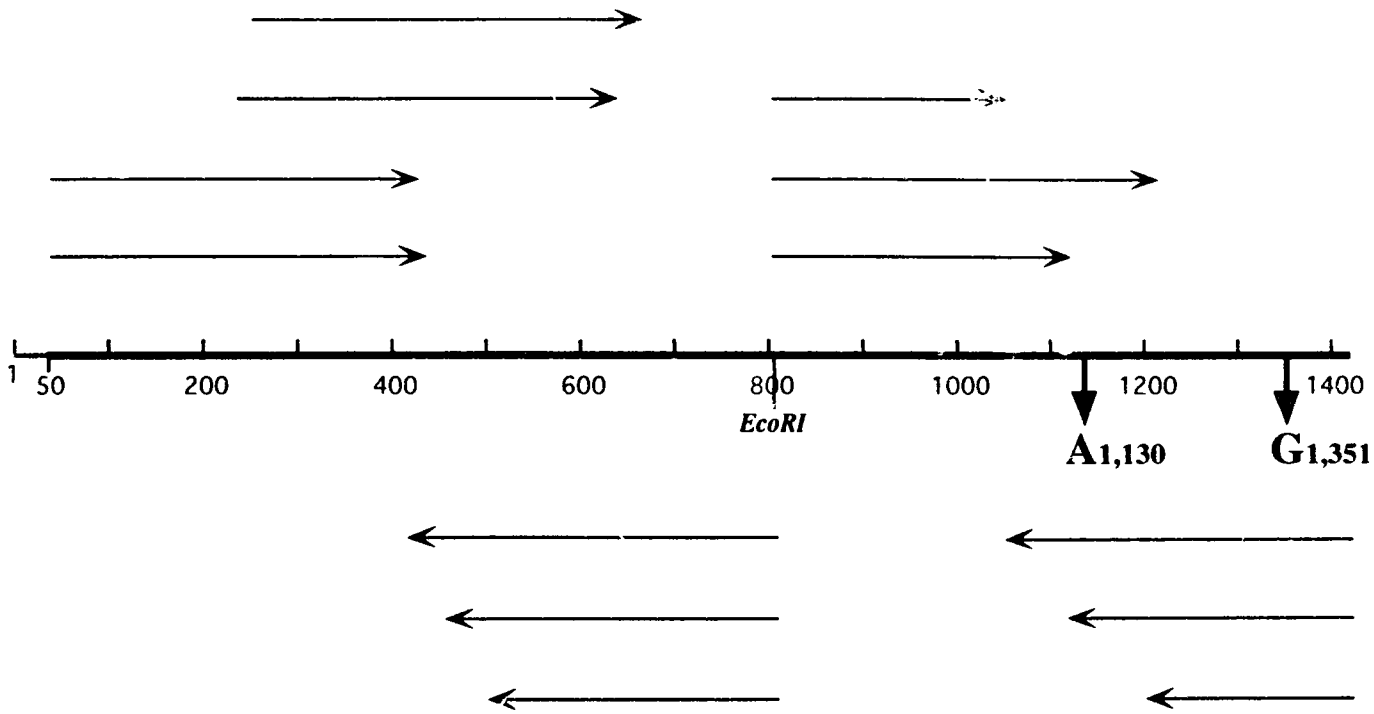

Figure 4.1 Schematic map of sequencing trials completed on the cDNA clones encoding the ß subunit of SCS from adult pig heart. Sequences were determined from Aß1 and Aß2 clones and are presented with respect to the representation of the Aß1 clone (base pair numbering is based upon newborn clones). All sequence information derived from these projects is identical to that obtained from the infant clones except for two transitions. Nucleotide residue 1,130, was found to be a G instead of A (no effect on amino acid sequence) and residue 1,351 in the 3' noncoding region was found to be A instead of G. These two silent substitutions are indicated by downward arrows.

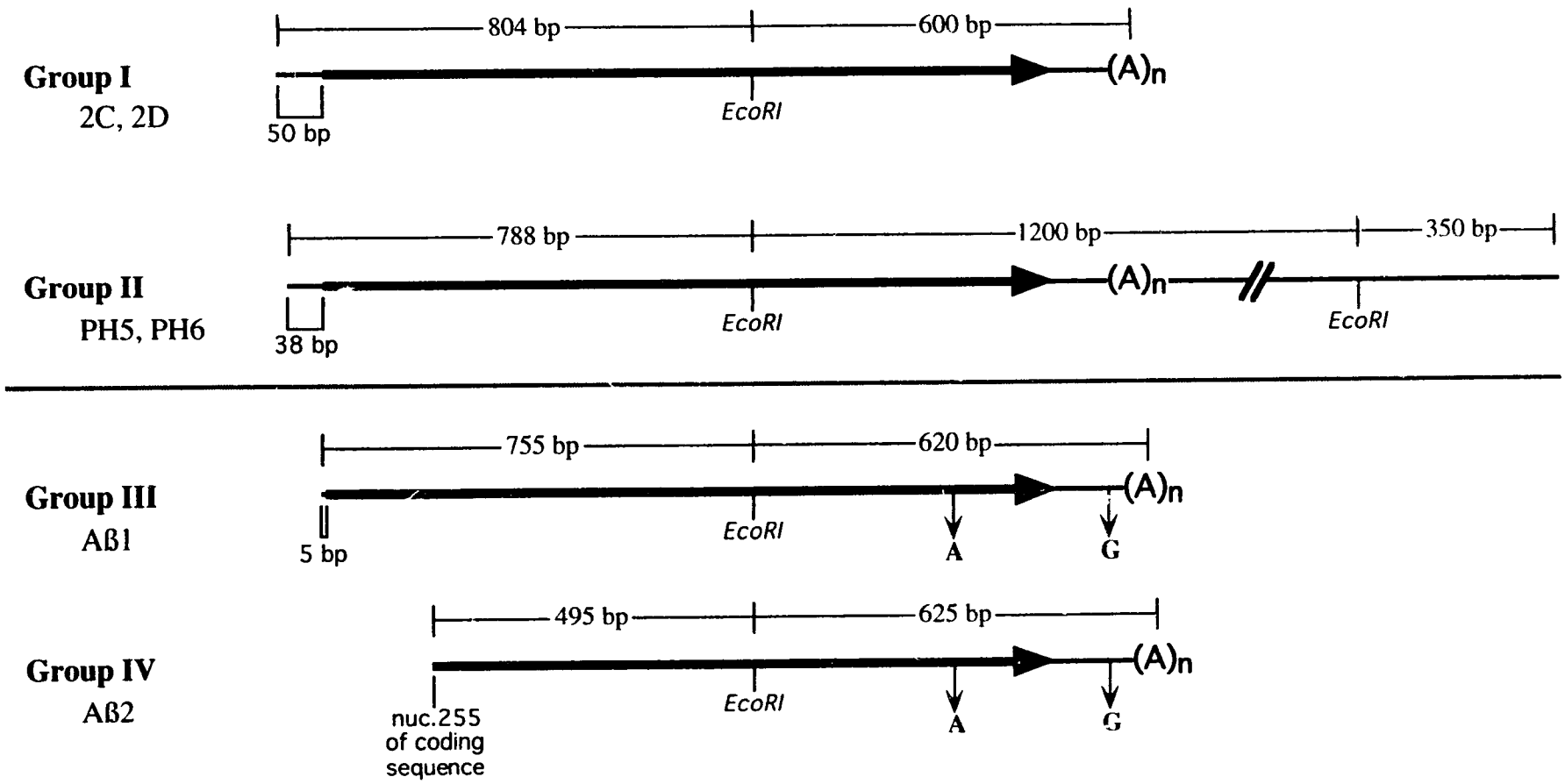

Figure 4.2 Group classification of independent ß subunit clones isolated from pig heart cDNA libraries. Each of t independent groups of cDNAs isolated are aligned based on sequence identity. Groups I and II originated from a newborn pig h DNA library, whereas Groups III and IV were isolated from an adult pig heart library. Representative clones from each group are li Open reading frames (5'→3') are depicted as thick arrows directed to the right. The length of *EcoRI* fragments (in base pairs) are listed ve each clone, while 5' untranslated lengths are listed below each representation. The placement of the two silent nucleotide substitutio served in the adult clones is indicated by arrows pointing downward. Polynucleotide A sequences for each clone are represented as $(A)_n$.

IV. DISCUSSION

It is entirely possible that a multiple gene system exists for the ß subunit in eukaryotes, and only one message has been repeatedly detected in both infant and mature pig heart tissues. However, in light of the preceding evidence gathered for the α subunit from this same tissue, it might be expected that if multiple isoforms existed for the Phß subunit, they could arise via a single gene system with alternative splicing of various exon cassettes. This would imply that at least some protein sequence would be shared among isoforms. If so, the screening technique applied here using randomly primed probes should have been sensitive enough to be able to detect any such related clones. Since none were isolated, it appears that, at least in pig heart, only one ß subunit exists for all SCS complexes throughout the differentiation from newborn to adult tissue. In addition, it might be suggested that the same cloned ß subunit is a component of a number of distinct SCS enzymes with different metabolic roles. For example, Weitzman *et al.* [8] have demonstrated that within a variety of mammalian tissues (including pig heart) discrete SCS enzymes exist which can be distinguished by their restricted nucleotide specificity for either GTP or ATP. The ß subunit cloned in this work is likely involved in one or possibly both of these enzymes, and future studies designed to assemble this protein with an α subunit of pig heart origin should clarify this issue.

It is surprising to note that from all the Phß subunit cDNAs isolated, none encoded a Trp residue in the mature protein coding sequence. The possibility that the codon was missed due to a sequencing error was unlikely since all clones were extensively sequenced in both directions and any compressions present were resolved using ITP as a sequencing label. Furthermore, all independent Phß clones (2C, PH5, Aß1, Aß2) from both newborn and adult sources have virtually identical nucleotide sequences. This fact excluded the possibility that all of the ß subunit clones arose from a similar mutated sequence during cDNA synthesis. Therefore, contrary to the published results of others [1] it was concluded that the ß subunit of SCS from pig heart does not

contain a tryptophan residue. The resolution of this contradiction is the subject of **Chapter 5**.

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Chapter 5

INVESTIGATION OF THE TRYPTOPHAN CONTENT OF PIG HEART SUCCINYL-CoA SYNTHETASE

I. INTRODUCTION

Nishimura and colleagues have previously demonstrated that SCS from pig heart tissue contains one tryptophan residue [1]. This was in keeping with the observation that the A_{280} for the enzyme was low (3.5 for a 10 mg/ml solution) [2]. They further reported that this Trp residue was found in the ß subunit. This was significant, because tryptophan residues had been detected in the *E. coli* enzyme [3], and all of these had been localized to the ß subunit where at least one acted as a fluorescent reporter group for the binding of substrates [4]. In this latter study, quenching of tryptophan fluorescence in the *E. coli* enzyme by acrylamide was affected significantly by the addition of substrates, most notably CoA and ATP [4]. Thus, it was proposed that at least one Trp residue was located at or near the active site. This suggestion was extended to the single Trp residue in the ß subunit of pig heart SCS based on initial fluorescence-quenching studies of this enzyme [5], even though the effects of substrates and other ligands on the fluorescence quenching of pig heart SCS were small in magnitude. Nevertheless, their results indicated that the tryptophan fluorescence is quite sensitive to these perturbations. These results suggested that the conservation of this single tryptophan residue near the active site is important to the catalytic activity of the enzyme.

In contrast to these reports, the DNA sequence of the isolated clones encoding the ß subunit of pig heart SCS contained no tryptophan (**Chapters 3 and 4**). As previously stated, there was confidence that the nucleotide sequence of the cDNAs was correct because both DNA strands of independent clones had been sequenced, including the use of ITP (in place of GTP) to resolve any compressions. Moreover, at sites where tryptophan occurred in the known sequences of the ß subunits from microbial sources (*E. coli*, *Trichomonas vaginalis* and *Thermus flavus*), conservative amino acid replacements were found in the corresponding pig heart sequence (see **Figure 3.4**, page 70: W → F_{58}; W → $Y_{6,191,225}$; W → M_{83}; W → I_{43}).

The amino acid sequences deduced from the cDNA clones encoding the α subunits from pig heart SCS [6] revealed the absence of any Trp residue as well. Taken together with the results for the ß subunit, the claim was extended to the whole enzyme: pig heart SCS contains no tryptophan. In order to explain this contradiction with the reports of Nishimura *et al.* [1], the author repeated the crucial experiments that gave evidence for the presence of the Trp residue. This chapter, therefore, focuses on the investigation of the tryptophan content of pig heart SCS through fluorescence and amino acid analyses.

II. MATERIALS AND METHODS

A. Enzyme isolation and purity

Pig heart SCS was isolated from fresh myocardium according to the procedure of Wolodko *et al.* [7]. The *E. coli* enzyme was purified from JM103 cells transformed with the overproducing expression plasmid pGS202 [8], according to methods previously described [9]. Succinyl-CoA synthetase activity was assayed by the direct spectrophotometric method based on the increase in A_{235} accompanying succinyl-CoA formation [10]. ATP was used as the substrate with *E. coli* SCS, and GTP was used for pig heart SCS. Homogeneous preparations, as estimated by SDS-PAGE analysis, of both SCS enzymes that were used in tryptophan fluorescence and amino acid compositional analyses had specific activities of 20 and 40 units/mg for the *E. coli* and pig heart enzymes, respectively. 'Non-homogeneous' pig heart SCS was prepared by identical purification methods except that only the final affinity chromatography step employing a resin of Cibracon-blue F3GA dye-agarose (Affi-Gel Blue, Bio-Rad) was omitted (see **Figure 5.1**).

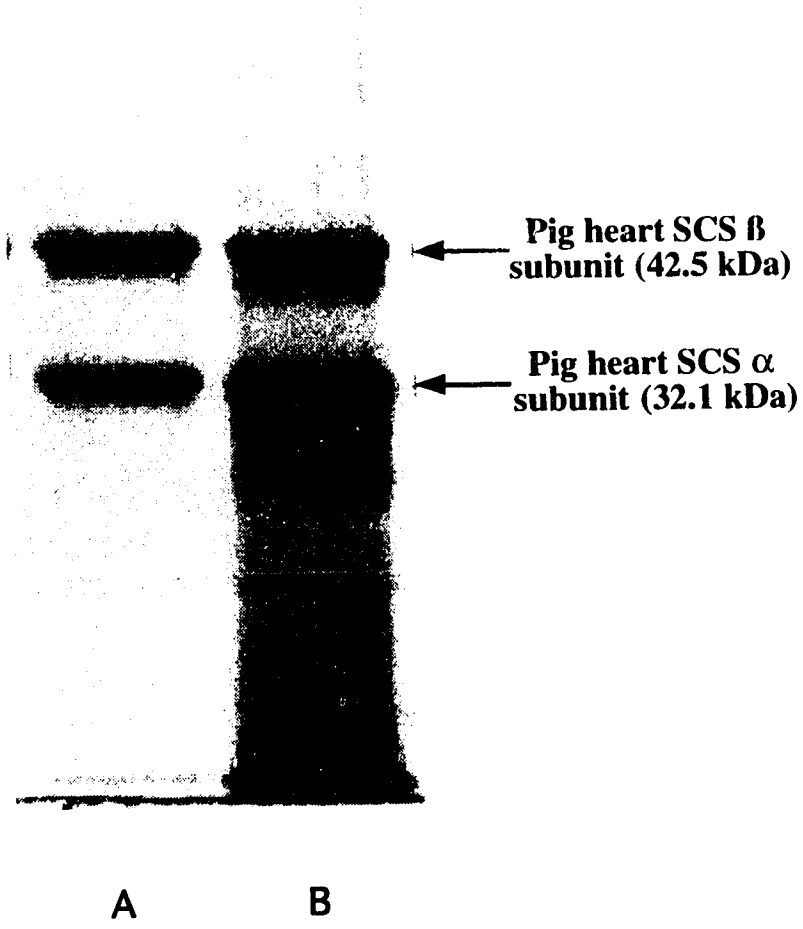

Figure 5.1 SDS-PAGE of pig heart SCS preparations. Gel electrophoresis was done in 10% (w/v) polyacrylamide and protein staining was done with Coomassie Blue R-250. Samples: **A**, SCS isolated from pig heart tissue by standard methods [7]; **B**, SCS purified from pig heart tissue prior to the final affinity chromatography step [7] employing Cibracon-blue F3GA dye-agarose (Affi-Blue Gel, Bio-Rad).

B. Fluorescence measurements

Samples of SCS from both pig heart and *E. coli* were dialyzed against 50 mM potassium phosphate (pH 7.4). The fluorescence of these samples was measured in a Perkin-Elmer MPF-44B fluorimeter, with excitation at 295 nm (Trp-specific excitation wavelength) using a 5 nm band width and a resultant emission peak of about 330 nm. Similar enzyme samples were denatured in 6M guanidine-HCl prior to measurement of the fluorescence. All measurements were done at 20 °C.

C. Amino acid compositional analysis

A procedure developed by Simpson *et al.* [11] was employed for analyzing the amino acid composition of SCS while preserving oxidatively sensitive Trp residues. Ten nmol samples of purified pig heart and *E. coli* SCS were each dialyzed extensively against distilled water at 4 °C and subsequently lyophilized in fired test tubes. In order to quantitate the tryptophan content (as well as other amino acids), samples were solubilized in 100 µl of 4 M methanesulphonic acid / 0.2% (w/v) tryptamine and hydrolyzed *in vacuo* for either 12 or 24 hours at 110 °C or 1 hour at 160 °C. Hydrolyzed samples were then neutralized with an equal volume of 3.5 M NaOH, and these were analyzed on a Beckman™ System 6300 amino acid analyzer using standard 12 cm column methodology. Typically, 5 µl of the neutralized hydrolysate was diluted in 45 µl Beckman™ sample dilution buffer and loaded onto the column for analysis. The amino acids derivatized with ninhydrin were eluted from the ion-exchange column using a stepwise gradient of increasing pH. Amino acid peaks eluted from the column were integrated to calculate the concentration of each amino acid. Mean values for the actual amount of digested protein were calculated from individual amino acid peak concentrations divided by the actual amino acid content of the cloned enzymes. In turn, quantities of each amino acid resolved in the analysis were calculated using the peak concentation of each amino acid divided by the mean quantity of protein hydrolyzed. For

the determination of tryptophan, an overloaded sample of 50 µl of the neutralized hydrolysate was applied directly for analysis.

III. RESULTS AND DISCUSSION

A. Tryptophan fluorescence of pig heart and *E. coli* SCS

The protein fluorescence of pig heart SCS was studied under conditions where the tryptophan fluorescence had been reported by Nishimura *et al.* [1]. As a positive control, parallel studies were carried out using purified SCS from *E. coli*, which was known to contain 6 Trp residues per native $\alpha_2\beta_2$ tetramer [12].

Samples of native pig heart and *E. coli* SCS were excited at 295 nm and their recorded emission spectra are shown in **Figure 5.2**. A 0.5 µM sample of *E. coli* SCS exhibited a large peak with a corresponding height (corrected for baseline) of 58 arbitrary fluorescence units. Considering the tryptophan content of the *E. coli* enzyme, this control experiment established that the sensitivity of the method was sufficient to detect one Trp residue per pig heart SCS dimer, if one existed. In order to make certain that the fluorescence of a single Trp residue could be detected, a higher concentration of 1.1 µM pig heart SCS was used for the analysis. Thus, it was calculated that if one Trp residue existed per SCS molecule, then an emission peak with a height of approximately 21 arbitrary units would result. As seen in **Figure 5.2**, the observed tryptophan-specific fluorescence response for this sample was a flat line, consistent with the absence of this amino acid in the protein.

It was conceivable, however, that if a Trp residue existed in the pig heart enzyme, its characterisic fluorescence may not be the same as those Trp residues of the *E. coli* enzyme. For example, a different local environment could have masked the fluorescence of this residue and thereby prevented its detection. It has been shown that denaturation with guanidine-HCl or urea allows measurements of tryptophan fluorescence which are

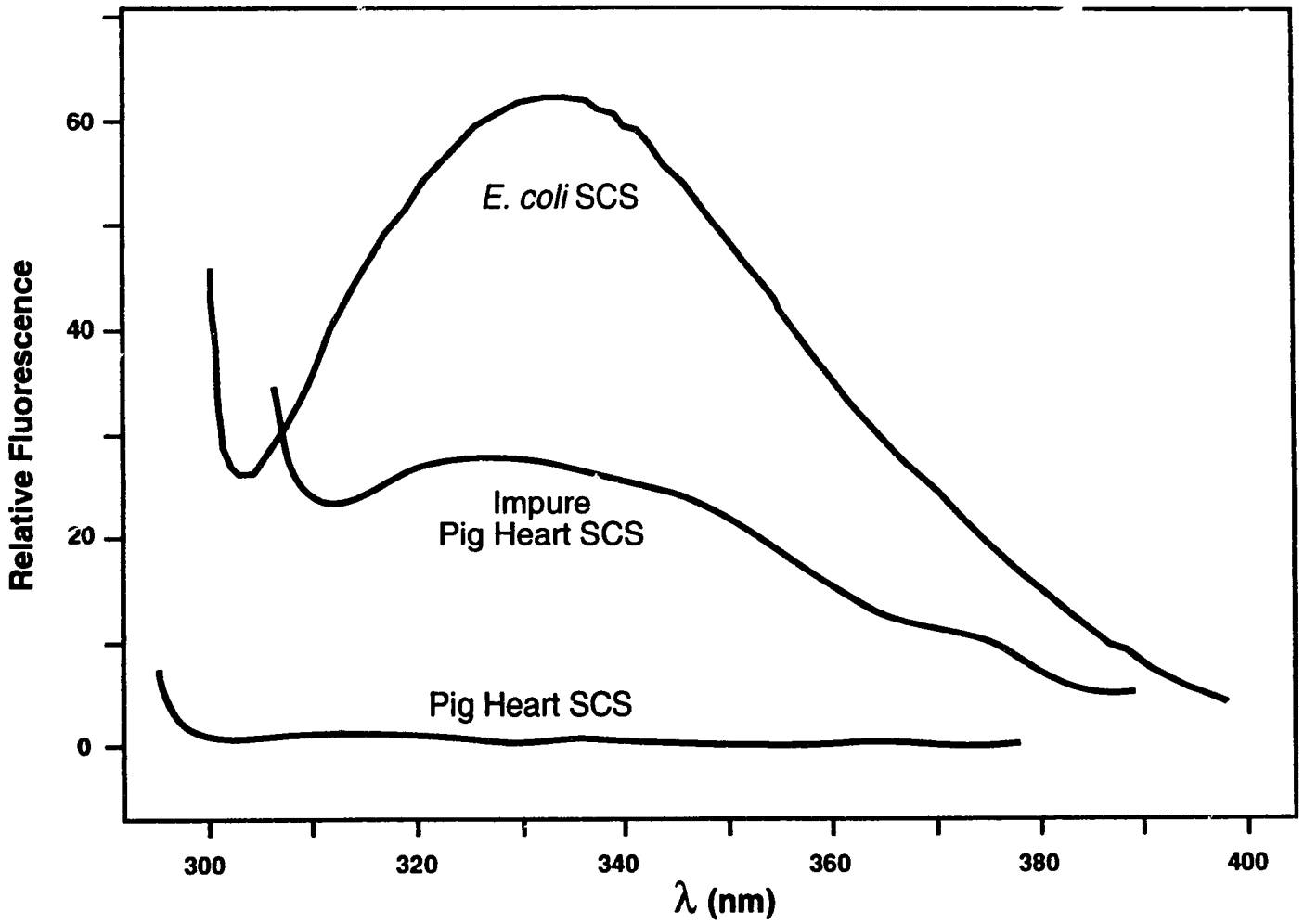

Figure 5.2 Fluorescence emission spectra of native SCS from pig heart tissue and *E. coli*. Proteins were dissolved in 50 mM potassium phosphate; excitation was at 295 nm. Concentrations of samples were: *E. coli* SCS = 0.5 μM; homogeneous pig heart SCS = 1.1 μM; and impure pig heart SCS = 1.3 μM. At 330 nm, the *E. coli* SCS sample demonstrated 58 arbitrary fluorescence units (baseline corrected), purified pig heart SCS, 1 unit, and impure pig heart SCS, 26 units.

independent of the environment in the native protein [13]. In order to obviate these potential shielding effects, SCS samples were treated with 6 M guanidine-HCl prior to excitation at 295 nm and measurement of the emission spectra. As shown in **Figure 5.3**, a 0.5 μM sample of denatured *E. coli* enzyme displayed a large fluorescence peak with a maximum shifted to 350 nm, consistent with emitting Trp residues being in contact with the solvent [13]. However, a 0.5 μM sample of denatured pig heart SCS gave a flat emission response (**Figure 5.3**), clearly indicating the lack of tryptopan in pig heart SCS. (Note: at this concentration, if there was one Trp residue per αß dimer, the emission yield would have been 9.5 arbitrary fluorescence units.)

B. Compositional amino acid analyses

In addition to fluorescence work, Nishimura *et al.* [1], used amino acid analysis to confirm their detection of a Trp residue in the ß subunit of pig heart SCS. The most frequently used method for hydrolyzing proteins to amino acids is by heating with 6 N HCl [14]. The determination of tryptophan by this protocol is, however, unproductive, since this amino acid is readily destroyed in HCl by oxidation. As an alternative to HCl, a nonoxidizing strong acid is used in order to protect the indole ring of tryptophan from oxidative destruction. Nishimura and colleagues followed the method developed by Simpson *et al.* [11] where protein hydrolysates were prepared by digestion with 4 N methanesulfonic acid/0.2% tryptamine, preserving tryptophan for subsequent detection. Nishimura's group reported one Trp residue in pig heart SCS residing in the ß subunit under conditions where hydrolysis occurred *in vacuo*, for 24 hours, at 110 °C [1].

In the present study, amino acid analyses were repeated employing the same experimental protocol but using pig heart SCS purified by the method of Wolodko *et al.* [7]. In order to control for the temperature and length of digestion, samples were hydrolyzed for either 12 or 24 hours at 110 °C, or for 1.25 hours at 160 °C, before being submitted amino acid compositional analysis. The results are presented in **Table 5.1**. Under conditions where up to 2.5 nmol of tryptophan should have been expected if the

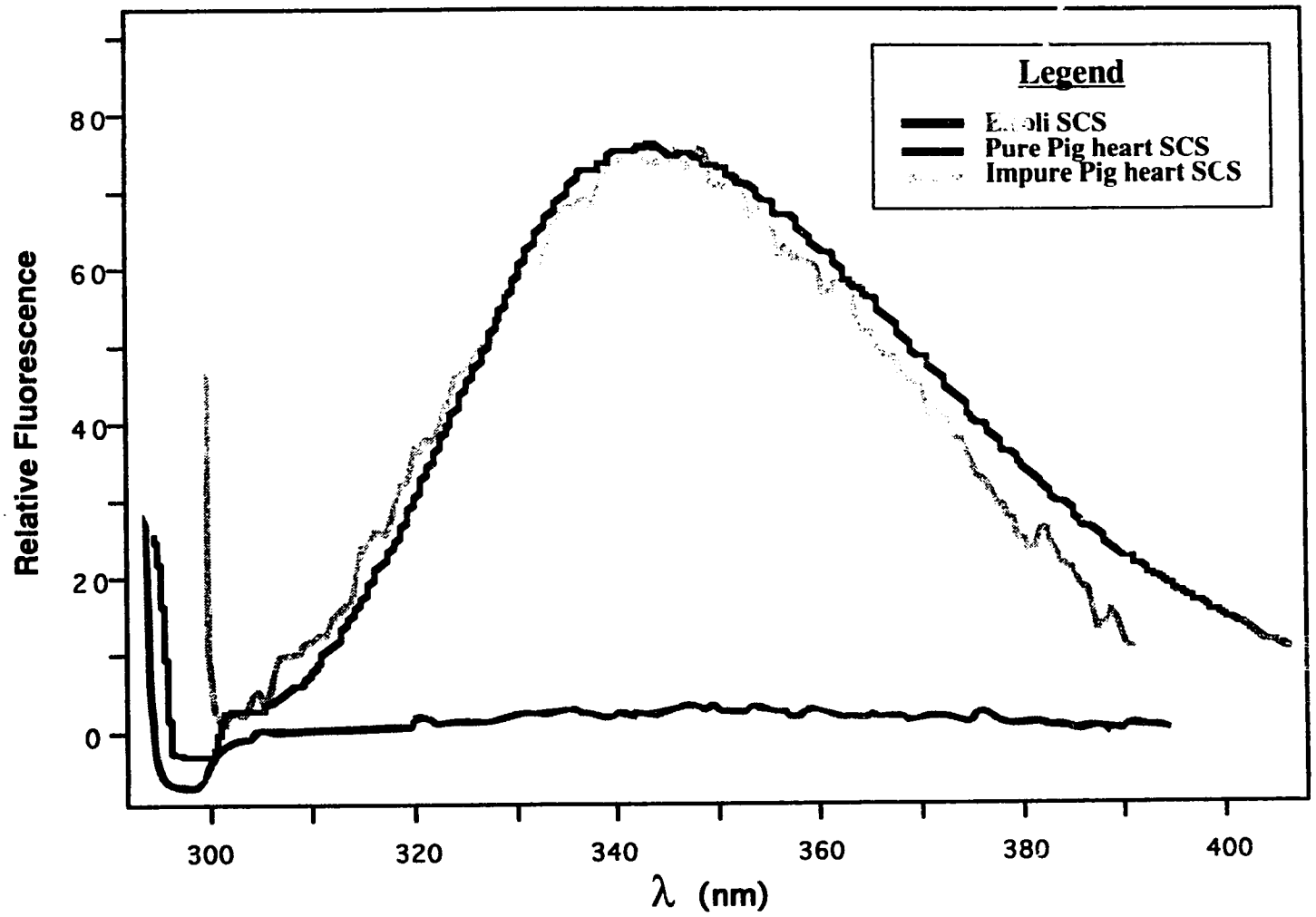

Figure 5.3 Fluorescence emission spectra of denatured SCS from pig heart and *E.coli*. Proteins were denatured in 6 M guanidine-HCl (pH 7.4) prior to excitation at 295 nm. Sample concentrations were: *E. coli* SCS = 0.5 μM; purified pig heart SCS = 0.37 μM; and impure pig heart SCS = 2.6 μM. Denaturation shifted the emission peaks from 330 to 350 nm.

Table 5.1

Amino acid composition of methanesulfonic acid hydrolysates of pig heart and E. coli *SCS*[1]

Amino acid	Pig heart SCS						*E. coli* SCS	
	Predicted from cDNA (per αβ)	1.25 h at 160 °C (per 74.6 kDa)	12 h at 110 °C (per 74.6 kDa)	24 h at 110 °C (per 74.6 kDa)	Impure sample 1.25 h at 160 °C (per 74.6 kDa)	From Nishimura *et al.*, (1988) (per 74.6 kDa)	Predicted from cDNA (per αβ)	24 h at 110 °C (per 71.7 kDa)
Lys	56	55.9	51.7	50.5	60.0	54.7	46	48.8
His	11	11.8	11.3	11.8	11.9	12.0	11	11.5
Arg	19	18.3	19.2	19.1	17.9	22.0	22	19.8
Asx	67	65.5	63.2	62.4	70.9	65.0	52	55.0
Thr	38	33.6	40.3	40.3	32.3	41.2	41	40.7
Ser	27	22.4	29.3	28.7	21.2	31.3	19	18.7
Ala	67	69.4	59.8	56.7	65.5	69.5	76	79.7
Val	55	54.0	46.6	46.9	62.7	53.4	62	60.3
Met	12	14.7	15.7	15.6	14.1	12.8	15	16.4
Ile	52	46.2	40.4	42.5	44.7	44.6	53	40.2
Leu	58	61.4	58.8	57.1	59.6	58.1	52	56.1
Tyr	13	13.3	13.8	14.2	12.8	14.5	13	13.1
Phe	26	27.3	27.4	27.7	26.3	25.5	18	19.0
Trp	0	**0.0**	**0.0**	**0.0**	**0.8**	**1.1**	**3**	**2.6**

[1] Samples were hydrolyzed in 4 M methanesulfonic acid / 0.2% (w/v) tryptamine under the conditions indicated, and analyses were performed on a Beckman System 6300 using standard 12 cm column methodology. The content of Pro and Cys were not quantifiable under the conditions used. Peaks corresponding to Glx and Gly were not well resolved and these data are also not included.

pig heart ß subunit contained a single Trp residue, no tryptophan peak was detected in any sample.[1] Although some variation existed among the three analyses with respect to some amino acids, these deviations were small, and the composition determined was entirely consistent with that predicted from the isolated Phß clone and the known α subunit sequence [6]. More importantly, regardless of the temperature or length of hydrolysis, in these independent, triplicate analyses no tryptophan was detected. As the control, a parallel analysis of the *E. coli* enzyme detected of 87% of the actual tryptophan content, confirming the reliability of the analysis. Therefore, this evidence corroborated the results of the tryptophan fluorescence work, and established the lack of tryptophan in the pig heart SCS samples that had been fully purified [7].

C. Interpretation with analyses of 'impure' pig heart SCS

The predicted amino acid sequence of all isolated Phß subunit cDNA clones indicated the absence of tryptophan, and this has been confirmed from the above experiments completed on the whole (αß) pig heart enzyme. These results contradict those reported by Nishimura *et al.* [1], that a single Trp residue was located in the ß subunit and that it could be used as a fluorescent probe. An explanation for the conflicting results might be that Nishimura's group studied a different variant of pig heart SCS. However, it is interesting to note that all independent samples of pig heart tissue that were examined for this thesis, including one newborn and two distinct adult sources, did not encode or contain tryptophan. Apart from subspecies variation, another possible cause for Nishimura's results might have been contamination by a Trp-containing impurity. To test this possibility, preparations of pig heart SCS that had been purified through all but the final affinity chromatography step (**Figure 5.1**) were examined by protein fluorescence and amino acid analyses for tryptophan content. A 1.3 μM sample

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[1] In order to assure detection of tryptophan, allowances were also made for incomplete recovery (75% of original quantities were expected from experimental techniques).

of this impure pig heart enzyme demonstrated substantial tryptophan-specific fluorescence under native conditions (**Figure 5.2**). Likewise, a 2.6 μM sample of this preparation gave similar results under denatured conditions (**Figure 5.3**). Through the outcome of these experiments, an estimation of one tryptophan per mole of enzyme was determined for this enzyme. Amino acid compositional analyses were completed on a sample of non-homogeneous pig heart SCS. While the observed content of amino acids approximated that predicted by the cloned subunits, these results also indicated the presence of a single Trp residue (**Table 5.1**). Therefore, a likely explanation for the observations made by Nishimura's group is the presence of a contamination in their preparations of pig heart SCS.

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Chapter 6

EXPRESSION, PURIFICATION AND FUNCTIONAL ANALYSIS OF CLONED PIG HEART SUCCINYL-CoA SYNTHETASE

I. INTRODUCTION

Succinyl-CoA synthetase plays a number of roles in different metabolic and biosynthetic processes. In addition to generating GTP within the citric acid cycle [1-3], mammalian SCS provides succinyl-CoA as a substrate in ketone body metabolism [4] and porphyrin synthesis [5]. In order to meet such opposing demands, it was postulated that two distinct enzymes would better serve cellular requirements by allowing succinyl-CoA or succinate to be available to opposing pathways [6].

Although mammalian SCS is widely known to be GTP-specific, work from Weitzman and colleagues has demonstrated the presence of ATP-specific SCS in most mammalian tissues [7, 8]. This is not a precedent since both ATP- and GTP-specific SCS activities have been detected in tissues from a wide variety of animal species [9, 10]. In addition, the ratio of these two activities varied depending on the tissue source [7]. Weitzman's group found that the ATP and GTP specificities were attributable to two distinct and separable proteins, establishing that there is more than one species of SCS functioning in various mammalian tissues. The existence of these mammalian isoforms of SCS was consistent with the different metabolic roles for the enzyme. One isoform would function in ketone body metabolism and porphyrin synthesis, and the other would participate in the citric acid cycle. Since in the mitochondria, the concentration ratio of GTP/GDP (~ 100:1) is much higher that of ATP/ADP (~ 1:1), GTP-specific SCS was suggested to be responsible for the production of succinyl-CoA driven by the higher chemical potential of GTP/GDP [9]. This enzyme would therefore be able to maintain favorably high levels of succinyl-CoA for the production of acetoacetyl-CoA in ketone body metabolism [4]. This proposal was supported by evidence showing an increase in GTP-specific SCS activity in several tissues of diabetic animals under ketosis [11]. From additional work, two distinct GTP-specific enzymes were characterized in mouse liver, the level one of which was elevated threefold in response to conditions of increased porphyrin synthesis [5]. Taken together, these results suggest that the GTP-specific

isoform of SCS is responsible for the production of succinyl-CoA necessary for ketone body metabolism and porphyrin synthesis. As a corollary, this implies that the ATP-specific enzyme is associated with the citric acid cycle and the conservation of energy. In support of this hypothesis, it has been observed that the conversion of the bloodstream form to procyclic form of *Trypanosoma brucei* [12] is accompanied by an increase in both the function of the citric acid cycle and the levels of its enzymes. Although the organism has both ATP- and GTP-specific enzymes, only the ATP isoform significantly increases.

Previous to this thesis work, the only mammalian SCS subunits that had been cloned were those of the α subunit from rat liver [13] and pig heart [14]. The cloning of the mammalian ß subunit (**Chapters 3 and 4**) has provided the first opportunity to express both subunits of a single dimeric species of SCS. In light of the different possible functions of mammalian SCS described above with respect to nucleotide specificity, studies were directed towards assessing the functional properties of one cloned isozyme of pig heart SCS. This report describes the expression of the Phß subunit, alone and in conjunction with a counterpart Phα subunit, and subsequently the biochemical characterization of this isozyme when purified.

II. MATERIALS AND METHODS

A. Bacterial strains and plasmids

The following *E. coli* strains were used in these studies. JM109 was routinely used for the construction and propagation of most plasmid derivatives. N99cI$^+$ carried the λ wild type repressor and was used for the isolation of recombinant pRIT2T clones [15]. N4830-1 carried the temperature sensitive λcI$_{857}$ repressor and was used for the expression of proteins from pRIT2T derivatives [16]. BL21(DE3) is a λ lysogen containing the gene for T7 RNA polymerase behind an inducible *lac*UV5 promoter: this

strain was used for the expression of T7 promoter driven plasmids (pT7-7 and pET-3b) [17]. TK3(D18) is a SCS null strain in which the genes for endogenous *E. coli* SCS have been deleted [18]. The genotypes of these strains can be found in **Appendix II**.

The plasmids and vectors used in construction of the recombinant expression vectors are as follows. M13mp11FXN was derived from M13mp11FX [19] where a NcoI linker was cloned into the StuI site of M13mp11FX. The vector, pRIT2T is a protein A fusion vector developed by Pharmacia [20]. The plasmids, pGP1-2 and pT7-7 were developed [21] and kindly provided by Dr. Stan Tabor; pGP1-2 contained a thermoinducible T7 RNA polymerase, whereas pT7-7 was an expression plasmid with a T7ø10 promoter upstream of a strong ribosome binding site and start codon. The vector, pET-3b is an expression plasmid containing the T7ø10 promoter, s*10* translational region for the gene *10* protein and Tø transcription termination signal [22].

B. Mutagenesis to create a mature Phß clone by PCR

The clone encoding the cytoplasmic ß subunit precursor of pig heart SCS (2C, see **Chapter 3**) was modified in order to remove the signal sequence and create a mature form when expressed *in vivo*. This was accomplished by the following PCR methods using the mismatch oligonucleotide PhßmatNco (see #5, **Appendix I** for sequence), which enabled the creation of an NcoI site (underlined) near the codon for the N-terminus of the mature ß subunit. DNA from the λ recombinant pig heart clone (2C) was amplified by PCR using the above oligonucleotide and the λgt11 vector primer, λF (see #1, **Appendix I**), for 15 cycles, with 3 units of the Klenow fragment of DNA polymerase I added after each cycle. A 100 µl reaction mixture, consisted of approximately 0.5 µg λ DNA, Klenow PCR buffer (30 mM Tris-acetate (pH 7.9), 60 mM sodium acetate, 10 mM magnesium acetate, and 10 mM dithiothreitol), 20 pmol of each primer, 50 µM dNTPs and 10 units of DNA polymerase I (Klenow fragment). The reaction was denatured initially at 94 °C for 30 seconds and then extended at 37 °C for 10 minutes. After six cycles, 5 µl of 2.5 mM dNTPs were added to the reaction mixture, and PCR was

continued for an additional nine cycles with an adjusted extension time of 25 minutes. Amplified DNA was recovered from the PCR reaction and subsequently cloned into expression vectors. The author is indebted to Dr. GuoHua Fang for his assistance in carrying out this work.

C. Construction of recombinant expression vectors

1. **pRIT2T/FXN·Phß** and **pRIT2T/FXN·Phα** - A DNA fragment encoding the mutagenized mature coding sequence of Phß (see above) was subcloned into the NcoI site of the M13 derivative of M13mp11FXN (**Figure 6.1**). This step effectively introduced a tetrapeptide recognition sequence for blood coagulation factor X_a N-terminally to the mutated mature start site of the Phß subunit. A BamHI fragment of this construct containing the nucleotide sequence coding for the FX_a cleavage site and the mature Phß subunit was ligated, in frame, downstream of the sequence that encoded a truncated form of staphylococcal protein A in the expression plasmid pRIT2T [20]. The resultant plasmid, pRIT2T/FXN·Phß, therefore codes for the expression a fusion protein of protein A fused to the FX_a recognition sequence followed by the mature form of the Phß subunit. An EcoRI fragment coding for the mature Phα subunit[1] was used to replace the Phß subunit gene of pRIT2T/FXN·Phß in order to create a similar fusion protein with Phα subunit. Here, the sticky ends of the NcoI/SalI digested pRIT2T/FXN vector were removed with mung bean nuclease before ligating with a Klenow-filled EcoRI fragment of the mature Phα clone. The recombinant expression vector, pRIT2T/FXN·Phα, was thus created with the Phα subunit gene in frame with the sequence for protein A and the FX_a cleavage site.

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[1] Two cDNA isoforms of pig heart SCS α subunit have been isolated, which are denoted α57 and α108 depending on an alternatively spliced internal exon [23]. The sequence used in all these expression constructs is that of α57, which is highly similar to the equivalent portion of the α subunit from both rat liver [13] and *E. coli* [24].

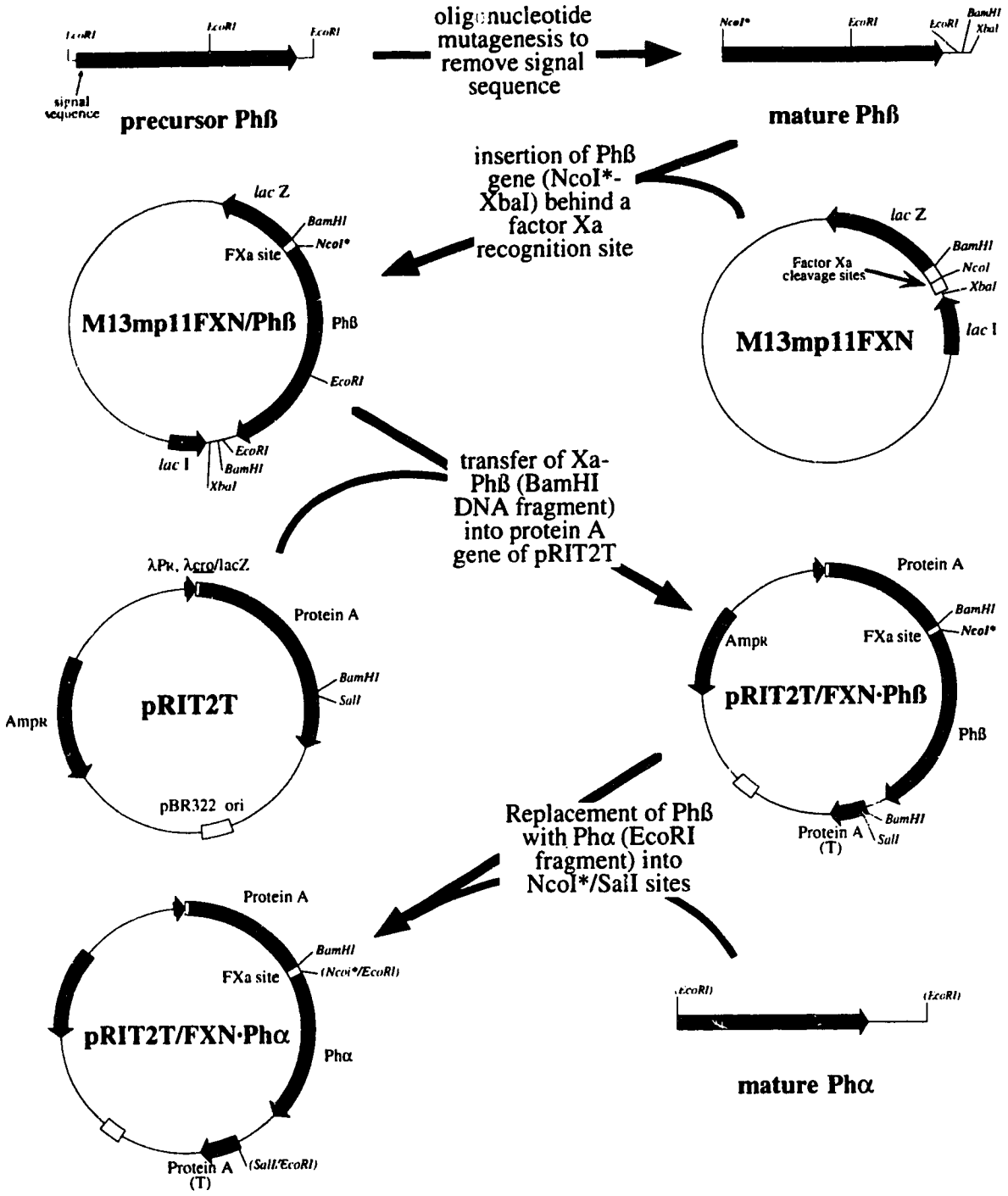


Figure 6.1 Construction of expression plasmids for the production of fusion proteins of protein A with mature pig heart SCS subunits. The precursor cDNA sequence of Phß was mutagenized to remove the signal sequence and to incorporate an NcoI* site at the mature N-terminus. This mature coding sequence was cloned into M13mp11FXN to fuse a IEGR (Factor Xa recognition) sequence, upstream and in frame with the Phß sequence. A DNA fragment of this fusion was transferred into the protein A gene of pRIT2T, creating pRIT2T/FXN·Phß. The plasmid, pRIT2T/FXN·Phα, was derived from pRIT2T/FXN·Phß, by replacing the Phß gene with a filled/blunt ended EcoRI DNA fragment encoding the mature Phα subunit.

2. **pT7-7/Phß** - The mature coding sequence of the Phß subunit was placed under the control of a T7ø10 promoter in the vector, pT7-7 [21], generating pT7-7/Phß (**Figure 6.2**). This was accomplished by a two step cloning procedure. First, an DNA fragment (bounded by a Klenow-filled NcoI site and an EcoRI site) encoding the N-terminus of the mature Phß subunit was ligated into the EcoRI and filled NdeI sites of pT7-7. Second, an EcoRI fragment of the Phß clone corresponding to the C-terminus of the Phß subunit was inserted to complete the construction.

3. **pT-P15AK/Phα** - A T7-controlled expression plasmid containing the kanamycin-resistance gene (Kan^R) and the P15A origin of replication was created by first ligating a PstI-BamHI fragment from pGP1-2 containing these elements, with similarly digested pT7-7 (**Figure 6.3**). From this hybrid vector, a HincII-BglII fragment encoding the *Col* EI origin of replication and part of the ß-lactamase gene was then removed. A NdeI-BamHI fragment of the mature Phα clone was then placed downstream and under control the T7ø10 promoter.

4. **pET-3b/Phß/α** - This expression construct was created by first subcloning a BstBI-BamHI fragment from pT7-7/Phß, consisting of the T7ø10 promoter and the mature Phß subunit coding gene, into the NruI/BglII digested site of the expression plasmid, pET-3b (**Figure 6.4**). A NdeI-BamHI fragment containing the mature Phα subunit coding sequence was then introduced downstream from the original pET-3b T7ø10 promoter resulting in the coexpression plasmid.

D. Bacterial expression

1. **Protein A fusions in N4830-1** - Following methods developed by Pharmacia™ [25], cultures of N4830-1 harboring pRIT2T derivative expression plasmids were grown at 30 °C in SOB medium (see **Appendix III** for composition) containing ampicillin (50 µg/ml) to early stationary phase (Abs_{600} of 2). Expression was induced by diluting the culture with an equal volume of media preincubated at 54 °C, thereby shifting the temperature of the whole culture to 42 °C. Protein expression was allowed to continue

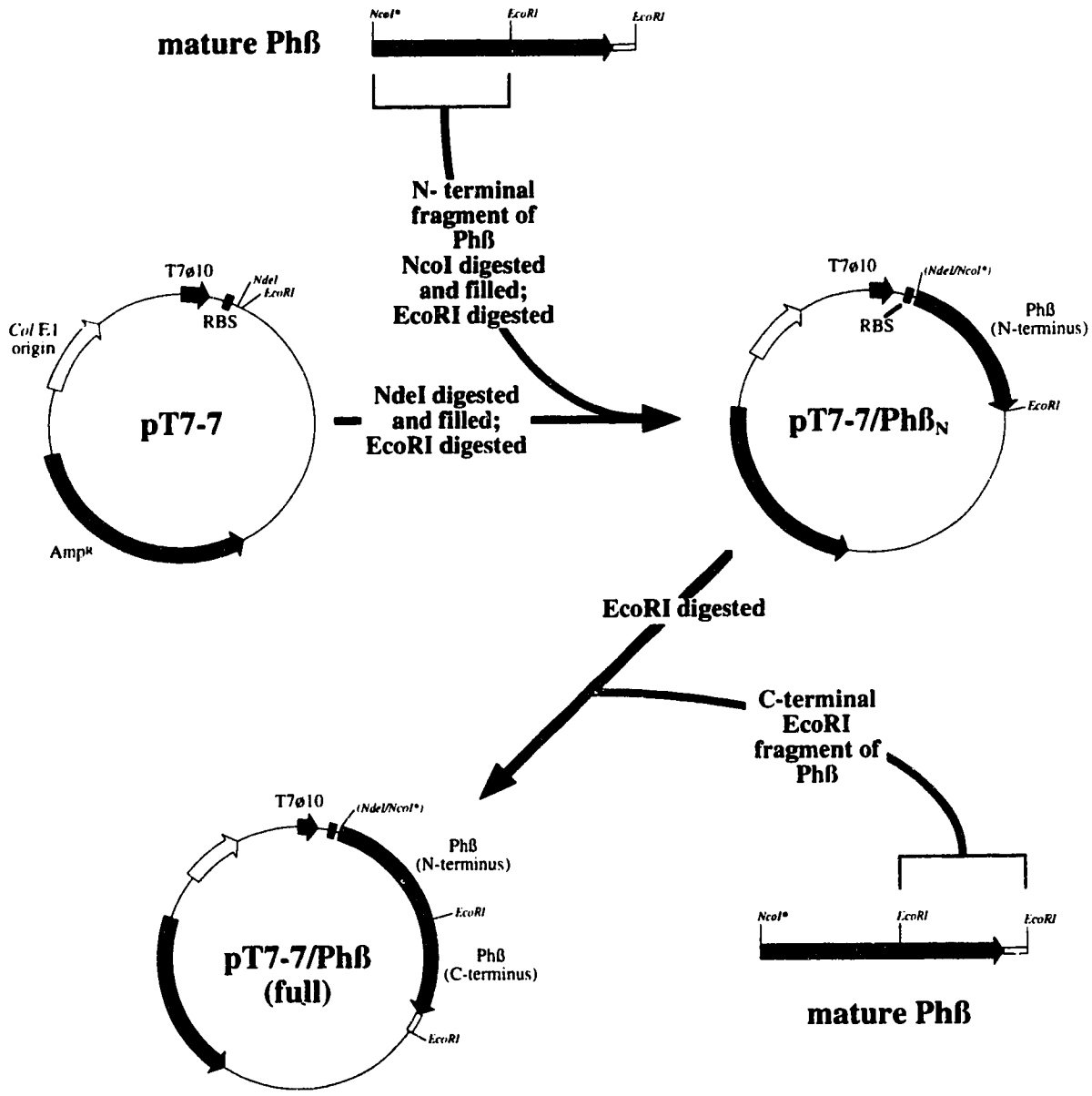


Figure 6.2 Construction of a T7 expression plasmid for the production of the ß subunit of pig heart SCS. The cDNA encoding the mature Phß subunit was cloned into pT7-7 in a two step procedure. First, an N-terminal NcoI(filled)-EcoRI fragment of the gene was cloned into similar sites of pT7-7, downstream of a strong ribosome binding site. The C-terminal EcoRI fragment of Phß was then cloned distally to complete the construction, thus placing the entire gene under the control of the strong T7ø10 promoter.

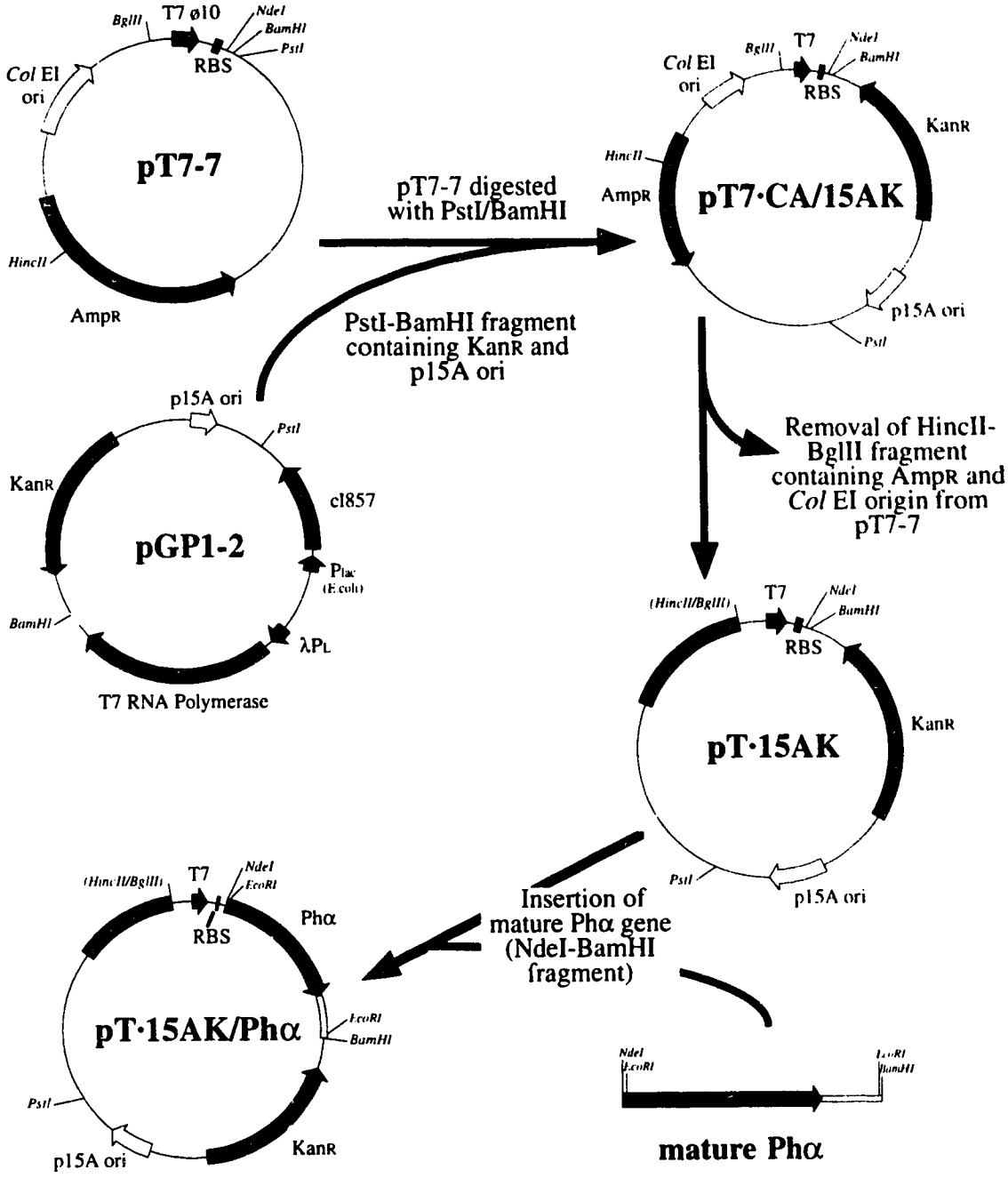


Figure 6.3 Construction of a T7 expression plasmid for the production of cloned pig heart α subunit. pT·15AK/Phα was designed to express the Phα subunit alone in BL21 cells and under conditions of coexpression with Phß from pT7-7/Phß. Since this latter plasmid contains a *Col* EI replicon and a Amp^R marker, the hybrid pT7·CA/15AK was first constructed to introduce a compatible p15A replicon and Kan^R gene into pT7-7. The ß-lactamase gene (Amp^R) and *Col* EI ori were removed from the hybrid, producing pT·15AK. The mature Phα gene was then placed downstream of the strong ribosome binding site, under the selective control of the active T7ø10 promoter.

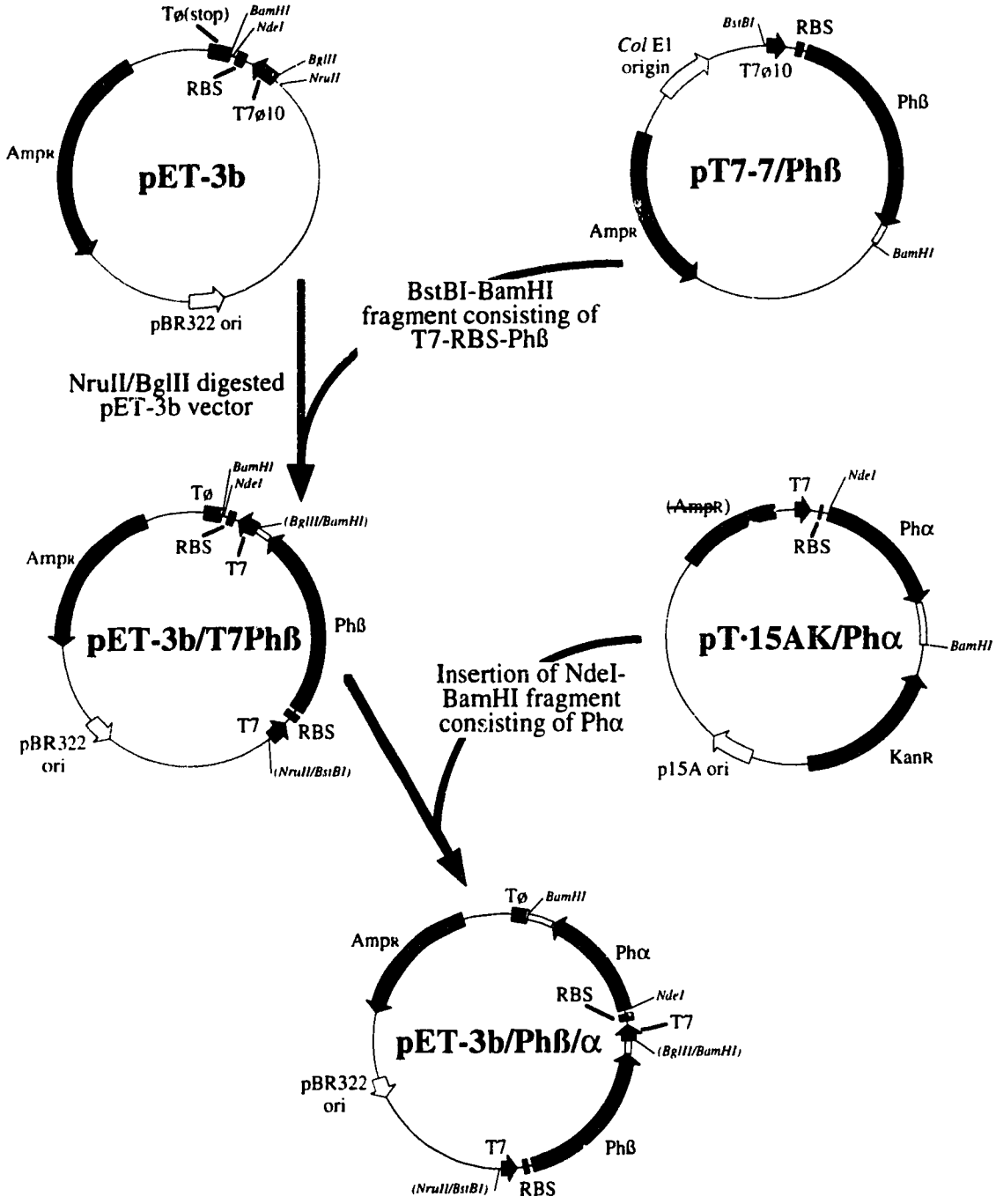


Figure 6.4 Construction of a T7-controlled coexpression plasmd for the bacterial synthesis of pig heart SCS α and ß subunits. A single plasmid, pET-3b/Phß/α, was developed to coexpress both pig heart SCS subunits from separate T7 promoters in a tandem fashion. First, the T7 promoter, ribosome binding site and Phß gene from pT7-7/Phß were transferred into pET-3b, upstream of its T7 promoter. The Phα gene was then introduced downstream from the original pET-3b T7ø10 promoter and ahead of the Tø transcription terminator, to complete the coexpression construct.

for 2 hours before cells were chilled and collected by centrifugation at 5 x 10^3 g for 5 minutes. The pellets were resuspended in 1/10 culture volume of cold TST buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl and 0.05% (v/v) Tween-20) and then sonicated three times for 3 minutes each. The resulting homogenates were centrifuged at 20 x 10^3 g for 20 minutes and the supernatant was diluted with 5 equal volumes of TST before applying to an IgG-Sepharose affinity column to purify expressed fusion proteins (see below).

2. **T7-controlled expression in BL21(DE3)** - Cultures of BL21(DE3) carrying expression plasmids were grown from newly transformed cells at 37 °C in LB broth supplemented with ampicillin (100 µg/ml) and kanamycin (75 µg/ml) as required [22]. When cultures reached an Abs_{600} of 0.6, expression was induced by the addition of isopropyl-ß-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Protein expression was allowed to continue for 4 hours at 37 °C following induction. Cells were harvested by centrifugation, frozen at -20 °C, thawed and resuspended in sonication buffer (10 mM potassium phosphate (pH7.4), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM EDTA) before being lysed. SCS activity was measured by the direct spectrophotometric method [26] according to methods previously outlined [27].

3. **T7-controlled expression in TK3(D18)** - TK3(D18) bacterial cells containing the plasmids pGP1-2 and pET-3b/Phß/α, were grown at 30 °C to an Abs_{600} of 1 in DLB broth (KCl was substituted for NaCl) augmented with 100 µg/ml ampicillin and 75 µg/ml kanamycin. T7 RNA polymerase synthesis was induced from pGP1-2 by raising the temperature to 42 °C for 45 minutes. T7-directed protein synthesis of SCS subunits was allowed to occur for a further 4 hours at 37 °C before cells were harvested and frozen. From this point on, TK3(D18) cells were treated in a manner identical to that described above for BL21 cells. Supernatants from sonicated lysates were also analyzed for SCS activity in a manner similar to that of BL21 expression lysates.

E. Purification of protein A fusion proteins

The protein A fusion proteins were purified chromatographically on IgG Sepharose 6 FF (Pharmacia) following methods developed by the manufacturer [25]. Cellular lysates containing proteins expressed from the plasmid, pRIT2T (see above), were applied to 2 ml columns of IgG Sepharose previously treated with 0.5 M acetic acid (pH 3.4) and neutralized with TST buffer. The columns were washed with 10 bed volumes of TST buffer followed by 2 volumes of 5 mM ammonium acetate (pH 5.0). Bound protein A fusion proteins were then eluted with 0.5 M acetic acid (pH 3.4) and peak fractions were analyzed by SDS-PAGE, and lyophilized prior to further use.

F. Factor X_a protease digestion of protein A fusion proteins

Purified protein A fusion proteins containing a cloned recognition sequence for factor X_a were treated with this restriction protease according to protocols determined by Nagai and Thøgersen [19]. Both the lyophilized peak fractions of fusion protein (see **D** above) and the bovine plasma factor X_a (Boehringer Mannheim) were resolubilized in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl and 1 mM $CaCl_2$ prior to digestion. The amount of enzyme used was 1/100 that of the substrate by weight, and protease cleavage was allowed to proceed overnight at either 4 °C or 25 °C.

G. Reconstitution of SCS activity using fusion proteins

Following established protocols [28], purified pig heart SCS [27] was denatured in order to isolate the component subunits. Purified fusion proteins of protein A with either Phα subunit or Phß subunit were refolded with their counterpart subunit isolated from pig heart enzyme using a modified method outlined by Nishimura *et al.* [28]. Proteins were diluted into refolding buffer (50 mM Tris-HCl (pH8.0), 25% (v/v) glycerol, 50 mM dithiothreitol and 0.1 mM EDTA) such that the final concentration of denaturant (urea) was reduced to 0.1 M or less. Resultant protein concentrations in the refolding mixtures were in the range of 25-100 µg/ml. After allowing refolding to occur for 1 hour

at 25 °C, samples were assayed for SCS activity by the direct spectrophotometric method [26] using both ATP or GTP as nucleotide substrates.

H. Pulse-chase labeling of expressed proteins with ^{35}S-methionine

The selective labeling of plasmid-encoded proteins using the T7 RNA polymerase system was carried out following the protocol described by Tabor [29] with the following modifications. The cultures of BL21(DE3) cells containing T7 driven expression vectors were grown to an Abs_{590} of 0.5 before a 1 ml aliquot was withdrawn and centrifuged. The resulting cell pellet was washed twice in 1 ml of M9 medium (see **Appendix III**) before being resuspended in 1 ml of M9 medium supplemented with 0.02% (w/v) Casamino acids depleted of methionine (Gibco). Cultures were incubated at 30 °C for 2 hours and then induced with 0.4 mM IPTG for 20 minutes. The host RNA polymerase was inactivated by the addition of rifampicin to a final concentration of 200 µg/ml. The cells were left standing at 37 °C for 30 minutes. To a 0.5 ml aliquot of cells, 10 µCi of [^{35}S]-methionine/cysteine (Trans-label, ICN) was added to radioactively label plasmid-expressed proteins. Radiolabeling occurred for 5 minutes before the addition of nonradioactive methionine at final concentration of 0.5% (w/v). Aliquots were removed both immediately prior to this chase and at various intervals afterwards. These aliquots were immediately centrifuged, the supernatants removed and the cell pellets were solubilized in SDS-PAGE sample loading buffer before being analyzed by SDS-PAGE and autoradiography.

I. Purification of expressed pig heart SCS in BL21(DE3)

A 10 liter culture of BL21(DE3) harboring the T7 coexpression plasmid pET-3b/Phß/α was employed in a similar manner outlined above for the expression of pig heart SCS. A typical yield of 25.5 grams (wet weight) of cells were harvested and then frozen overnight at -20 °C prior to resuspension in a 200 ml of sonication buffer (0.1 M Tris-HCl, 0.1 M KH_2PO_4, 0.1 M KCl, 0.2 mM EDTA, 1.0 mM PMSF, 1 µg/ml pepstatin,

and 1 mM benzamidine (pH 7.4). The suspension was sonicated, and the resulting lysate was centrifuged at 15 x 10^3 rpm for 30 minutes at 4 °C. The supernatant was recovered and 20 ml of 5% (w/v) protamine sulphate was added and gently stirred before repeating the centrifugation. Proteins in the supernatant were subsequently precipitated for 2 hours on crushed ice by 20% (w/v) ammonium sulfate, and were removed by centrifugation at 15 x 10^3 rpm. The resultant supernatant fraction was brought to a final concentration of 50% (w/v) ammonium sulfate and left overnight at 4 °C. The precipitated proteins were collected by centrifugation and solubilized in 10 mM KH_2PO_4, 1 mM PMSF, 5 mM ß-mercaptoethanol, 0.1 mM EDTA and 0.5% (v/v) glycerol (pH7.4). The sample was desalted by gel filtration chromatography through a 5 x 45 cm Sephadex G-25 (Coarse) column using the solubilization buffer as the elution buffer. Fractions containing high enzyme activity and devoid of salt (as measured by conductivity) were pooled and loaded onto a 5.0 x 20 cm hydroxyapatite affinity column (Bio-Gel HTP Gel, Bio-Rad) previously equilibrated with the same buffer. The 'loaded' column was washed with 800 ml of buffer before eluting the bound protein with a 2 liter salt gradient of 10 mM to 400 mM KH_2PO_4 in the equilibration buffer. Peak fractions exhibiting GTP-specific SCS activity were pooled and precipitated with 50% (w/v) ammonium sulfate overnight at 4 °C. The precipitated protein was resuspended in the solubilization buffer listed above along with 10 mM $MgCl_2$ and 10 mM sodium succinate (pH7.4), and then subsequently phosphorylated with 0.1 mM GTP. This protein sample was loaded onto a 2.5 x 120 cm Bio-Gel A 0.5M (200-400 mesh - Fine, Bio-Rad) affinity column equilibrated with 20 mM KH_2PO_4, 0.1 mM EDTA, 0.1 mM PMSF, 5% (v/v) ß-mercaptoethanol and 0.5% (v/v) glycerol. Using this buffer to elute protein from the column, active peak fractions were then collected and assessed for their purity by SDS-PAGE analysis.

III. RESULTS

A. Expression of Pig heart SCS subunits as fusion proteins

An expression system was sought to produce high levels of the cloned pig heart SCS mature subunits. The prospect of expressing the subunits as C-terminally joined fusion proteins with a highly expressed bacterial protein was considered advantageous for several reasons [19]. One, the use of such systems offered a level of stability to the foreign protein and protection from degradation. Two, the expression of these fusion proteins was optimized since the bacterial protein was located N-terminally. In addition, this methodology eliminated any potential interference on translation initiation caused by the 5' end of the foreign gene. Just such problems have been observed during initial attempts to express the rat liver α subunit of SCS [30]. Therefore, the cloned, mature pig heart α and ß subunits were chosen to be expressed as fusion proteins with the IgG-affinity domains of staphylococcal protein A [20]. An added advantage of the protein A system was that these fusion proteins could be rapidly purified by a single step using IgG Sepharose affinity resin. Further, to facilitate the isolation of the pig heart subunits alone, the fusion protein constructs were 'engineered' with a tetrapeptide recognition site (Ile-Glu-Gly-Arg) for factor X_a protease [19] between protein A and the pig heart subunits (**Figure 6.5**). The proteolytic cleavage of the fusion proteins followed by an additional passage of the digestion mixture through the IgG column, would bind the protein A to the affinity matrix, and would elute the pig heart subunits in the flow-through.

Extracts were prepared from N4830-1 cells expressing the protein A-fusion proteins; these fusion proteins are referred to hereafter as PA-Phα and PA-Phß (see Lane 1, **Figure 6.6**). A single elution step from the affinity column seemed to purify the PA-Phß fusion protein in excess of 95%. Note: repeated application of the PA-Phß (or PA-Phα) fusion protein to the IgG column increased the sample purity only marginally (results not shown).

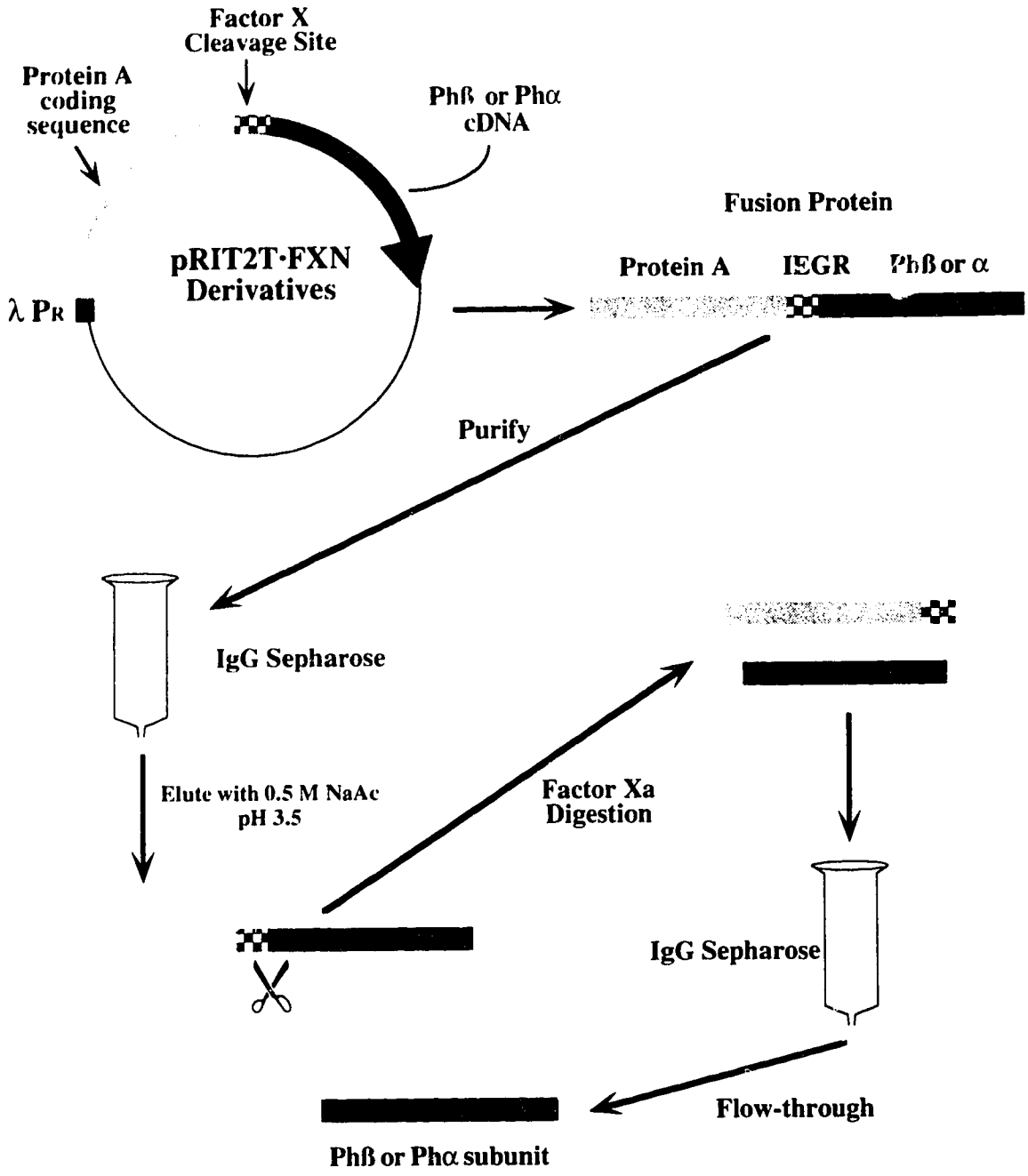


Figure 6.5 Purification scheme for fusion proteins and liberated subunits. Expression constructs were created in the vector pRIT2T·FXN to produce fusion proteins. These consisted of the protein A affinity tail linked to either the pig heart SCS α or ß subunit through a factor Xa cleavage site (IEGR). Fusion proteins were expressed in N4830-1 cells. Cellular extracts were then prepared and applied to affinity columns made of IgG Sepharose. Once eluted with acetic acid, fusion proteins could be treated with factor Xa in order to remove the protein A tag. An additional passage over the affinity column serves to purify the expressed SCS subunits.

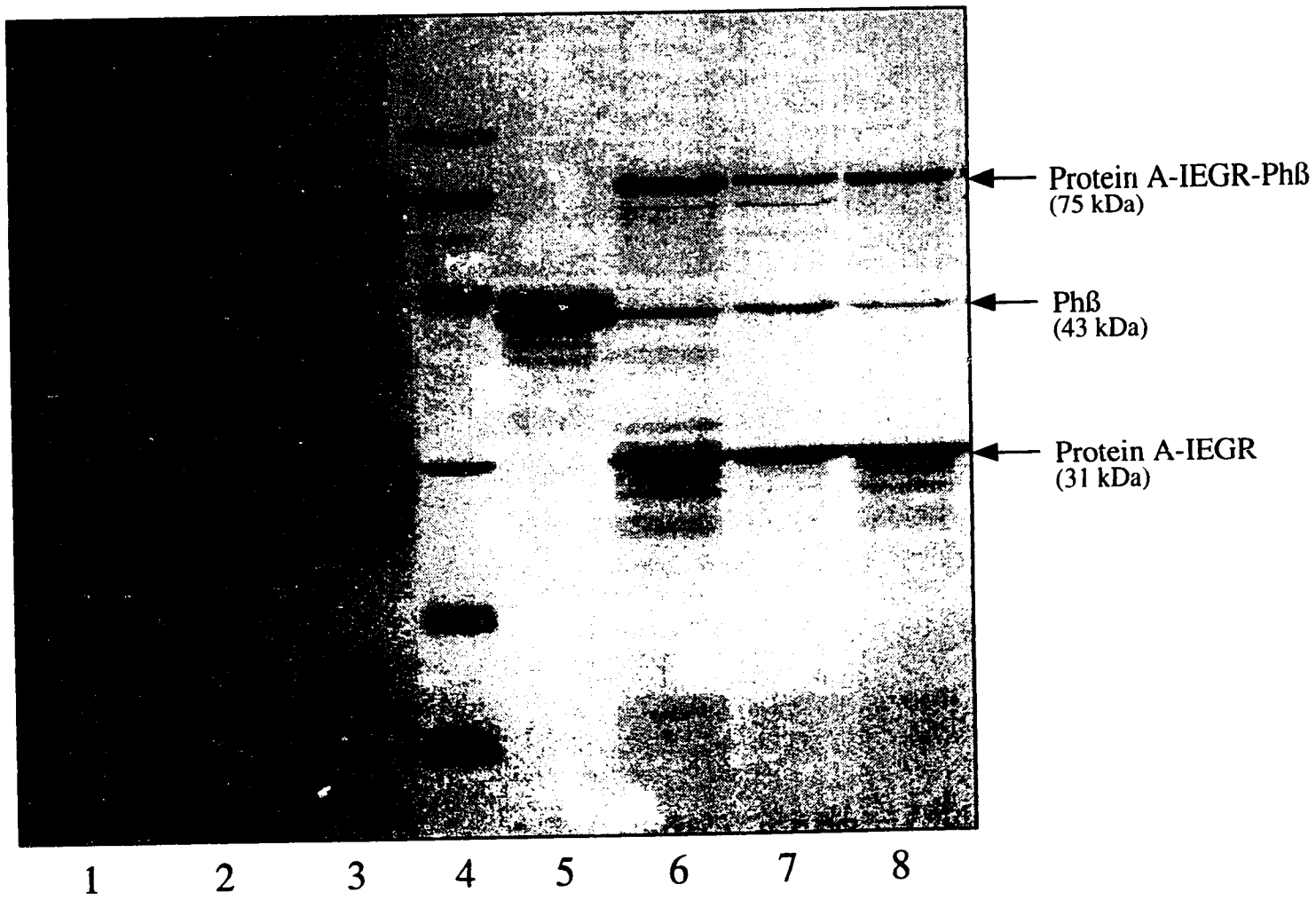


Figure 6.6 SDS-PAGE analysis of Protein A-Phß fusion protein expression, purification and factor Xa cleavage. Lane 1, cell lysate of N4830-1 cells expressing Protein A-IEGR-Phß fusion protein; Lane 2, flow through from IgG Sepharose column after application of cell lysate; Lane 3, purified fusion protein eluted from column with 0.5 M acetic acid (pH 3.4); Lane 4, protein standards (97.4 kDa, 66.2 kDa, 45 kDa, 31 kDa, 21.5 kDa, 14.4 kDa); Lane 5, ß subunit of SCS purified from pig heart tissue according to standard methods [27]; Lane 6, factor Xa digestion of purified fusion protein (10 µg) with factor Xa for 2 hours at 25 °C; Lane 7, same as Lane 6, using 5.5 µg of the fusion protein for 24 hours; Lane 8, same as Lane 7, but digestion was at 4 °C.

The molecular mass of the PA-Phß fusion protein was observed to be 75 kDa, consistent with that predicted from the sequence (Lane 3, **Figure 6.6**). An observed molecular mass of 65 kDa for the PA-Phα fusion protein was also in agreement with the predicted value.

Although the purified fusion proteins were obtained at levels of approximately 3-5 mg per liter of culture, subsequent proteolytic digestion with factor X_a under optimized conditions achieved only partial cleavage of the recognition site linking the protein A and the Phß subunit domains (Lane 6, **Figure 6.6**). In spite of this low digestion level, the observed molecular weight of the liberated Phß subunit agreed with its predicted value of 43 kDa. The results with the digestion of the PA-Phα fusion protein were even more frustrating since the protease recognized and cleaved a site within the α subunit thereby fragmenting the mature protein (results not shown). These results unfortunately limited the use of the fusion proteins to prepare feasible amounts of expressed pig heart SCS subunits necessary for further characterization. Since this was the ultimate goal of these studies, no further investigation was made as to why the proteolytic cleavage was unproductive.

B. Reconstitution of active SCS from fusion proteins

It is known that both *E. coli* and pig heart SCS enzymes can be assembled, *in vitro*, from mixtures of purified subunits [28, 31-33]. Unlike the *E. coli* enzyme, reconstitution of pig heart SCS requires the presence of 15-25% (w/v) polyethylene glycol or glycerol, is not dependent on nucleotides and is more inefficient *in vitro* [28]. Nevertheless, yields of 60% recovery of wild type activity have been reported for the refolding of pig heart SCS from isolated subunits. It was originally intended that the production of fusion proteins be used to prepare large amounts of purified mature subunits and the subsequent refolding to prepare active SCS complexes. However, since the cleavage step was unproductive, attempts were limited to reconstituting SCS activity using these purified fusion proteins themselves. **Table 6.1** shows the results of these efforts. When PA-Phα fusion protein was refolded with the ß subunit isolated from pig

Table 6.1

Refolding and assembly of fusion proteins into active SCS complexes

Subunit composition of refold mixture[1]		Reconstituted SCS activity (units/ml)	
ß subunit	α subunit	ATP	GTP
iPhß	-	0	0
-	iPhα	0	0
iPhß	iPhα	0	0.364
prot.A-Phß	-	0	0
-	prot.A-Phα	0	0
prot.A-Phß	prot.A-Phα	0	0
iPhß	prot.A-Phα	0	0.036
prot.A-Phß	iPhα	0	0
X_a(prot.A-Phß)	iPhα	0	0.038

[1] Refolded proteins were either: subunits isolated from purified pig heart SCS (iPhß, or iPHα); fusion protein of protein A with cloned pig heart subunits (prot.A-Phß, or prot.A-Phα); or fusion protein treated with factor X_a (X_a(prot.A-Phß)). Where applicable, equimolar samples of proteins were diluted into refolding buffer (50 mM Tris-HCl (pH 8.0), 25% (v/v) glycerol, 50 mM dithiothreitol, and 0.1 mM EDTA) such that the final concentration of denaturant (urea) was reduced to 0.1 M or less. Reconstitutions were carried out for 1 hour at 25 °C after which samples were assayed for enzymatic activity using either ATP or GTP as the substrate nucleotide.

heart SCS, the complex exhibited GTP activity. Although this recovered activity was low, only 1/10 that of wild type, its significance was suggestive. This reconstituted activity implied that the Phα subunit portion of the fusion protein had folded into a 'native' conformation, despite its protein A tag, and was capable of assembling into an active complex with its counterpart subunit. The refolding of the intact protein A-Phß fusion protein with the isolated Phα subunit did not exhibit any SCS activity. However, it was remarkable that after cleavage with factor Xa, the released Phß subunits did reconstitute substantial GTP activity. This indicated improper folding of the Phß subunit while fused to protein A. It appeared that after release from its structural constraints, the once tethered Phß subunit was able to proceed down a productive pathway of folding to achieve its native conformation which was catalytically competent. Since only GTP activity was observed in all these reconstitution trials, regardless of the subunit combination, it was inferred that the cloned pig heart subunits defined GTP specificity.

C. Pulse radiolabeling of T7 expressed pig heart subunits

In light of the failure to isolate pig heart subunits from fusion proteins, a T7 RNA polymerase/promoter system was employed for the expression of mature SCS proteins directly. This system was advantageous due to the highly processive nature of T7 RNA polymerase and its high selectivity for initiation at its own promoter sequences [21]. Thus, sequences encoding the mature pig heart subunits were cloned downstream of T7ø10 promoters in the expression plasmids pT7-7, pT·15AK, and pET-3b. The resultant recombinant vectors were transfected into one of two bacterial strains for subsequent expression. T7 RNA polymerase was provided endogenously in the bacterial strain BL21(DE3), or exogenously from the plasmid pGP1-2 when expression occurred in the SCS null strain TK3(D18).

To assess the stability of the mature subunits when produced *in vivo* in BL21(DE3), the expressed proteins were pulse labeled with [^{35}S]-methionine and observed over time. An additional advantage of using the T7 expression system lay in

the fact that the T7 RNA polymerase was resistant to antibiotics such as rifampicin that inhibit the *E.coli* RNA polymerase. Consequently, transcripts produced under the control of the T7 promoter in the presence of rifampicin could be selectively enriched, allowing exclusive labeling of translated proteins. Cultures expressing either the mature Phß or Phα subunit alone, or together, were subjected to short pulses of [^{35}S]-methionine labeling (see **MATERIALS AND METHODS**). The results are presented in **Figure 6.7**. The molecular masses of these selectively labeled proteins agreed with that predicted for the mature pig heart subunits: that of the expressed Phß subunit was approximately 43 kDa, whereas that of the mature Phα subunit was about 33 kDa. As seen in panels A and B, when expressed alone, both the mature ß and the α subunits accumulated to significant levels and were stable over the observed time periods. The high stability of these expressed proteins is likely the result of unpaired subunit aggregation and sequestration as protease resistant inclusion bodies. Evidence for this has been reported for several SCS subunits expressed by the T7 system [34].

In contrast to these results, when both subunits were coexpressed from pET-3b/Phß/Phα, an appreciable level of degradation was noted (panel C, **Figure 6.7**). Over the course of 1 hour, approximately half the amount of the pulse labeled subunits were proteolytically broken down. Moreover, the extent of degradation of the α subunit appeared to be greater than that of the ß subunit. The observed breakdown of the pig heart proteins under these conditions suggests that these subunits are able to form a soluble complex which is susceptible to degradation. Since the focus of this work was not directed at an in depth analysis of subunit stability, but rather to gain a general understanding for it, these studies were pursued no further.

D. Coexpression of mature Phß and Phα subunits

Two distinct T7-controlled coexpression systems were designed to assess the function of cloned pig heart SCS expressed in BL21(DE3) cells. In the first system, a single coexpression plasmid, pET-3b/Phß/α, was constructed in which both the mature

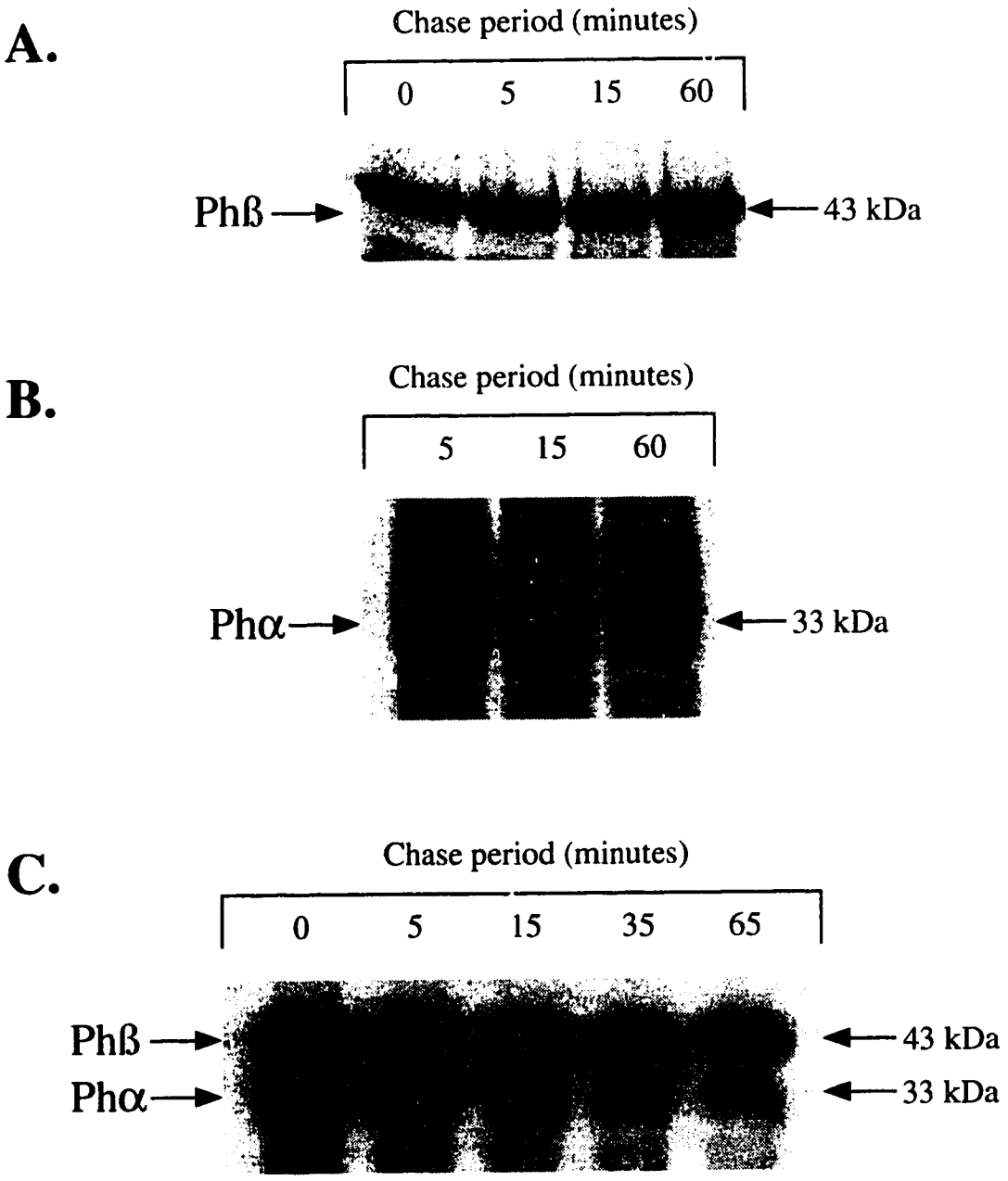


Figure 6.7 Stability of cloned pig heart SCS subunits expressed in BL21(DE3) bacterial cells. Cultures expressing either pig heart ß or α subunits alone or together, were grown to an Abs_{590} of 0.5 before labeling the expressed proteins with [^{35}S]-methionine in the presence of rifampicin. After a 5 minutes pulse labeling period, excess nonradioactive methionine was added to a final concentration of 0.5% (w/v). Aliquots were removed at various chase periods and immediately solubilized in SDS-PAGE sample buffer. These protein samples were then analysed by SDS-PAGE and autoradiography. **A**, labeling of Phß expressed from pT7-7/Phß. **B**, Phα expressed from pT·15AK/Phα. **C**, Phß and Phα coexpressed from pET-3b/Phß/α.

Phß and Phα genes were placed downstream of separate T7 promoters in a tandem fashion. The second system was created to independently synthesize the mature Phα and Phß subunits from the two separate, yet compatible, plasmids, pT-P15AK/Phα and pT7-7/Phß. This latter two-plasmid system was developed not only to corroborate results obtained from the single coexpression plasmid, but also to compensate for subunit excesses which may result from the tandem promoter construction in the dicistronic plasmid (see **MATERIALS AND METHODS**). Cellular lysates were prepared from BL21 cultures expressing proteins from either the one- or two-plasmid systems, and these extracts were assayed for SCS activity with ATP or GTP as the nucleotide substrate. The results are tabulated in panel A of **Table 6.2**. In the single-plasmid system, a definite increase in GTP-specific activity over background was observed in crude extracts containing expressed pig heart SCS. A smaller increase was found for the two-plasmid system, possibly indicating a limiting copy number for one of the plasmids. However, these same expression lysates also exhibited marginal increases in ATP-specific activity as well. Therefore, although suggestive, definitive conclusions could not be made concerning the specificity of this expressed isozyme under these conditions where a high background existed.

The endogenous activity of SCS in wild-type bacterial cells co[illegible] determination of the nucleotide specificity in the expressed pig heart isozyme. To resolve the issue, an SCS null strain was used as a host for the expression of pig heart SCS. Here, TK3(D18) cells were transfected with the coexpression plasmid pET-3b/Phß/α, and pGP1-2, a plasmid which provided an exogenous source of T7 RNA polymerase. Under conditions of SCS expression, cellular extracts were prepared and assayed for activity. As shown in panel B of **Table 6.2**, the use of TK3 cells as a host provided an indication that the combination of the cloned pig heart SCS subunits formed an active complex which was GTP-specific.

Table 6.2

Coexpression of cloned α and β subunits from pig heart SCS

A. Coexpression in BL21(DE3)

Subunit expression mechanism	Expression culture assayed[1] (+/- plasmids employed)	Average SCS activity (units/mg cell protein) ATP	GTP
-	BL21 alone	0.877	0.086
two plasmid system	pT·15AK/Phα + pT7-7/Phß	0.950 [+8.3%]	0.188 [+120%]
single plasmid system	pET·3b/Phß/α	1.09 [+24.3%]	0.472 [+449%]

B. Coexpression in TK3(D18) with pGP1-2

Expression culture assayed[2] (+/- plasmids)	Average SCS activity (units/mg cell protein) ATP	GTP
TK3	0	0
pET·3b/Phß/α	0	0.077

[1] BL21 cultures harboring either one, two or no plasmids were grown under conditions to coexpress cloned pig heart SCS subunits. Cells were harvested, sonicated and centrifuged before cleared cellular extracts were assayed for SCS activity using either ATP or GTP. Note: numbers in square brackets represent percent increases in either ATP- or GTP-specific SCS activity over background levels.

[2] TK3 cultures bearing pGP1-2 (for exogenous T7 RNA polymerase) and possibly pET·3b/Phß/α were grown and treated under similar conditions to those of BL21 cultures in order to determine SCS activity levels.

E. Purification of expressed pig heart SCS yields an active isozyme which is GTP-specific

The results obtained from the expression of the pig heart isozyme in the null strain remained troublesome. The consistently low activities observed for the assembled complex (approximately 0.08 units/mg) were not representative of the highly active forms that could be purified routinely from pig heart tissue (approximately 30 units/mg) [27]. This could be rationalized considering crude extracts were used to measure enzyme activity and that the deletion mutant TK3(D18) can support only low levels of foreign protein expression. Both of these factors have lowered the assayed specific activity in such preparations. However, the possibility remained that the cloned isozyme might demonstrate ATP specificity in addition to, or at the expense of, GTP specificity, if examined in a more purified form. This concept had merit noting the increases in both GTP- and ATP-supported SCS activity during coexpression of the pig heart subunits in BL21 cultures.

Purification of the expressed, dimeric pig heart enzyme provided a means to resolve the issue of nucleotide specificity. BL21(DE3) cells were chosen instead of TK3(D18) as the expression host since higher yields of protein production were routinely obtained. Cellular extracts containing pig heart SCS expressed from pET-3b/Phß/α were prepared and further purified according to the protocol presented in **MATERIALS AND METHODS**. A hydroxyapatite affinity resin was found to be effective in fractionating endogenous SCS away from the expressed enzyme (**Figure 6.8**). Using a linear gradient of KH_2PO_4, the *E. coli* enzyme was eluted at 110 mM phosphate, whereas the pig heart enzyme eluted at 275 mM. The positions of both of these enzymes in this elution profile corresponded to those previously observed with purified *E. coli* SCS and pig heart enzyme isolated from tissue, using the same column. In addition, only GTP supported activity was associated with this latter peak. As shown in panel B, **Figure 6.9**, further purification over a Bio-Gel A column served both to increase the specific activity to 20

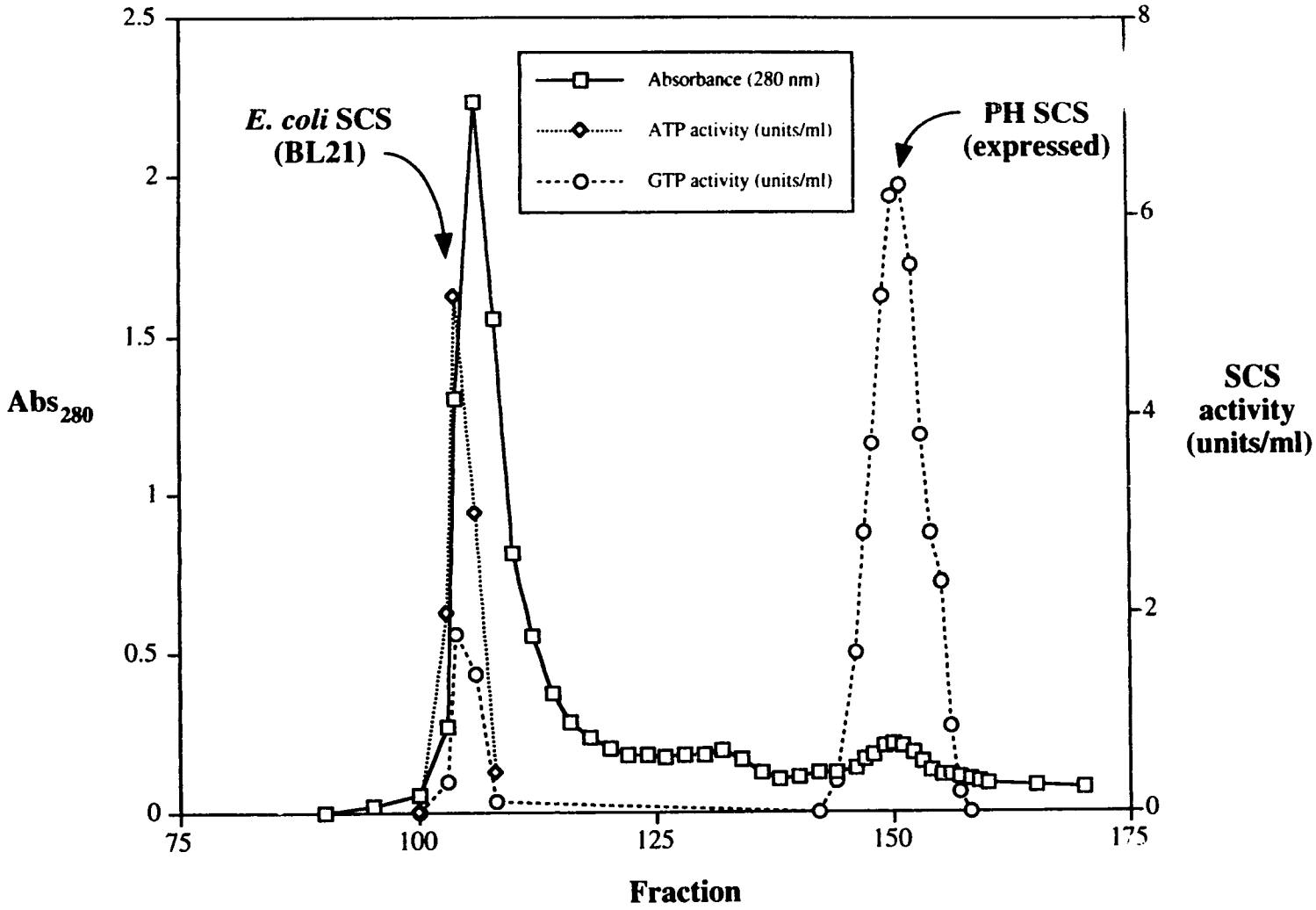
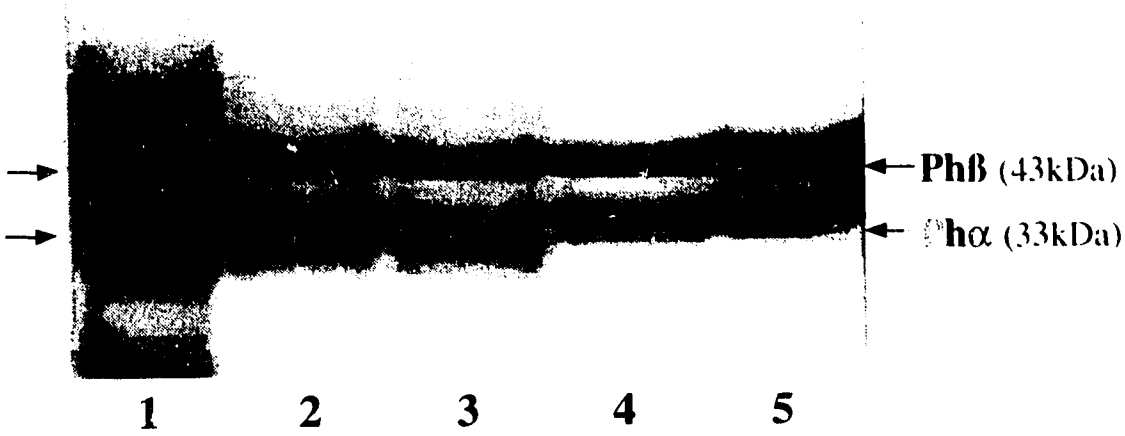


Figure 6.8 Elution profile of hydroxyapatite fractionation of pig heart SCS expressed in BL21(DE3) cells. Previously treated extracts (see text for details) of BL21 cells containing coexpressed Phα and Phß subunits were applied onto a 5.0 x 20 cm Bio-Gel HTP column (Bio-Rad) using a loading buffer composed of 10 mM KH_2PO_4, 1 mM PMSF, 5 mM ß-mercaptoethanol, 0.1 mM EDTA and 0.5% (v/v) glycerol. After washing the column with loading buffer, protein was eluted with a linear gradient of 10 to 400 mM KH_2PO_4 in loading buffer. Fractions were assayed for absorbance at 280 nm (Abs_{280}) and also SCS activity using both ATP or GTP.

A.



B.

Purification Stage	SCS Activity (units/ml) ATP	SCS Activity (units/ml) GTP	GTP specific SCS activity (units/mg)
Cell Extract **(lane 1)**	800	280	n/a
Protamine sulfate **(lane 2)**	774	408	n/a
Ammonium sulfate 20%	299	621	n/a
50%	595	1258	n/a
G-25 (desalting) **(lane 3)**	500	1000	n/a
HTP chromatography peak pool **(lane 4)**		460	**11.9**
phosphorylated		663	**14.0**
Bio-Gel A 0.5 M (fine) chromatography **(lane 5)**		400	**20.0**

Figure 6.9 Purification of pig heart SCS from an *E. coli* expression system. Extracts of BL21 cells coexpressing pig heart SCS subunits (from pET-3b/Phß/α) were prepared in order to purify and characterize a cloned isozyme of pig heart SCS. **A. SDS-PAGE profile through purification of expressed pig heart SCS.** Lane 1, crude extract of expression lysates; Lane 2, after protamine sulfate treatment; Lane 3, 50% ammonium sulfate precipitate desalted on Sephadex G-25; Lane 4, peak fraction from HTP chromatography; and Lane 5, overloaded sample after final purification through Bio-Gel A 0.5 M (fine) resin. **B. SCS activities (ATP and GTP) through purification.** Note: GTP-specific activities of purified pig heart SCS could not be determined in early purification stages due to endogenous, contaminating SCS activity from bL21 cells. Numbers in brackets at various purification stages correspond to above lanes in SDS-PAGE (A). n/a - not available.

units/mg and to provide a homogeneous preparation of the enzyme (as indicated by an overloaded sample on SDS-PAGE, panel A, Lane 5, **Figure 6.9**). The purification of the expressed pig heart enzyme has demonstrated that the combination of these cloned SCS subunits produced a highly active isozyme which was strictly GTP-specific.

IV. DISCUSSION

Work presented here describes the bacterial expression of two cloned subunits from pig heart SCS. When expressed individually, the Phα and Phß subunits appear to be stable proteins, likely the result of being sequestered as inclusion bodies resistant to protease activity [34]. However, when coexpressed with the Phß subunit, the Phα subunit demonstrated a greater susceptibility to degradation. This observation may be due to an excess of unpaired Phα subunit arising from the expression conditions. Unlike the upstream Phß gene, the transcription of the downstream Phα gene was driven by two promoters instead of one (see **Figure 6.4**). Subsequent translation likely led to a predominance of Phα subunits over Phß subunits. Similar claims have been made of an analogous coexpression system producing vaccinia mRNA capping enzyme subunits [35]. Considering surpluses of one SCS subunit were not normally found in cellular extracts [36], it is possible that Phα subunits not complexed with Phß subunits in a stable enzymatic form may have been susceptible to proteolytic degradation. Moreover, labeled Phα subunits were likely to have been diluted more quickly with newly synthesized, unlabeled subunit relative to Phß subunits. Combination of these two factors could thereby give rise to the apparent decreased stability of the Phα subunit when compared to the Phß subunit.

An additional possibility for the greater instability of the Phα subunit may have been due to an extension of 6 amino acids at the N-terminus of this mature subunit when expressed. This may reflect the observation that precursors of the rat liver α subunit containing signal sequences were more unstable than the mature forms of the same

subunit [34]. Furthermore, this N-terminal addition may have been responsible for preventing the expressed pig heart enzyme from achieving specific activity levels of 30 plus units/mg as measured for enzyme when purified from tissue [27].

Although the specific activity of the expressed pig heart enzyme (20 units/mg) was nearly wild-type, it was possible that this isozyme was impaired with respect to its ability to fold and assemble into its most active conformation. Moreover, a potential cause for not attaining maximal activity was the lack of appropriate mitochondrial chaperone(s) required to mediate such events as proper folding. Perhaps even the foreign environment of the bacterial cytoplasm may have itself prevented the attainment of full potential activity. These explanations are in keeping with earlier studies [28] showing only limited ability to artificially reconstitute pig heart SCS from its isolated subunits in an *in vitro* milieu.

Notwithstanding the fact that the fusion proteins of the pig heart subunits with protein A were unsuitable for further study, they gave the first indication that the cloned pig heart enzyme was GTP-specific. It was remarkable that the Phα subunit was able to form an active complex with an isolated Phß counterpart while fused to the protein A affinity tail. In the three dimensional structure of *E. coli* SCS, the N-terminus of the α subunit lies on the surface of the protein (see **Figure 1.7**, page 30) [37]. It was likely that the Phα subunit retained a similar structure, given the high degree of similarity between the two amino acid sequences. If so, enough flexibility in N-terminus would have allowed the Phα subunit to obtain a catalytically competent conformation while fused to protein A. Considering the PA-Phß fusion protein, again from the published structure, the N-terminus of the ß subunit in the *E. coli* SCS was observed to be buried [37]. Thus, if the N-terminus of the ß subunit was buried in the pig heart protein, it would be unlikely that the fusion protein would be able to reconstitute a proper dimeric structure let alone SCS activity when refolded with an isolated Phα subunit. Consistent with this prediction, this was observed experimentally. Moreover, the proteolytic cleavage of the fusion

protein in the intervening sequence by protease X_a, and thus release of the Phß subunit from protein A, allowed proper subunit interactions and enzymatic activity to be recovered when refolded with Phα. Therefore, from these results it appears that predictions regarding the structure and function relationships between pig heart and *E. coli* SCS may be valid.

It has been suggested that GTP-specific SCS functions in mammalian ketone body metabolism and porphyrin synthesis, while a unique ATP-specific isoform serves the citric acid cycle [4, 5]. The characterization of a cloned pig heart isozyme displaying GTP specificity is consistent with this theory when considering the relative importance of ketone body metabolism in this tissue. Heart muscle preferentially uses acetoacetate over glucose as an energy source [38]. Thus, a primary role of myocardial SCS is likely to supply a high level of succinyl-CoA necessary for the metabolic consumption of this ·tone body. It is, therefore, conceivable that a GTP-specific complex formed from the mbination of the Phß and Phα57 subunit is responsible for the maintenance of the ketone pathway in pig heart tissue.

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Chapter 7

DISRUPTION OF THE *E. coli* SCS TETRAMER: A MUTAGENESIS STUDY BASED ON THE PUBLISHED QUATERNARY STRUCTURE

I. INTRODUCTION

An interesting property exemplified by succinyl-CoA synthetases from various sources is the heterogeneity of quaternary structure. 'Small' forms of the enzyme, corresponding to αß dimers, are found in Gram-positive bacteria [1, 2], yeast [1, 3] and mammalian mitochondria [4, 5]. The only known sources of 'large' forms of SCS, which are composed of an $\alpha_2\beta_2$ tetrameric structure with two active sites, are *Escherichia coli* [6, 7], and other Gram-negative bacteria [1, 2, 8]. The rationale of this larger quaternary structure composed of a dimer of dimers has remained unclear. A number of possible roles for the quaternary structure of oligomeric enzymes have been outlined [9, 10]: they include formation of a shared active site, increased solubility, increased stability, decreased osmotic pressure in the cell, substrate channeling, and the requirement of quaternary structure for the active conformation of one functional unit (subunit, dimer, etc.). In addition, cooperation between multiple active sites has been observed to accelerate some catalyzed reactions [11].

Several studies have suggested that the tetrameric structure of *E. coli* SCS may have a catalytic mechanism of alternating sites cooperativity. Evidence, including half-sites reactivity with respect to enzyme phosphorylation [12-14], and that from ^{31}P NMR studies [15, 16], hybrid enzyme formation [17, 18], and ^{18}O exchange kinetics [11], have been taken as supporting the cooperative interactions between the two αß halves of the tetramer. Here, the two active sites were proposed to function in alternating fashion, with the phosphorylation of the histidine residue at one active site promoting a less favorable step in the catalysis at the neighboring site [19]. However, a number of studies have called into question the interpretation of these results or provided contradictory evidence to suggest the *E. coli* tetramer is comprised of two independently active dimer enzymes associated to form a 'dimer of dimers' [20-23].

The crystal structure of *E. coli* SCS indicated that the catalytic histidine residues of both active sites were phosphorylated [24]. This was contrary to the theory of 'half-of-

the-sites' phosphorylation which had supported a mechanism of cooperative alternating sites as the rationale for the quaternary structure in this enzyme. Moreover, CoA was bound at each of the two active sites, and little asymmetry was noted between the two halves of the enzyme.

Due to the symmetrical relationships within the crystal structure of *E. coli* SCS, two arrangements of αß dimers are possible for the overall tetrameric structure. (An alternative quaternary structure is explored in **Chapter 8**.) In the tetramer chosen by Wolodko *et al.* [24], it is worth noting that the two composite dimers are associated almost exclusively through their ß subunits. Therefore, any 'communication' between the active sites in this structure must be mediated through these ß subunits. To determine whether or not the catalytic activity of tetrameric SCS is linked to its quaternary structure, mutants were designed to disrupt the ß subunit interface between the two αß dimers of the heterotetramer. This chapter, therefore, focuses on the attempts made to split the tetrameric SCS into a dimeric form as a test for the quaternary relations proposed for *E. coli* SCS [24], and as an initial effort to rationalize the oligomeric structure of this enzyme.

II. MATERIALS AND METHODS

A. Bacterial strains, plasmids and vectors

Two *E. coli* strains were used for expression in these studies. JM103 was routinely used for the construction and propagation of all mutated vectors and plasmid derivatives. TK3(D18), a SCS null strain in which the genes for endogenous *E. coli* SCS have been deleted, was used for the expression of all mutant forms of *E. coli* SCS. CJ236, a strain deficient for dUTPase and N-uracil glycosylase, was used in the mutagenesis studies [25]. The genotypes of these strains can be found in **Appendix II**.

The plasmids and vectors used in the development and expression of mutant *E. coli* SCS proteins were as follows. M13mp18 [26] was used as a vector for the mutagenesis of the *E. coli* sucC/D genes encoding SCS from this source. The vector, pGS202 [27], is a derivative of the thermoinducible expression vector pJLA503 [28], containing the *E. coli* sucC/D genes.

B. Construction and mutagenesis of vectors and expression plasmids

A general mutagenic vector was created by introducing the genes encoding both the ß and α subunits of *E. coli* SCS into M13mp18 by standard methods [29]. More specifically, M13mp18.202 was created by transferring a XhoI-EcoRI fragment, consisting of the *E. coli* SCS *sucC/D* genes (coding for the ß and α subunits, respectively), from the expression vector pGS202, into the SalI and EcoRI sites of M13mp18 (**Figure 7.1**). The resultant recombinant was propagated in the *dut⁻ ung⁻ E. coli* strain, CJ236, to create uracil-enriched single stranded template used in subsequent mutagenesis [25]. As detailed in **Materials and General Methods**, site-directed mutagenesis was carried out following the method of Kunkel *et al.* [25] using a variety of mutagenic oligonucleotides (see **Appendix I**). The uracil-containing parental strand of the mutated hybrid was selectively degraded in the *ung*$^+$ host, JM103, after transformation and propagation. Plaques were selected, and stocks were developed before isolating both the double and single stranded forms of the mutated M13mp18.202 DNA. The single stranded DNA was sequenced to identify the mutants and to ensure no mutations occurred elsewhere in the sequence. After doing so, a DNA fragment containing the mutation was removed from the double stranded form of M13mp18.202 and used to replace the similar, non-mutated, segment of pGS202. Typically, an NdeI-ClaI fragment comprising the mutated *sucC* gene (ß subunit) was used as the exchanged DNA cassette.

The ß subunit mutations were combined by one of three different methods. If at all possible, the restriction endonuclease sites lying between the mutated gene sequences

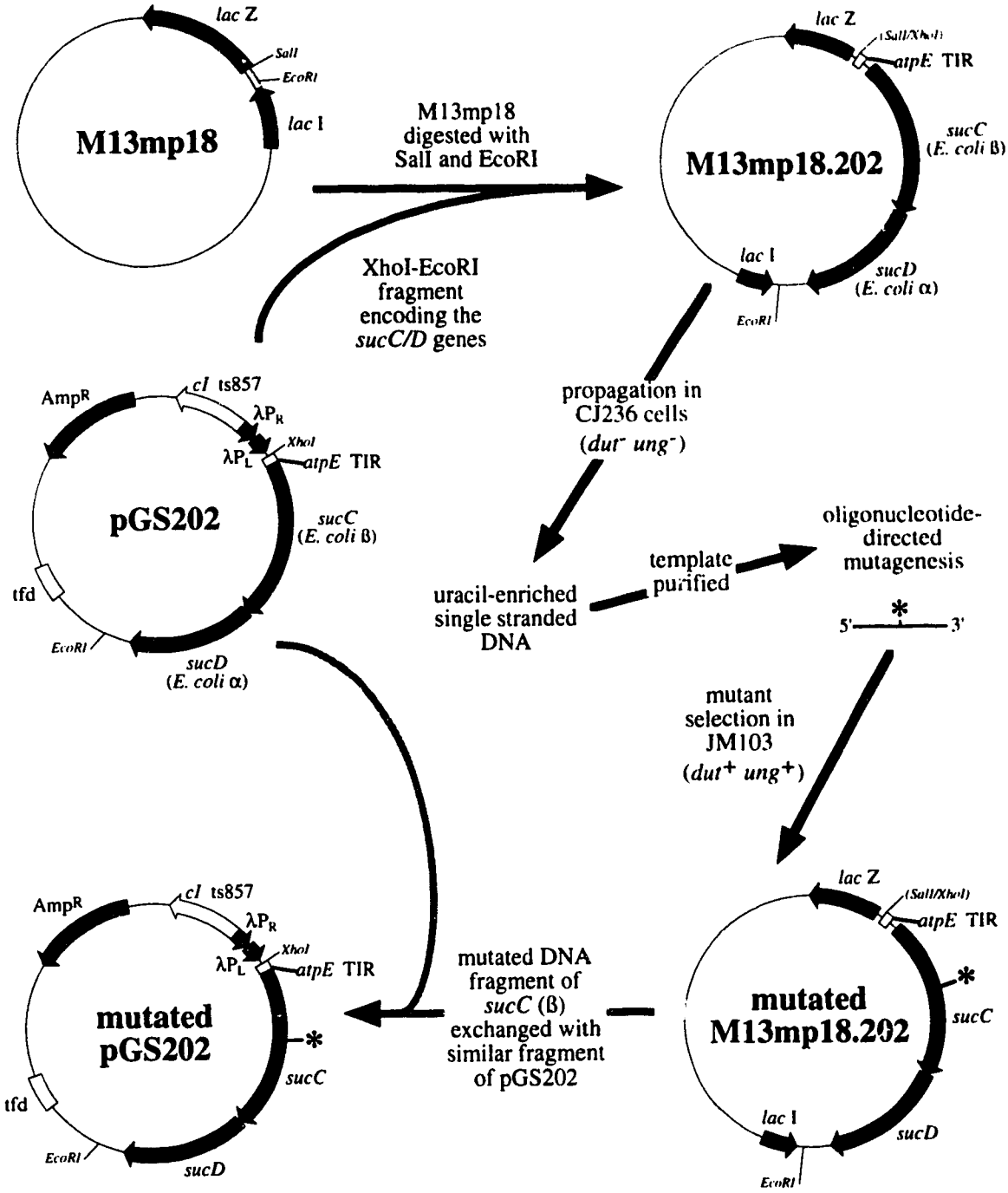


Figure 7.1 Construction of mutagenesis vectors and expression plasmids for the production of mutated forms of *E. coli* SCS. The general mutagenesis vector, M13mp18.202, was created by introducing the *E. coli sucC* and *sucD* genes (coding for SCS ß and α subunits respectively) into M13mp18. This vector was used for the production of uracil-enriched, single stranded template required for oligonucleotide-directed mutagenesis. Clones were selected and characterized by DNA sequencing analysis before DNA fragments containing the mutations were transferred into pGS202 for subsequent protein production. {*cI* ts857 - temperature sensitive λ repsessor; λPR, λPL - right and left λ promoters; *atpE* TIR - translation initiation region of the *atpE* gene; tfd - bacteriophage fd transcription terminator.}

were used to combine the DNA fragments incorporating these mutations in pGS202. A second method employed the use of two different mutagenic oligonucleotide primers in the mutagenesis reaction. Here, the frequency of the mutants that were detected in the small populations assessed (5-6 plaques were analyzed at one time) was approximately 25-50%. Finally, a third method used the mutagenized DNA itself as a uracil-template for the creation of additional mutations. All methods used were thoroughly tested for mutant authenticity by DNA sequencing prior to the expression of the gene-altered SCS subunits.

C. Bacterial expression of mutant *E. coli* SCS

The pGS202 plasmids expressing the mutant forms of *E. coli* SCS were transformed into the SCS null strain,TK3(D18) for subsequent expression. Culture media (50 ml) was prepared consisting of DLB (LB with KCl substituted for NaCl (pH 7.0), see **Appendix III**) supplemented with 100 µg/ml ampicillin, 50 mM L-glutamic acid, and 0.4 µg/ml thiamine (vitamin B_1). Cultures were inoculated with 1/50 x volume of an overnight culture, and resultant cultures were grown at 30 °C until an OD_{600} of approximately 0.4 was reached. The cultures were then shifted to 42 °C for 5 to 6 hours in order to allow plasmid-directed expression of SCS subunits to occur. The cells were harvested and frozen at -20 °C before thawing and resuspending in 0.4 culture volumes of KPCEP buffer (50 mM KH_2PO_4, 50 mM KCl, 1 mM EDTA (pH 7.4) and 0.1 mM PMSF). These suspensions were sonicated, and the lysates were centrifuged at 20 x 10^3 g for 20 minutes to obtain cleared cellular extracts. Samples were assayed for SCS activity. The remainder was treated with 50% (w/v) ammonium sulfate in order to precipitate and concentrate the proteins for further analysis.

D. Analysis of mutant quaternary structure and function

The initial assessment of the subunit structure of *E. coli* SCS mutants was made through gel filtration chromatography. It was determined that efficient and reproducible

analyses of the quaternary structure could be obtained with an FPLC system (Pharmacia) utilizing a Superose™12 HR 10/30 column (Pharmacia). The gel properties of this system allowed for the distinct and unequivocal separation of the tetrameric (142 kDa) and anticipated dimeric (71 kDa) species of *E. coli* SCS.

Samples for analyses were prepared as follows. Proteins precipitated with 50% (w/v) ammonium sulfate (usually cell extracts) were collected by centrifugation at 20 x 10^3 g for 30 minutes and resuspended in a small volume (1 ml) of KPCEP buffer. Centrifugation was repeated once more for 15 minutes. This was followed by filtration of the sample through a 0.22 µm filter (Millipore GV - low protein binding) to remove any particulate matter.

The filtered samples (either 50 µl, 200 µl or 500 µl) were applied to the column using KPCEP buffer as the solvent phase. FPLC conditions used for the maximal separation of the tetrameric (*E. coli* SCS wild type, 142 kDa) and dimeric (approximated by pig heart SCS, 75 kDa) species of SCS consisted of a flow rate of 0.2 ml/minute with fractions collected in 1.5 minute (0.3 ml) intervals. Samples of these fractions were qualitatively analyzed for protein content by SDS-PAGE and for SCS activity by the direct spectrophotometric method [30] according to methods previously outlined [31].

E. Analysis of mutant stability

The stability of the *E. coli* SCS mutants were characterized with respect to urea denaturation, pH and ionic strength. The samples that were analyzed in these experiments originated from FPLC peak fractions and were of high purity.

Urea denaturation entailed diluting the mutant SCS with KPCEP buffer and varying concentrations of urea: from 1 M to 4 M (final concentration). Incubation was carried out for hour at 25 °C. Aliquots were then assayed for activity in the standard fashion.

The pH effects were studied by diluting the mutant SCS 1 to 10 (v/v) in KPCEP buffer which ranged in pH from 4 to 11. These mixtures were incubated at 0 °C until assayed.

Mutant stability with regard to ionic strength was investigated by a 1 to 10 dilution of the protein in KPCEP buffer with various concentrations of KCl from 50 mM to 2.0 M. Samples were assayed for enzymatic activity shortly after mixing the protein and buffer.

III. RESULTS AND DISCUSSION

A. Substitution of non-conserved pig heart residues at the dimeric interface of *E. coli* SCS

It was clear from the crystal structure of *E. coli* SCS that the non-dissociating tetrameric structure [31] was due to the interactions between the ß subunits, linking one αß dimer with another (see **Figure 1.5**, page 28). The residues of the ß subunit that were evident in such contacts are highlighted in **Figure 7.2**. Since the amino acid sequence of the ß subunit of dimeric pig heart SCS had recently been determined (see **Chapters 3 and 4**), a comparison of the relevant sequences revealed that the residues which contribute the contacts at the ß-ß subunit interface in the tetramer of *E. coli* SCS, were not conserved in the pig heart protein. It is possible that these residues which hold the two dimers together in the *E. coli* enzyme by hydrogen bond or ion pairing have changed through evolution in order to prevent eukaryotic forms of SCS from assuming a tetrameric structure. As shown in Panel A of **Table 7.1**, one or both members of the five hydrogen-bond or salt bridge associations found at the ß-ß subunit interface of *E. coli* SCS were changed in the corresponding pig heart sequence. These equivalent non-conserved residues in the pig heart sequence were chosen to replace those in the *E. coli* sequence which appeared responsible for tetramer stabilization and specificity of dimer

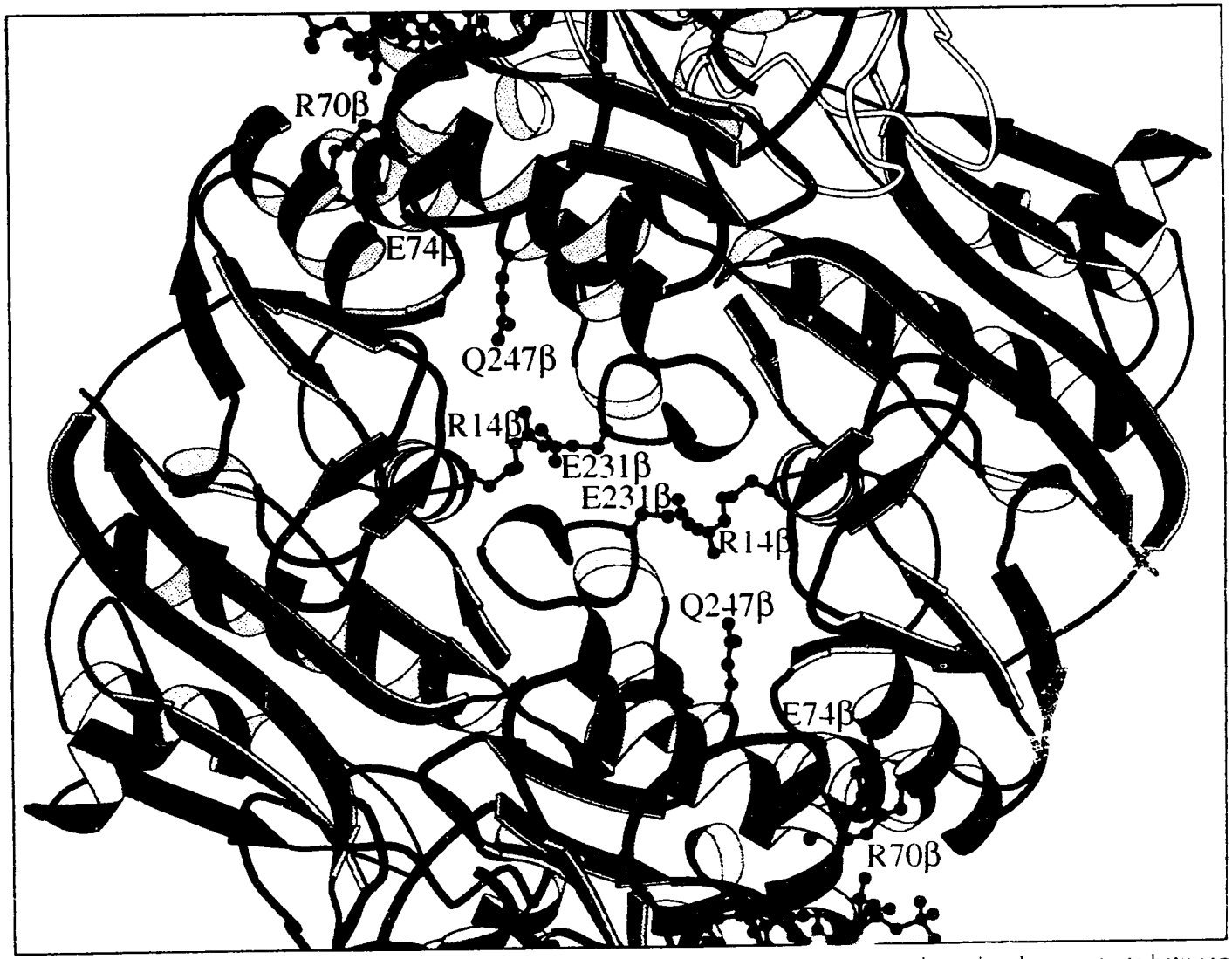


Figure 7.2 Schematic representation of the αß dimer interface in the *E. coli* SCS tetramer ...ated are the close contacts between the α1β1-dimer (yellow/green) and the α2β2-dimer (blue/magenta). This view is the same as that shown in **Figure 1.**. Dimer-dimer associations are mediated through the ß subunits. Residues which are responsible for inter-ß subunit interactions, and are not conserved when compared to equivalent positions in the pig heart ß subunit sequence, are depicted as ball-and-stick models in the color of their respective ß subunit. The other member(s) of these salt bridge or hydrogen bond pairings have been omitted for clarity. Note: the non-crystallographic twofold axis runs perpendicular to the page through the center of the figure. The interactions between the ß subunits in the top left region are therefore repeated in the lower right region. This graphic was generated by M. Fraser for [42].

Table 7.1

A. E. coli ß *subunit residues and interactions responsible for αß dimer associations and comparison to equivalent pig heart residues*

	Interacting *E. coli* ß Subunit Residues	Type of Interaction	Equivalent Pig Heart ß Subunit Residues[1]
1)	$R14ß_1$ ↕ $D228ß_2$	Electrostatic	**D[14ß]** × D[228ß]
2)	$R70ß_1$ ↕ $E249ß_2$	Electrostatic	**G[70ß]** × D[249ß]
3)	$E74ß_1$ ↕ $R297ß_2$	Electrostatic	**K[74ß]** × Q[297ß]
4)	$E231ß_1$ ↕ $Y6ß_2/Q7ß_2$	Hydrogen Bond	**A[231ß]** × Y[6ß]/Q[7ß]
5)	$Q247ß_1$ ↕ $N94ß_2$	Hydrogen Bond	**K[247ß]** × N[94ß]

B. *Mutations designed to disrupt the tetrameric quaternary structure: substitution of nonconserved pig heart residues*

Single Mutants	Multiple Mutants
R14ß→D	R14ß→D / E231ß→A
R70ß→G	R70ß→G / E74ß→K
E74ß→K	R14ß→D / R70ß→G / E74ß→K
E231ß→A	R70ß→G / E74ß→K / Q247ß→K
Q247ß→K	All 5 (single mutants combined)

[1] Nonconserved pig heart residues shown in bold are the equivalent residues of each pair observed to be changed most dramatically and were, therefore, used in the design of the tetramer disruption mutants (see Panel B).

interactions (Panel B, **Table 7.1**). Through site-directed mutagenesis, each mutation was created in the thermoinducible *E. coli* SCS expression plasmid, pGS202 (see **Figure 7.1**). In the likelihood that more than one of the electrostatic or hydrogen-bond partnerships was required for the interactions between dimers, the mutations were created in combination as well. The substitutions, R14ß→D and E231ß→A were combined as were R70ß→G, E74ß→K and Q247ß→K in order to maximize the disruptive effects of removing the stabilizing interactions of these spatially related residues. All five mutations were combined to fulfill the possibility that every relevant change noted in the pig heart ß subunit sequence was necessary for establishing the dimeric species of SCS.

The mutant enzymes were synthesized *in vivo* in two bacterical strains. The strain, JM103, was used for general cloning purposes and initial characterization of the expressed proteins. An SCS null strain, TK3(D18), was used to avoid the added complication of endogenous wild type *E. coli* SCS during qualitative analyses. Nearly 50% of all the soluble proteins in the cleared extracts of these cells were expressed mutant *E. coli* SCS subunits (see **Figure 7.3**). Through measurement of the SCS activity, this entire class of pig heart substitution mutants was found to display levels approximating the wild type control (**Table 7.2**). While this result was consistent with the concept of independent dimers associating in a single large complex, the quaternary structural analysis of these mutants revealed some surprising results. When subjected to FPLC analysis, every mutant, including the All5 mutant, eluted from a Superose™12 sizing column at a position corresponding to wild type, tetrameric SCS (see **Figure 7.4**). Thus, these mutations, acting alone or in combination, failed to disrupt the tetrameric structure of the enzyme. Clearly then, sources other than, or, in addition to, the electrostatic and hydrogen-bonds investigated here were responsible for the potential dimer-dimer interactions in *E. coli* SCS.

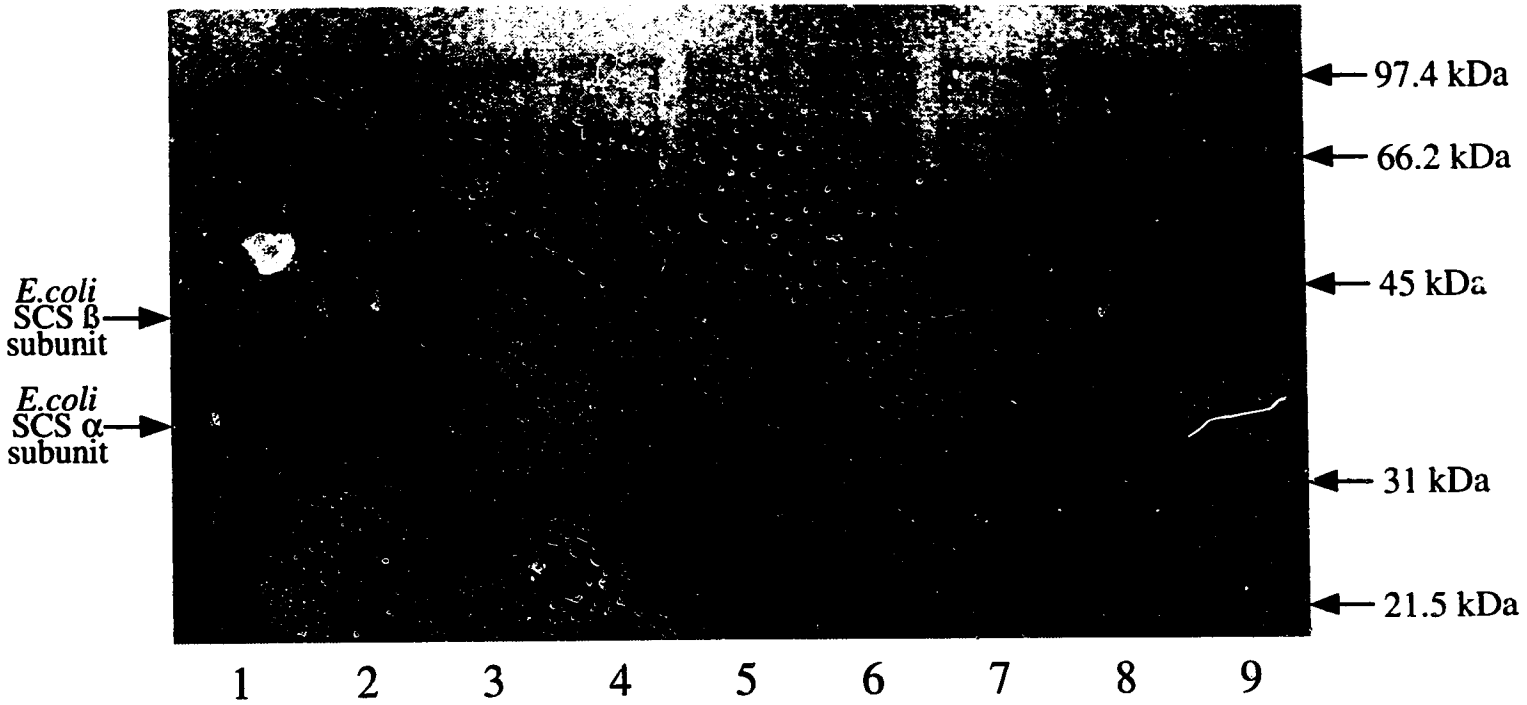


Figure 7.3 SDS-PAGE of expressed *E. coli* SCS mutants with substitutions of nonconserved pig heart residues at the αß dimer-dimer interface. The SCS subunit proteins were synthesized in JM103 cells from the expression plasmid, pGS202. The cells were lysed by sonication, and extracts were cleared of pelletable debris by centrifugation as described in **MATERALS AND METHODS**. Samples of the resultant supernatants were analysed by SDS-PAGE. Shown here are all the single (and one double) pig heart substitution mutants of *E. coli* SCS. The levels of the expressed SCS subunits for these mutants are typical for all mutants that were created. Lane 1, purified *E. coli* SCS; Lane 2, lysate of JM103 bearing pGS202 (wild type expressed E. coli SCS); Lane 3, protein standards (molecular weights listed at right); Lane 4, JM103 lysate of R14ßD; Lane 5, R70ßG; Lane 6, E74ßK; Lane 7, E231ßA; Lane 8, Q247ßK; Lane 9, R70ßG/E74ßK.

Table 7.2

Levels of enzymatic activity of the E. coli SCS mutants with substitutions of nonconserved pig heart residues at the αß dimer-dimer interface[1]

A. Single Mutants

Negative Control (units/mg cell protein)	Positive Control (units/mg cell protein)	Single Mutants (units/mg cell protein) (Expression in JM103)				
JM103	pGS202 / JM103	R14ßD	R70ßG	E74ßK	E231ßA	Q247ßK
0.71	37.4	26.5	39.3	34.4	29.5	33.1

B. Multiple Mutants

Negative Control (units/mg cell protein)	Positive Control (units/mg cell protein)	Multiple Mutants (units/mg cell protein)				
JM103	pGS202 / JM103	R14ßD / E231ßA	R70ßG / E74ßK	R14ßD / R70ßG / E74ßK	R70ßG / E74ßK / Q247ßK	All 5
0.71	23.9	21.4	21.5	23.8	24.8	25.7
TK3 (D18)	pGS202 / TK3	R14ßD / E231ßA	R70ßG / E74ßK	R14ßD / R70ßG / E74ßK	R70ßG / E74ßK / Q247ßK	All 5
0.006	28.2	22.5	n/a[3]	24.3	24.4	23.9

[1] The cells expressing the SCS proteins were lysed by sonication and cleared of cellular debris by centrifugation as described in **MATERIALS AND METHODS**. The supernatant extracts were assayed for SCS activity using the direct spectrophotometric method [27, 28] and ATP as the nucleotide substrate.

[2] Expression in: JM103 [top row]; TK3 [bottom row].

[3] n/a - not available

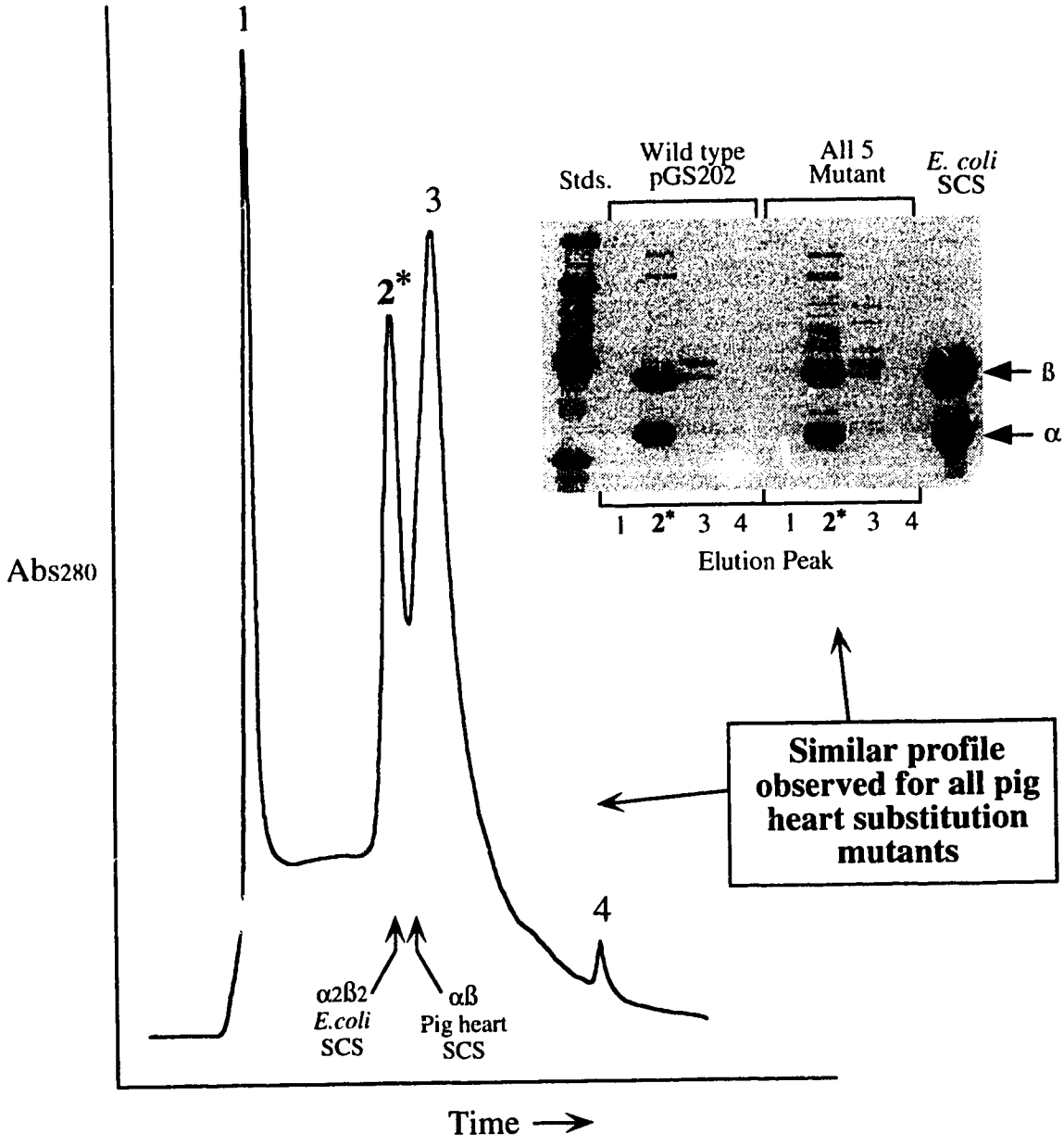


Figure 7.4 FPLC-quaternary structure analysis of *E. coli* SCS mutants substituted with nonconserved pig heart residues at the αß dimer-dimer interface. Shown is the elution profile of the All5 mutant (R14ßD/R70ßG/E74ßK/E231ßA/Q247ßK) from a Superose™12 column monitored at 280 nm. This profile was virtually identical for all the pig heart substitution mutants of *E. coli* SCS and for the wild type enzyme. Indicated at the bottom of the profile are the relative positions where tetrameric (α2ß2, *E.coli*) and dimeric (αß, pig heart) SCS eluted on this molecular sizing system. The cleared supernatants of the cellular lysate containing either the expressed wild type or mutant SCS proteins were applied to the sizing column using KPCEP buffer as a solvent phase and a flow rate of 0.2 ml/minute as described in **MATERIALS AND METHODS**. The peak fractions at 280 nm were collected and analyzed by SDS-PAGE (see inset). As deduced from a similar pattern on the SDS-PAGE, both wild type *E. coli* SCS and the All5 mutant elute at the same position (within peak **2***). As well, no difference from the wild type molecular size was detected for any of the other mutants in this class.

B. Removal of ß subunit interactions with CoA binding site of neighboring dimer

In the deduced structure [24], each ß subunit of *E. coli* SCS made contact with both an α subunit in the creation of an active site, and the ß subunit of the second αß dimer (see **Figure 1.6**, page 29). Along with these associations, the ß subunit was also seen to interact with the coenzyme A bound in the neighboring dimer (**Figure 7.5**). The contributions of four ß subunit residues (Arg-29ß, Glu-33ß, Ser-36ß and Lys-66ß) appeared to assist the α subunit of the opposing dimer in binding the adenosine end of CoA [24]. These residues (with the exception of Glu-33ß) were not conserved in the pig heart ß subunit. An attractive rationale linking the quaternary structure of the *E. coli* enzyme with catalysis was therefore provided by the role of the second ß subunit in CoA binding. The ß subunits of both dimers within the tetramer appeared to be required for the assembly of one complete active site.

To determine what extent the second ß subunit served in contributing to the nearby active site, mutations were made to remove the potential effects of residues involved in binding CoA at the neighboring dimer. Through site-directed mutagenesis, the residues R29ß, E33ß, S36ß and K66ß were changed to alanines. Arginine-29 was the only ß subunit residue of the second αß dimer which was observed to interact directly with the α subunit of the other dimer. This electrostatic interaction ($R29ß_1 \Leftrightarrow E97α_2$) was removed by changing R29ß→D, thereby creating a region of potential charge repulsion. Combinations of the alanine mutations were also made to cause greater potential disruption if the single mutations proved ineffective. As shown in **Table 7.3**, the SCS activities of these expressed mutants were not different from the wild type *E. coli* enzyme. Moreover, the tetrameric structure of such mutant enzymes, as observed by FPLC, remained intact. It appeared that the contributions of these ß subunit residues were unimportant catalytically. In sum, the ß subunit may have added little more than a small stabilizing effect to this substrate binding site. In addition, any 'cooperation' between the two active sites in this structure must, therefore, have occurred entirely by an

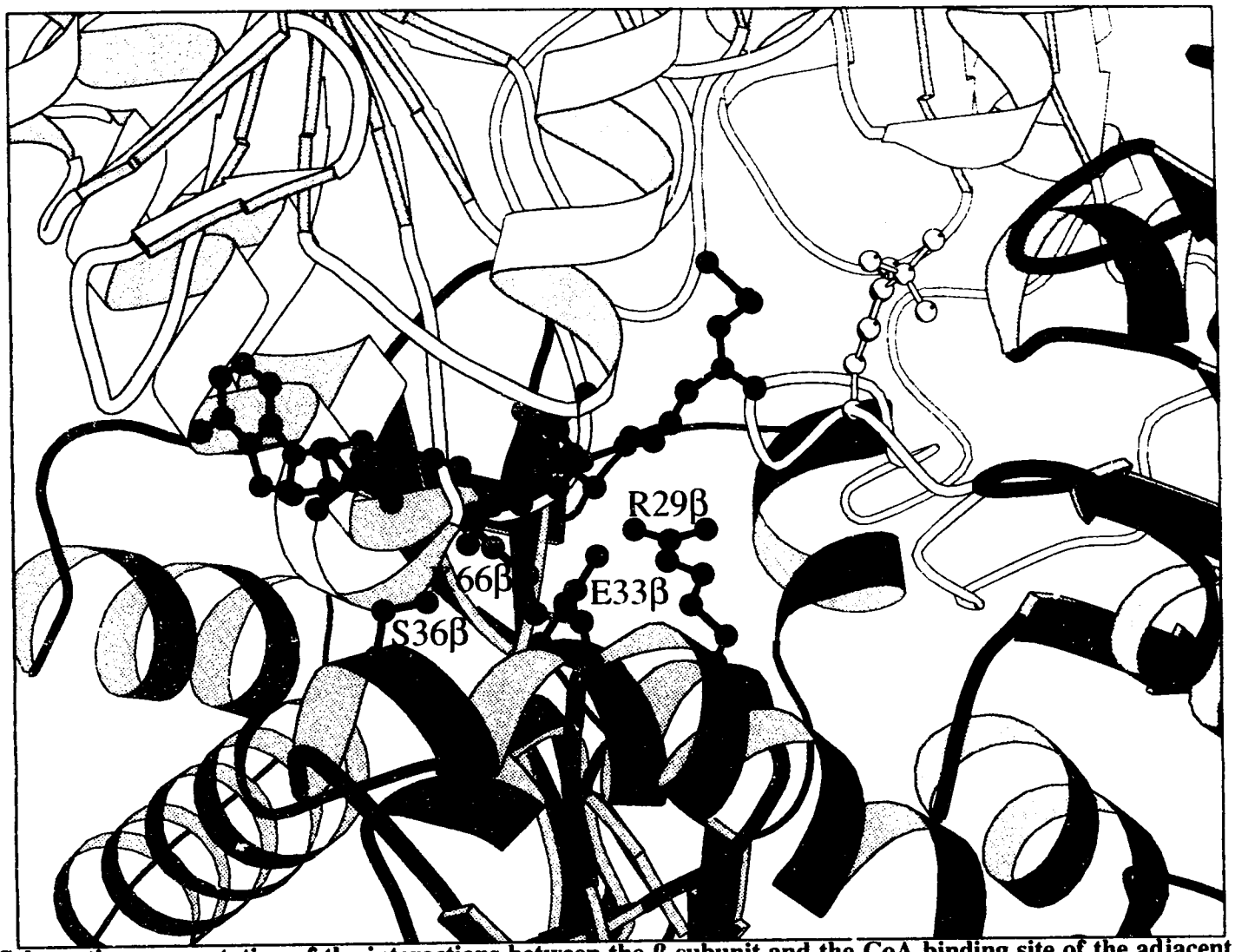


Figure 7.5 Schematic representation of the interactions between the ß subunit and the CoA binding site of the adjacent dimer in *E. coli* SCS. Shown in ball-and-stick models are the residues of the ß2 subunit (magenta) which interact with the molecule of CoA (red) bound to the α1 subunit (yellow) of the neighboring α1ß1-dimer (yellow/green). This view is the same as that shown in **Figure 1.6**. The catalytic phosphohistidine residue, His246α, is depicted as a yellow ball-and-stick model. E33ß, S36ß and K66ß form electrostatic and hydrogen bonds with CoA directly, whereas R29ß associates with E97α (not shown). This graphic was generated by M. Fraser for [42].

Table 7.3

CoA binding site mutations in the ß subunits of E. coli *SCS: SCS activity and quaternary structure*

Mutation(s)	SCS activity (units/mg cell protein)[1]	Quaternary Structure[2]
Positive control pGS202 / TK3	30.8	Tetramer
R29ßD	29.9	Tetramer
K66ßA	25.9	Tetramer
E33ßA / S36ßA	30.1	Tetramer
R29ßA / E33ßA / S36ßA	27.0	Tetramer
E33ßA / S36ßA / K66ßA	31.1	Tetramer
R29ßA / E33ßA / S36ßA / K66ßA	31.8	Tetramer

[1] Cleared extracts of TK3 cells expressing SCS proteins were prepared and assayed for enzymatic activity using ATP as a substrate. See **MATERIALS AND METHODS**.

[2] The quaternary structure of each mutant was qualified by FPLC analysis using a Superose™12 sizing column.

indirect mechanism mediated through the ß subunits which linked one αß dimer to its partner in the tetramer.

Perhaps the combination of these CoA binding site mutations with the pig heart dimer interface substitutions might augment one another, leading to the desired disruption on the quaternary structure. To this end, the above ß-CoA binding site mutants were constructed using the All5 mutant as a template. The resultant combination of mutations failed to produce any alterations in both the SCS activity and the tetrameric structure of the enzyme (**Table 7.4**). Considering the above results, the nature of the crystallographically-determined dimer interface must include other forces which are responsible for tetrameric stability.

C. Tryptophan mutagenesis at the ß subunit interface.

The preceeding work attempting to disrupt the dimer-dimer interface may have failed because van der Waals' and hydrophobic forces existing between ß subunits were ignored. Although van der Waals' forces are small when measured individually (< 1 kcal/mol), they are additive over interacting regions which exhibit steric complementarity [32]. When considering the large surface area between the αß dimers (1363 $Å^2$), these dispersion forces would constitute a substantial amount of binding energy that would contribute to the stabilization of the tetramer. In addition, the importance of hydrophobic forces in burying nonpolar surfaces has been suggested to account for nearly the entire association constants observed in many protein complexes [32]. Considering that 73% of the contact surface area between the ß subunits in the observed tetramer was hydrophobic in nature, this force may play a large role in the stabilization of the overall quaternary structure.

The changes made by substituting pig heart residues along the dimer-dimer interface served to remove local electrostatic and hydrogen bonds, but they did not address existing hydrophobic and van der Waals' forces. To do so, a series of bulky

Table 7.4

E. coli *SCS mutants combining the pig heart substitutions at the dimer interface with the CoA binding site mutations in the ß subunit: SCS activity and quaternary structure*

Combination mutant[1]	Overall number of reciprocal (symmetrical) mutations[2]	SCS activity (units/mg cell protein)[3]	Quaternary structure[4]
Positive control pGS202 / TK3	-	28.0	Tetramer
R29ßD + All5	(1+5) x 2 = 12	26.3	Tetramer
R29ßA + All5	12	25.9	Tetramer
K66ßA + All5	12	27.1	Tetramer
K66ßE + All5	12	27.4	Tetramer
E33ßA + All5	12	25.8	Tetramer
E33ßA / S36ßA + All5	(2+5) x 2 = 14	25.7	Tetramer
R29ßA / E33ßA / S36ßA + All5	(3 + 5) x 2 = 16	27.2	Tetramer
R29ßA / E33ßA / K66ßA + All5	16	23.7	Tetramer
R29ßA / E33ßA / S36ßA / K66ßA + All5	(4 + 5) x 2 = 18	25.2	Tetramer

[1] Mutants were designed to combine CoA binding site mutation(s) in the ß subunit with the All5 mutant containing the the five pig heart substitutions at the dimer-dimer interface (R14ßD, R70ßG, E74ßK, E231ßA and Q247ßK).

[2] The twofold symmetrical interactions of the αß dimers in tetrameric *E. coli* SCS implies a doubling effect of the introduced mutations: single site mutations would result in two overall changes.

[3] Cleared extracts of TK3 cells expressing SCS proteins were assayed for enzymatic activity using ATP as a substrate. See **MATERIALS AND METHODS**.

[4] FPLC analysis using a Superose™12 sizing column was employed to characterize the quaternary structure of each mutant. See **MATERIALS AND METHODS**.

tryptophan residues were introduced at regions of close contact between the ß subunits. It was hoped that the steric interference created would produce a physical separation of the associating subunit faces. This would increase the contact distances, thereby decreasing the van der Waals' forces between dimers. The interruption of the packing of the αß dimers against one another would enable water molecules to penetrate, thereby decreasing the buried surface area [107]. As well, the substitutions of tryptophan at intimate contact sites would eliminate the salt bridges and hydrogen bonds at these positions. This disruptive approach has been successful in other systems: the introduction of bulky residues has been shown to have sterically disrupted the subunit interfaces in both glutathione reductase [33] and malate dehydrogenase [34].

Two regions were considered in designing such tryptophan-interfering mutants. The first site occurred between the C-domain of first ß subunit and the N-domain of the second ß subunit where intersubunit contacts were made between R70ß$_1$ ⇔ E249ß$_2$, E74ß$_1$ ⇔ R297ß$_2$, E27ß$_1$ ⇔ ß-carbonQ247ß$_2$ and E94ß$_1$ ⇔ Q247ß$_2$. Substitution of two Trp residues at Q247ß→W and E249ß→W (see **Figure 7.6**), would result in a WWW tripeptide in this region since a Trp residue exists at position 248 in the ß subunit. The second site chosen to maximize the disruptive effect of introducing Trp residues was near the center of the tetramer (and ß subunits) where E231ß$_1$ interacts with both Y6ß$_2$ and Q7ß$_2$. The disruption effect of mutating E231ß→W should have been additive since the substituted indole side chain would have been in a position opposite that of the counterpart substituted Trp in the second ß subunit (see **Figure 7.6**).

These mutations (E231ßW and Q247ßW/E249ßW) were created as single mutations or in tandem. They were also combined with the ß-CoA binding site mutations and the remnant of the pig heart substitutions (R14ßD, R70ßG and E74ßK) to ensure that all evident interactions stabilizing the observed tetramer were removed or perturbed. The results of activity measurements and quaternary structure analyses for this series of mutants is presented in **Table 7.5**. These Trp mutations did appear to be disruptive since

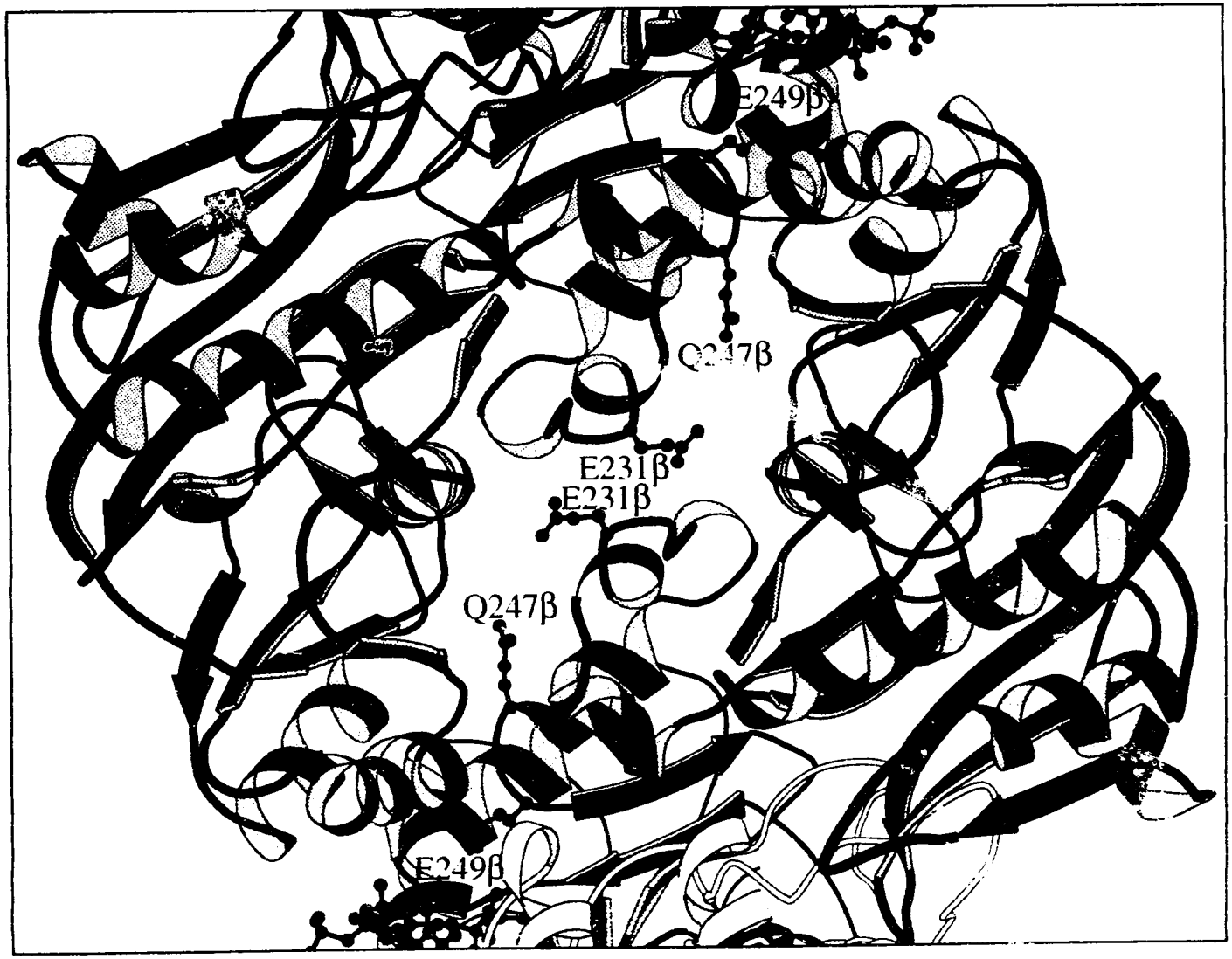


Figure 7.6 Schematic representation of the dimer interface sites targeted for tryptophan-disruption mutagenesis. Shown are the ß1 (green) - ß2 (magenta) subunit associations which mediate tetramerization in *E. coli* SCS. The ß subunit residues E231ß, Q247ß and E249ß were chosen as sites for the mutagenic relacement with tryprotphan residues. This graphic was generated by M. Fraser for [42].

Table 7.5

Tryptophan disruption mutants at the αß dimer-dimer interface of E. coli SCS: alone and in combination with pig heart substitutions or ß-CoA binding site mutations

Mutant[1]	SCS activity (units/mg cell protein)[2]		Percentage of wild type activity[3]	Quaternary structure[4]
	TK3 lysate extract	FPLC purified protein		
Positive control pGS202	34.2	41.1	**100%**	Tetramer
Trp mutants alone				
E231ßW	15.1	16.6	**42%**	Tetramer
Q247ßW / E249ßW	35.1	42.2	**98%**	Tetramer
E231ßW / Q247ßW / E249ßW	8.5	9.75	**24%**	Tetramer
Trp mutants in combination with pig heart substitutions				
E231ßW + Phsub	29.7	40.8	**89%**	Tetramer
Q247ßW / E249ßW + Phsub	20.0	26.2	**61%**	Tetramer
E231ßW / Q247ßW / E249ßW + Phsub	7.7	n/a	**22%**	Tetramer
Trp mutants in combination with ß-CoA binding site mutations				
E231ßW + CoA	26.0	37.7	**81%**	Tetramer
Q247ßW / E249ßW + CoA	22.6	18.5	**60%**	Tetramer
E231ßW / Q247ßW / E249ßW + CoA	12.0	9.4	**29%**	Tetramer

[1] Tryptophan disruption mutants were created as single mutations or in combination with the remainder of the pig heart substitutions (Phsub = R14ßD, R70ßG and E74ßK) or with the ß-CoA binding site mutations (CoA = R29ßA, E33ßA, S36ßA and K66ßA).

[2] SCS activity of mutant enzymes was assayed twice from either TK3 expression lysate extracts or FPLC fractions of mutant proteins purified from a Superose™12 sizing column. See **MATERIALS AND METHODS.**

[3] Percentages indicated refer to an average of mutant activity relative to wild type (pGS202).

[4] Overall quaternary structure of each mutant was estimated by FPLC analysis using a Superose™12 sizing column.

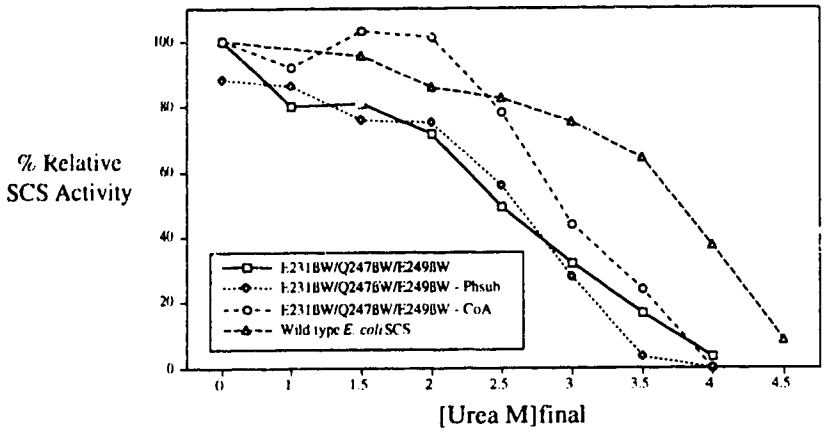
the SCS activities of some mutants were decreased to 20% that of wild type levels. Although E231ßW and Q247ßW/E249ßW seemed to be additive in perturbing the catalytic function of the enzyme, these structural changes did not interfere with the tetrameric subunit structure as demonstrated by FPLC analysis. Therefore, these disruption mutants were effective only in preventing the enzyme from attaining a conformation that was necessary for full catalytic competency.

It was possible that the structure of these Trp mutants had been somewhat destabilized or conformationally 'loosened', but that these changes were insufficient to produce a dimeric species of the *E. coli* enzyme. To determine if this was the case, stability tests were performed on the mutant proteins to discern whether they behaved differently from the wild type enzyme. Such effects had been observed in dimeric triosephosphate isomerase which was mutated at a single residue (Asn-78→Asp): an increased susceptibility to urea denaturation was coupled with a equilibrium shift from dimer to monomer for this mutant [35]. The substitution of a single tyrptophan residue at the dimeric interface of glutathione reductase caused thermoinstability as well as the development of cooperativity in this enzyme [33]. Nonetheless, in the present study, the Trp disruption mutants of *E. coli* SCS were essentially wild type in nature with respect to urea denaturation, pH and ionic strength tests of stability (see **Figure 7.7**).

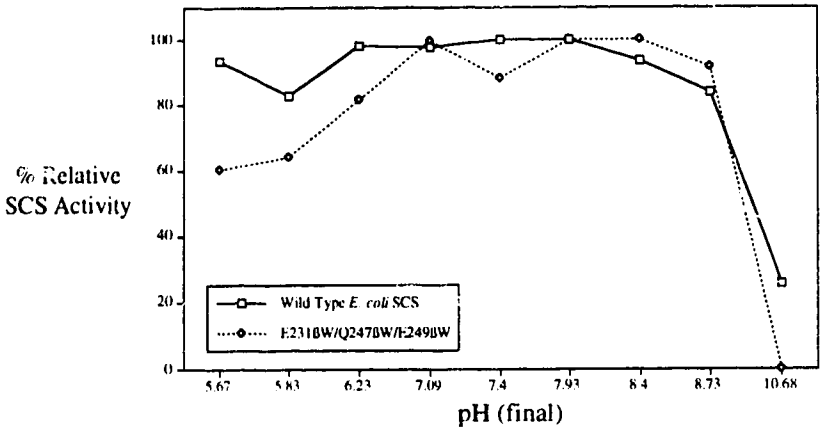
D. Interpretation of the ineffective mutagenesis to disrupt the *E. coli* SCS tetramer

Disruption mutants were created which were believed to remove interactions linking the two dimers in the observed tetrameric structure of *E. coli* SCS. These included: electrostatic and hydrogen bonds at the ß-ß subunit interface; interactions of the ß subunit to CoA bound in the neighboring dimer; and, hydrophobic and van der Waals' forces between the two dimers. However, none of the mutants created alone, or in combination, had any effect on the quaternary structure of the enzyme. This was surprising for several reasons. Since ß subunits associate with reciprocal twofold symmetry, two binding sets of interactions existed between them. Therefore, all

A. Urea Denaturation: Wild type *E. coli* SCS vs. Trp disruption mutants



B. pH Stability: Wild Type *E. coli* SCS vs. Trp disruption mutant



C. Ionic Strength: Wild type *E. coli* SCS vs. Trp disruption mutant

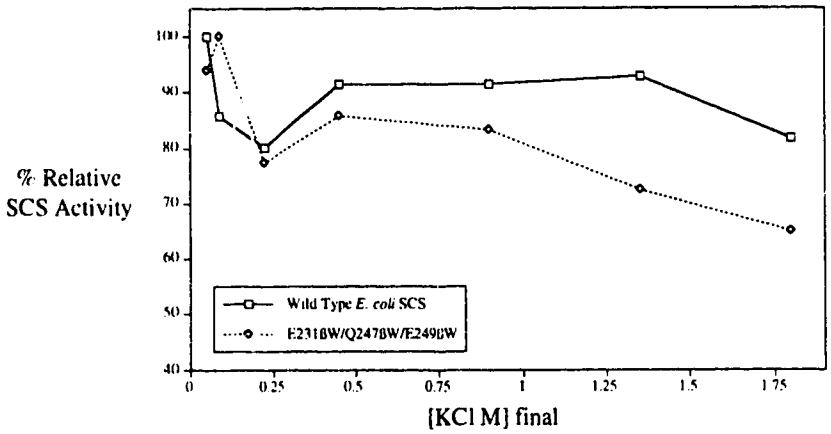


Figure 7.7 Stability of *E. coli* SCS tryptophan disruption mutants with respect to urea denaturation, pH, and ionic strength. Tryptophan disruption mutants, purified by FPLC, were assayed for SCS activity following treatment with: **A**, varying concentrations of urea; **B**, differing conditions of pH; and **C**, altered ionic strength (see **MATERIALS AND METHODS**). SCS activities are plotted as percentages relative to that observed at 0 M urea, pH 7.4 and 0 M KCl.

mutations which were introduced were multiplied twofold. For example, in the case of the mutant where all 5 of the pig heart substitutions were combined with all 4 of the CoA binding site mutations, 9 x 2 = 18 ion pairs and hydrogen bonds were actually removed. To retain a tetrameric structure, 12 unneutralized charged residues would have been at the dimer interface. Energetically this would have been highly unfavourable since estimates of single charges buried in hydrophobic environments of proteins are on the order of 10 kcal/mol [10]. Furthermore, the ineffectiveness of these combined mutations was inconsistent with a number of other reports indicating that the disruption subunit interfaces in multimeric proteins were achieved by a small number (usually 1) of mutations [33, 34, 36-40].

The ineffectiveness of the mutations, in light of the above considerations, suggested that the assignment of αß dimers constituting the tetramer in the unit cell, crystallographically, was made incorrectly. This would imply that the interactions that were thought to hold the tetramer together were inappropriate, and justified the unproductive nature of the mutations that were examined to disrupt this structure. In addition, refinement of the crystal structure has indicated that several of the interactions between ß subunits are not as significant as originally believed [41]. To sum, these results suggested that a reevaluation of the quaternary arrangement of *E. coli* SCS was in order. The following chapter addresses this matter and deals with the mutagenic attempts at tetramer disruption based on an alternative quaternary structure.

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Chapter 8

SUCCESSFUL DISRUPTION OF THE *E. coli* SCS TETRAMER: MUTAGENESIS BASED UPON AN ALTERNATIVE QUATERNARY STRUCTURE

I. INTRODUCTION

Using the published crystallographic structure of *E. coli* SCS [1] as a guide, thorough site-directed mutagenesis directed to the dimer interface was unsuccessful in disrupting the tetrameric structure (**Chapter 7**). The removal of electrostatic and hydrogen bonding interactions by replacement with non-conserved residues from a dimeric SCS species (pig heart), failed to perturb the quaternary structure. Likewise, substitution of bulky Trp residues at similar sites appeared not to affect the hydrophobic and van der Waals' forces linking the two dimers. These results were unexpected considering that the entropy-driven association of subunits in multimeric proteins has been found to be quite susceptible to minimal interference at associating interfaces [2-8]. Furthermore, the interactions the ß subunit makes to the active site of the neighboring dimer were found to possess little functional or structural significance.

Due to the ineffectiveness of these mutagenic studies, conclusions regarding a rationale for the tetrameric structure could not be made. However, these studies were crucial in providing an indication that the assignment of αß dimers in the quaternary structure of *E. coli* SCS was incorrect.

A discussion of the molecular packing of *E. coli* SCS tetramers within the crystal used to solve the structure is relevant to this investigation. Within the unit cell of the SCS crystals defined by a $P4_322$ space group, the smallest repeating asymmetric unit was a tetramer [9]. However, the repeating unit within the unit cell was actually an 'octamer' (see Panel I, **Figure 8.1**). This octamer was composed of two tetramers related by two non-crystallographic twofold axes (labeled *a* and *b*) that were 90° from a crystallographic twofold axis (labeled *c*). The nature of this latter axis (which coincides with a structural twofold axis) dictates that the αß dimers which were related by it (colored yellow/green or blue/magenta for the two respective unique αß dimers in **Figure 8.1**) were necessarily identical in the structure. Two unique sets of αß dimer-dimer contacts existed in this crystal form of *E. coli* SCS, thus providing two possibilities for the designation of the

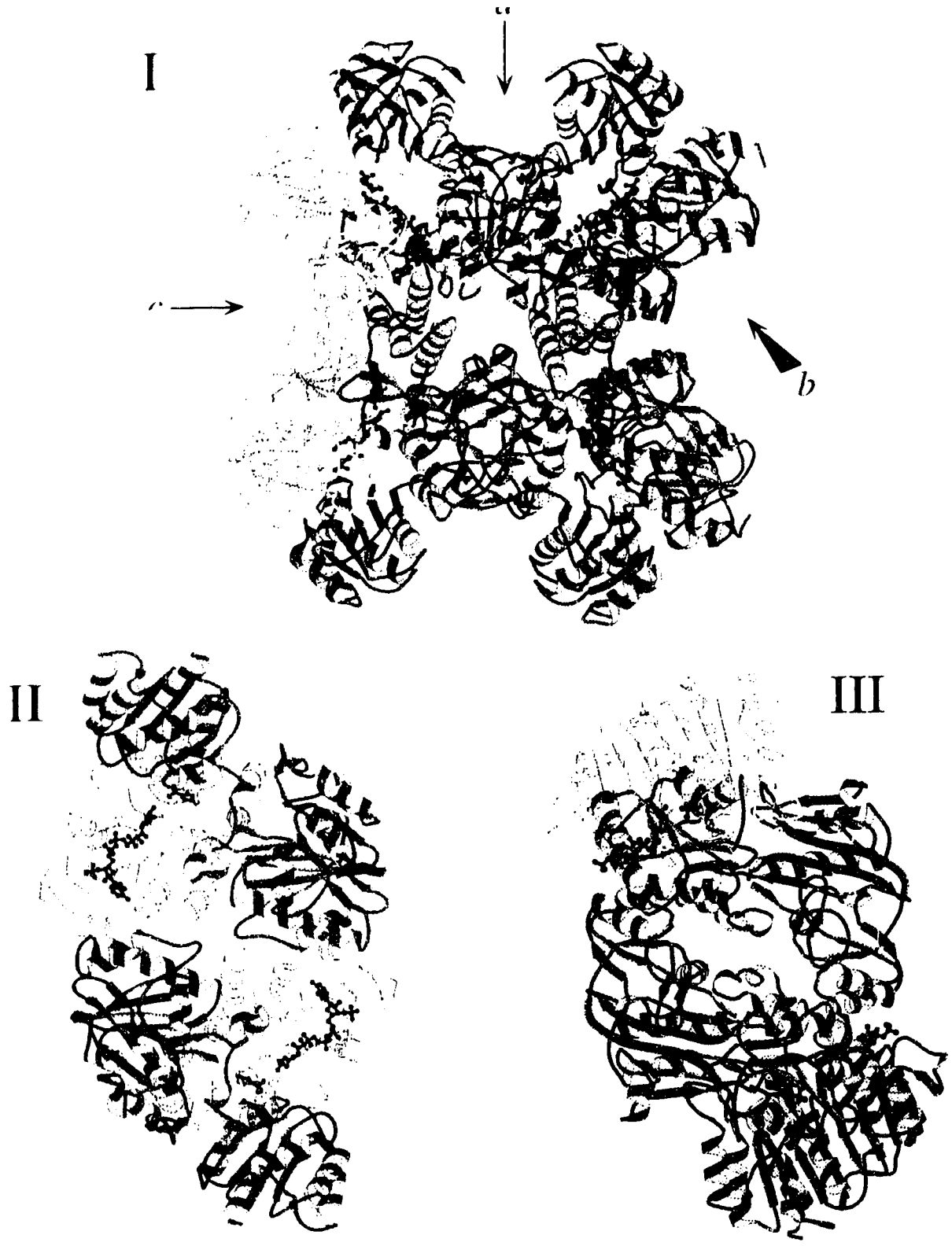


Figure 8.1 The octamer observed in the crystal packing of *E. coli* SCS, and the two possible structures for the tetramer. The octamer, composed of two *E. coli* SCS tetramers, is schematically presented in panel **I** and is the repeating feature within the unit cell. The α subunits are colored in yellow and blue, while the ß subunits are depicted in green and magenta. The two non-crystallographic axes of symmetry, labeled *a* (vertical in the page) and *b* (perpendicular to the page) relate αß dimers colored yellow/green to those colored magenta/blue. The crystallographic axis, labeled *c*, relates dimers colored yellow/green or magenta/blue to identical αß dimers of the same color. Two possibilities exist for the structure of the tetramer as viewed along the *c* and *a* axes, and these are presented in panels **II** and **III**, respectively. The tetramer shown in panel **III** was chosen for publication by Wolodko *et al.* [1]. This graphic was generated by M. Fraser for [36].

tetrameric structure. The first of these related 'unique' dimers across the non-crystallographic twofold axis (*a*) in the octamer (Panel I, **Figure 8.1**) resulting in the tetrameric arrangement chosen for publication (Panel III, **Figure 8.1**; comprised of distinct yellow/green and blue/magenta αß dimers). The alternative choice for the tetramer was formed from interacting dimers which were related by the crystallographic twofold axis (*c*), consisting of either 2 yellow/green αß dimers or 2 blue/magenta αß dimers (Panel II, **Figure 8.1**). By this symmetry related operation, these crystallographically defined tetramers were comprised of identical dimers. In the absence of other discriminating sources, two equally viable choices therefore existed for the tetrameric asymmetric unit.

The quaternary structure chosen for the tetramer was assigned by Wolodko *et al.* [1] to relate unique dimers across the non-crystallographic twofold axis (*a*) (Panel III, **Figure 8.1**, and **Figure 1.5**, page 28). Their selection was based on the following criteria. Several lines of evidence suggested that *E. coli* SCS operated by a mechanism of alternating sites cooperativity [10-17]. This concept provided a rationale for the tetrameric structure of the enzyme. Inherent in this catalytic model was the necessity for asymmetry between the two halves of the enzyme which reciprocally changed in conformation as a substrate interacted with one active site and promoted catalytic events at the other site [14]. In order to observe potential differences between the two halves of the enzyme, αß dimers displaying some asymmetry were chosen to constitute the tetramer. Thus, αß dimers related in the octamer of the crystal by a non-crystallographic twofold axis were selected instead of identical αß dimers related by a crystallographic twofold axis. The resulting compact, globular protein supported physico-chemical studies which predicted the overall shape and size of the enzyme [18]. The associations between the αß dimers in this model were mediated by ß-ß subunit interactions. Considering this, the observation that the ß subunits tended to aggregate under nondenaturing conditions [15], was also consistent with the ß subunit contacts in this

quaternary structure arrangement. The fact that interactions of the ß subunit were made to the neighboring CoA binding site only strengthened this assignment (see **Figure 7.5**, page 152). These interactions appeared to provide an additional rationale for the tetrameric structure in the function of the enzyme; the formation of the active site required different regions of each of the two ß subunits.

While the above evidence was supportive, two sources of investigation question the assignments made for the published quaternary structure of *E. coli* SCS. First, systematic mutagenesis based on this structure and designed to modify the interface of αß dimers and the CoA binding site contributions of the ß subunit was ineffective in disrupting the tetrameric structure (**Chapter 7**). Second, refinement of the crystal structure of *E. coli* SCS to a higher resolution of 2.3 Å [19] indicated that: 1) the contact surface area between αß dimers in the alternative quaternary arrangement was determined to be larger than that found between the two ß subunits mediating αß dimer interactions in the published structure; and, 2) several close contact distances between residues from the two interacting αß dimers in the published tetramer were observed to be increased. This evidence suggests that the alternative structure is the correct structural assignment of dimers which constitute the tetramer (see **Figures 8.2, 8.3**). In support of this proposal, cross-linking studies on *E. coli* SCS produced protein species which were consistent with the configuration of subunits observed in the alternative structure [20]. Here, α_2ß trimers, which were possible with the alternative structure, were detected; cross-linked $ß_2$ dimers predicted for the published structure were noticeably absent.

One method of testing this possibility, lies in analyzing the effect of disruption mutations directed to the dimer interface of the alternative quaternary structure. This chapter focuses on such investigations in order: 1) to determine if the proposed alternative quaternary structure of *E. coli* SCS is the correct one; and, 2) to help deduce the rationale for the tetrameric structure.

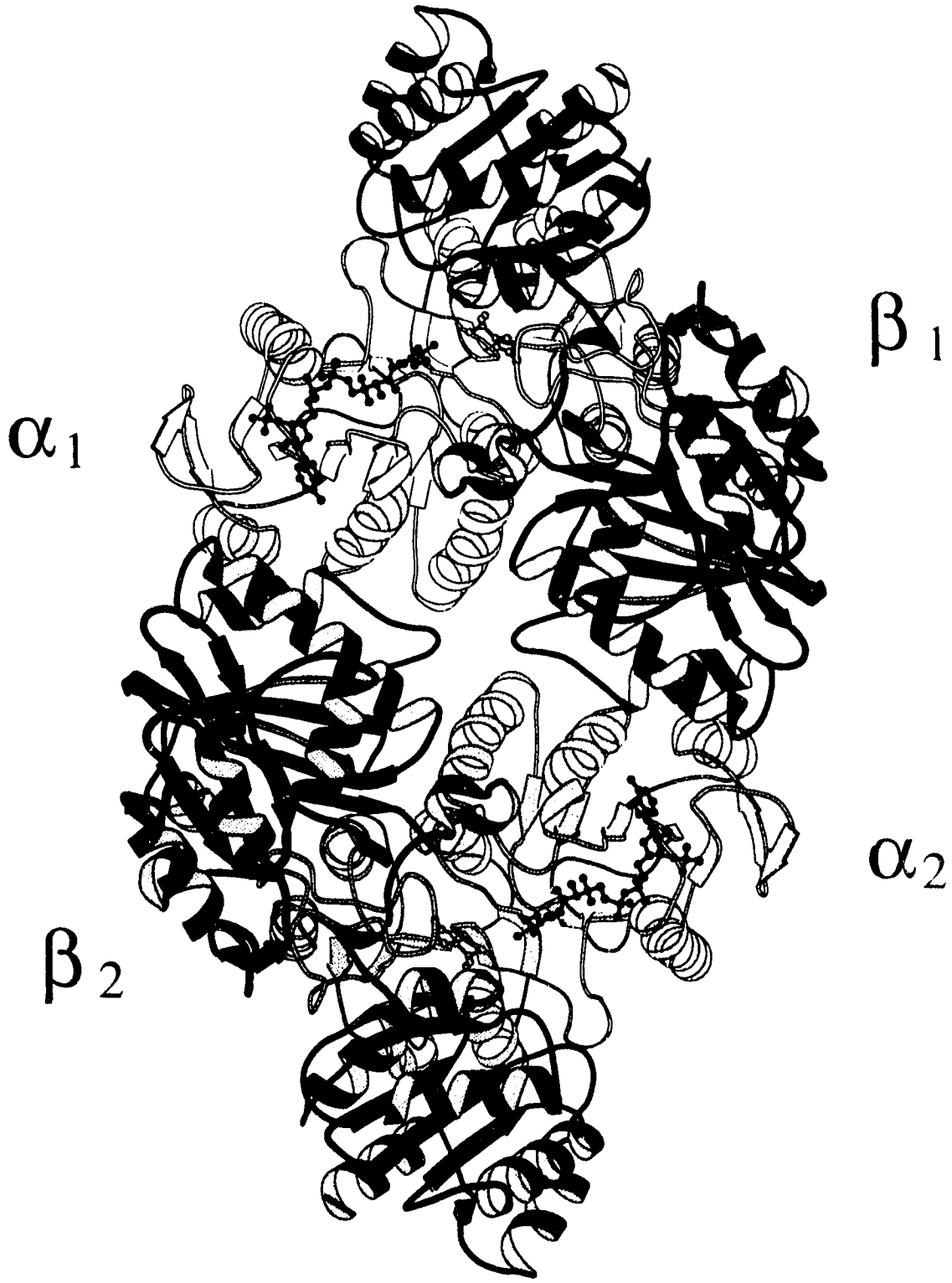


Figure 8.2 Schematic representation of the 'alternative' tetrameric structure of *E. coli* SCS (A). This view is the same as that shown in panel II of **Figure 8.1**. The coloring of the second dimer ($\alpha_2\beta_2$) has been changed for clarity: α_2 is changed from yellow to gold and β_2 is changed from green to aqua. The crystallographic twofold axis of symmetry relating the $\alpha\beta$ dimers is perpendicular to the page. Inter-dimer associations occur through the interactions of a single loop and helix in the β subunit with three α-helices of the α subunit in the neighboring dimer. Stick-and-ball models of CoA (colored red) and the phosphohistidine residues (in their respective α subunit colors) are also indicated. This graphic was generated by M. Fraser for [36].

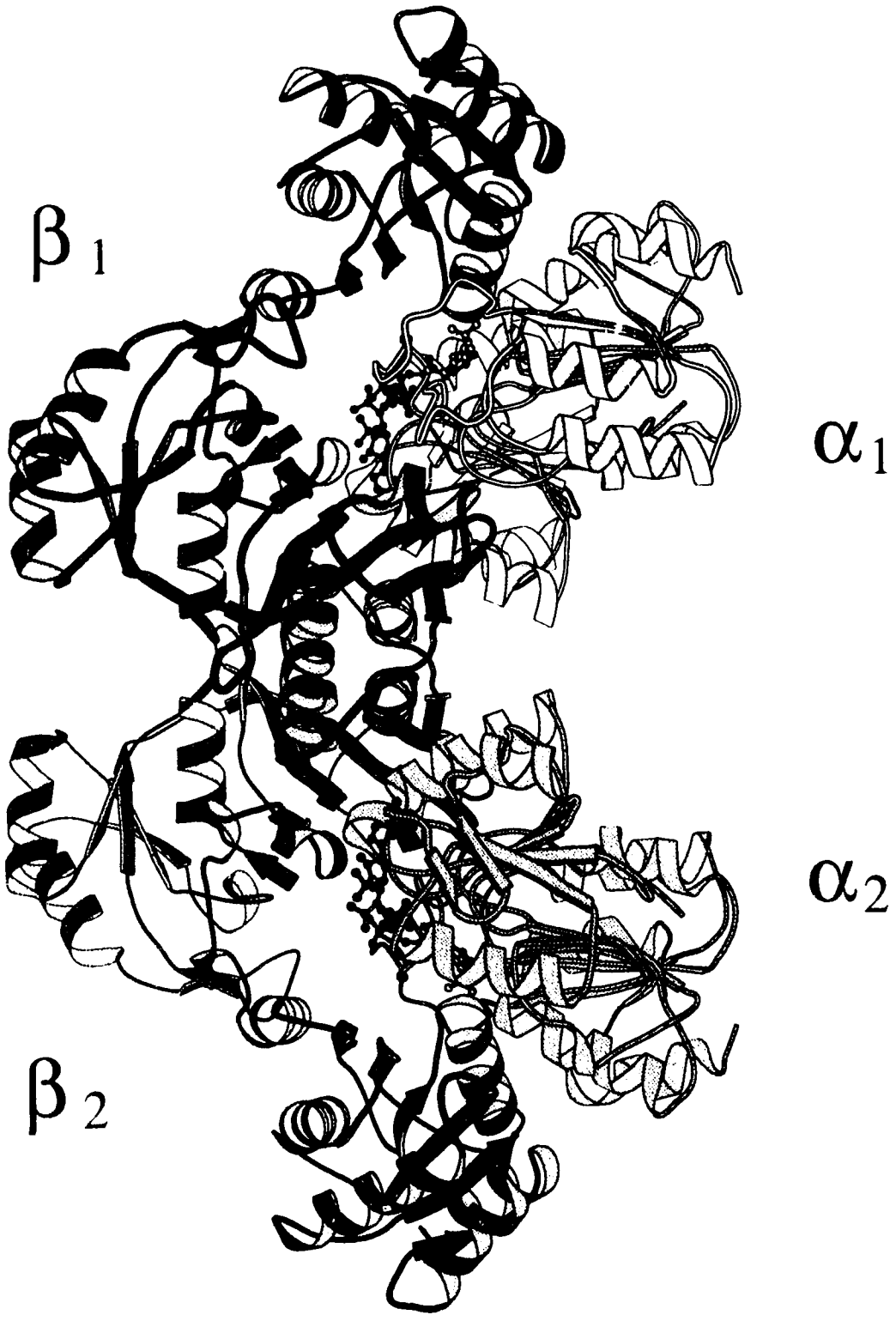


Figure 8.3 Schematic representation of the 'alternative' tetrameric structure of *E. coli* SCS (B). This view of the alternative structure proposed for the *E. coli* SCS tetramer is related to that shown in **Figure 8.2** by a rotation of 90° to the left, along a vertical axis in the page. The extended, open configuration of the alternative quaternary structure is emphasized from this viewpoint. This graphic was generated by M. Fraser for [36].

II. MATERIALS AND METHODS

A. Bacterial strains, plasmids and vectors

The bacterial strains, plasmids and vectors used in these studies were the same as those described in **Chapter 7**.

B. Construction and mutagenesis of vectors and expression plasmids

The construction and mutagenesis of vectors and expression plasmids were essentially the same as those described in **Chapter 7**.

For the construction of the Ph/ER *E. coli* SCS mutant (see **Results** for details), a mutated NdeI-BstXI DNA fragment of M13mp18.202 mutagenized with the DB22 oligonucleotide (see **Appendix I** for sequence), was used to replace a similar non-mutated segment of pGS202. A mutated NdeI-ClaI fragment of M13mp18.202 mutagenized with the DB23 primer (see **Appendix I**), was shuffled with the same non-mutated sequence in pGS202 to create the DDER mutant.

C. Bacterial expression of mutant *E. coli* SCS

The bacterial expression of mutant *E. coli* SCS proteins was identical to that described in **Chaper 7**.

D. Analysis of mutant quaternary structure and function

The analysis of quaternary structure and function of the *E. coli* SCS mutants was the same as that described in **Chaper 7**.

E. Enzyme isolation

The *E. coli* SCS was purified from JM103 cells transformed by the overproducing expression plasmid pGS202 [21], according to methods previously described [1]. Pig heart SCS was expressed and isolated from *E. coli* BL21 cells harboring the plasmid pET-3b/Phß/α by methods described in **Chapter 6**. Succinyl-CoA synthetase activity

was assayed by the direct spectrophotometric method [22]. ATP was used as a substrate with *E. coli* SCS, and GTP was used with pig heart SCS. Preparations of both SCS enzymes used in 'Blue Native PAGE', Western analysis and ultracentrifugation had specific activities of 30 and 40 units/mg for the pig heart and *E. coli* enzymes respectively. The purity of these enzymes was assessed by SDS-PAGE.

F. 'Blue Native PAGE' and Western analysis

A modification of the method of 'Blue Native polyacrylamide gel electrophoresis', originally developed by Schägger and Jagow [23], was employed to characterize the native quaternary structure of the wild type and mutated *E. coli* SCS proteins. Discontinuous gradient polyacrylamide gels (7 - 16.5% (w/v)) were cast using an acrylamide/bis-acrylamide (A-B) mixture of 49.5% total concentration of both monomers (acrylamide and bis-acrylamide) in grams/100ml with 3% bis-acrylamide/acrylamide (w/w). The separating gel solutions were buffered with 0.5 M 6-aminocaproic acid and 50 mM BisTris (HCl) pH 7.0 and cross-linking was catalyzed with 0.05% (w/v) ammonium persulfate and TEMED. The stacking gel was formed using 4% (w/v) A-B mixture and similar buffer and cross-linking components. Cathode buffer consisted of 50 mM tricine, 15 mM BisTris (pH 7.0) and 0.02% (w/v) Serva Blue G, whereas anode buffer was composed of 50 mM BisTris (pH 7.0). Running buffers and polymerized gels were cooled at 4 °C prior to electrophoresis. Protein samples (either purified proteins or FPLC peak fractions) were phosphorylated with 1 mM ATP before the addition of 0.25% (w/v) Serva Blue G in 0.5 M aminocaproic acid and 75% (v/v) glycerol. These chilled samples were electrophoresed into the stacking gel at 50 V (<10 mA) and when the proteins had entered the separating gel, the voltage was increased to 200 V for the duration of electrophoresis. At a point where proteins had migrated one third of the distance through the separating gel, the cathode buffer was removed and replaced with a similar buffer which excluded the Serva Blue G dye. This was done to remove excess dye from the gel.

At the completion of native gel electrophoresis, proteins were either fixed in gels with 45% (v/v) methanol and 10% (v/v) acetic acid followed by staining with Coomassie Brilliant Blue G-250, or, transferred onto Immobilon-P (Millipore) membranes. In the latter process, gels were first equilibrated with transfer buffer (cathode buffer with no Serva Blue G). Immobilon membranes were wetted with methanol and soaked in transfer buffer before assembling the transfer sandwhich. Here, six Whatman 3mm membranes, soaked in buffer, were placed on either side of the equilibrated gel and the membrane. A semi-dry transblot apparatus (Tyler Research Corp.) was used and protein transfer onto the membrane was allowed to occur for 1 hour at 350 mA. Following the transfer, steps including blocking of the membrane, binding of primary antibodies (polyclonal, SCS specific) and secondary antibodies (goat anti-rabbit IgG-alkaline phosphatase conjugates) and all washing steps were performed according to those detailed in the section, 'Screening with antibodies', found in **Materials and General Methods, Chapter 2**. After this point, blots were then equilibrated in alkaline phosphatase buffer (0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl and 50 mM $MgCl_2$) for 5 minutes prior to the addition of the chemiluminescent substrate, Lumi-phos 530 (Boehringer Mannheim). Blots sealed in bags were incubated at 37 °C for 15 minutes before exposure to x-ray film for 30 minutes and development of the subsequent fluorographs.

G. Sedimentation velocity analyses

Sedimentation coefficients were determined for pig heart SCS, wild type and mutant *E. coli* SCS following experiments described by Wolodko *et al.* [18]. Ultracentrifugation of the protein samples at concentrations of 1.1 or 2 mg/ml was carried out using a Beckman Spinco Model E analytical centrifuge equipped with RTIC, electronic speed control units, and a schlieren optical system. A titanium AnH rotor was used with wedge window cells in order to superimpose two scanner patterns. Sedimentation velocity determinations were conducted at 60 x 10^3 rpm at 20 °C. The

buffer used in these analyses for all the proteins was composed of 50 mM KH_2PO_4, 50 mM KCl and 1 mM EDTA (pH 7.4). Sedimentation coefficients were determined from the midpoint measurements of boundary positions. The partial specific volume used for the sedimentation coefficient calculation of the *E. coli* SCS mutant was 0.759 cm^3/g, which corresponded to the wild type enzyme [18].

H. Purification scheme for the DDER mutant of *E. coli* SCS

The DDER mutant form of *E. coli* SCS was expressed and purified from TK3(D18) cells harboring the mutated expression plasmid, pGS202-DDER. A 6 liter culture of DLB (see **Appendix III**) supplemented with 100 µg/ml ampicillin, 50 mM L-glutamic acid and 0.4 µg/ml thiamine was inoculated with 400 ml of a culture of the above cell line and was grown at 30 °C until the OD_{600} reached 0.5. Plasmid-directed protein expression was then induced by shifting the temperature to 42 °C and continuing the growth for an additional 5 hours to a final OD_{600} of 1.0. The cells were harvested by centrifugation at 10 x 10^3 rpm and then frozen overnight at -20 °C. The thawed cell pellet was then resuspended in sonication buffer consisting of 75 mM KH_2PO_4, 0.2 mM EDTA, 10 mM ß-mercaptoethanol, 0.2 mM PMSF, 1 mM benzamidine and 1 µg/ml leupeptin (pH 7.4). After sonication (2 minutes), the resulting lysate was centrifuged 40 minutes at 10 x 10^3 rpm at 4 °C. The supernatant extract was recovered and treated with a 1/10 volume of a 5% (w/v) solution of protamine sulfate by stirring at 4 °C for 30 minutes. After an additional centrifugation step at 15 x 10^3 rpm, the mutant *E. coli* SCS enzyme in the supernatant was phosphorylated for 30 minutes at 4 °C by the addition of 1 mM ATP and 10 mM $MgCl_2$. This phosphorylation mixture was then applied to a 5.0 x 15 cm column of DEAE-Sephacel previously equilibrated with sonication buffer. The column was washed with 2 column volumes of this buffer prior to eluting the SCS proteins with 250 mM KCl in equilibration buffer. The peak fractions containing SCS activity were pooled and for each 100 ml volume 50 g of ammonium sulfate was then

added, gently mixed and left overnight at 4 °C. The precipiated proteins were collected by centifugation at 15 x 10^3 rpm for 30 minutes and solubilized in 50 mM KH_2PO_4, 0.2 mM EDTA, 10 mM ß-mercaptoethanol, 0.2 mM PMSF and 1 mM benzamidine (pH 7.4). This solution was then loaded onto a 2.5 x 120 cm column of SS300 HR and fractionated by gel filtration using the same buffer. The peak fractions eluting from this column displaying SCS activity were collected and pooled.

I. Thio- and de-thiophosphorylation experiments on native and dimeric *E. coli* SCS

1. *Thio[illegible]phorylation of wild type and dimeric* E. coli *SCS* - Both purified proteins stored [illegible]monium sulfate precipitates were collected by centrifugation at 20 x 10^3 g for 30 minutes at 4 °C and dissolved in KPCE buffer (50 mM KH_2PO_4, 50 mM KCl and 1 mM EDTA (pH 7.4)). These resuspended proteins were dialyzed overnight at 4 °C against this buffer in order to remove traces of ammonium sulfate. The specific enzymatic activities were 34 and 45 units/mg for the native tetramer and the DDER dimeric mutant, respectively, at this time. The enzymes were then dephosphorylated for 15 minutes at room temperature (22 °C) in a reaction consisting of 50 mM sodium succinate, 0.5 mM CoA, 5 mM $MgCl_2$ and 5 mM DTT (pH 7.4) [24]. The dephosphorylated proteins were dialyzed overnight against KPCE buffer at 4 °C and the resultant activities of the dialysates were now: 47 units/mg - wild type *E. coli* SCS and 50 units/mg - DDER dimer. Thiophosphorylation of each enz[illegible]e was accomplished by employing a modified protocol used by Wolodko *et al.* [12]. A 200 µl incubation mixtures consisted of 0.5 mM ATPγS (adenosine 5'-*O*-(3-thio)-triphosphate; containing [^{35}S]ATPγS of specific radioactivity of 600 µCi/mmol or greater), 10 mM $MgCl_2$, 50 mM Tris-HCl and 10-15 mM KH_2PO_4, pH 7.4, and 200 ng of SCS (final concentation = 1 mg/ml). Thiophosphorylation was allowed to occur for greater than 2 hours before the nucleotide and small molecules were separated from the protein by gel filtration on a 2.5 x 30 cm column of G-50 Sephadex using KPCE buffer as the solvent. The eluted fractions containing protein were monitiored at A_{280}, and the radioactivity associated

with these fractions was counted by scintillation. Fractions containing radioactively labeled protein were pooled and confirmed to be free of nucleotide by measurement of the the ratio of absorbance at 280 nm to that of 260 nm.

2. ***Discharge of thiophosphate*** - Thiophosphorylated SCS enzymes used subsequently for observing the rates of de-thiophosphorylation were prepared either in the presence or absence of phosphate. In the case of the former preparation, KPCE buffer, which contains 50 mM KH_2PO_4, was used as the buffer. In the latter preparation, the thiophosphorylated SCS was dialyzed against a buffer consisting of 50 mM Tris-HCl, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT and 0.1 mM EDTA (pH 7.4) to render the phosphate concentration less that 50 µM. Reactions following the discharge of phosphate consisted of a 650 µl mixture of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM DTT and where applicable, 10 mM sodium succinate, 0.5 mM CoA, 1.0 mM ATP and 25-44 mM (final concentration) KH_2PO_4. The reaction was initiated by the addition of [^{35}S]-thiophosphorylated SCS (16-33 mg/ml final concentration) and stopped at timed intervals by transferring 100 µl aliquots into 1 ml of phenol (buffered with 10 mM Na_2HPO_4, pH 8.0) with 10 mM EDTA. Following the method of Boyer and Bieber [25], [^{35}S]-thiophosphate was separated from [^{35}S]-thiophosphorylated SCS by repeated extraction from the phenol layer into an aqueous buffer consisting of 10 mM Na_2HPO_4, 10 mM EDTA and 10 mM $Na_4P_2O_7$ (pH 8.0) saturated with buffered phenol. After 6 extractions with this 'washing buffer' (2 ml each wash), the entire phenol layer was counted for radioactivity associated with the remnant [^{35}S]-thiophosphorylated SCS.

III. RESULTS AND DISCUSSION

A. Design of tetramer disruption mutants based on an alternative quaternary structure

Examination of the alternative quaternary structure proposed for *E. coli* SCS revealed some differences in the nature of the αß dimer-dimer interface when compared to that of the published structure (see **Figure 8.1**). Instead of the association of scattered residues from 5 helices in the ß subunits (see **Figure 7.2**, page 145), the only close contacts between αß dimers in the alternative structure were formed by a single contiguous coil and short α-helix in the ß subunit (D150ß-F165ß) which interacted with 3 helices of the α subunit of the neighboring dimer (see **Figure 8.2**). Closer inspection of this region showed an interface between the two αß dimers with several hydrogen bonds and electrostatic interactions (**Figures 8.4, 8.5**). Observed were the interactions between the ß subunit of one dimer and the α subunit of the second dimer. Because of the symmetrical arrangement of dimer-dimer interactions in the tetramer, there were two identical sets of interactions. These close contacts are listed in **Table 8.1**. A comparison of residues in the equivalent positions from both the α and ß subunits of pig heart SCS is also shown. The α subunit residues responsible for dimer-dimer interactions in *E. coli* SCS were found to be more conserved with the corresponding pig heart residues (63% similar) than were the ß subunit residues (33% similar). As well, it was observed that two of the α-helices from the α subunit which contact the neighboring dimer are also involved in the binding site for the adenine end of CoA (see **Figure 8.4**). In consideration of these points, interface mutations were not directed to α subunit residues in order to avoid inactivation of a potentially active dimeric *E. coli* SCS species. Therefore all mutations were confined to the ß subunit. A short continuous sequence of this subunit was responsible for all dimer connections in this alternative structure. This made the design of disruption mutants easier than if the same number of mutations to be made were spaced further apart. Moreover, this region of the *E. coli* ß subunit was not

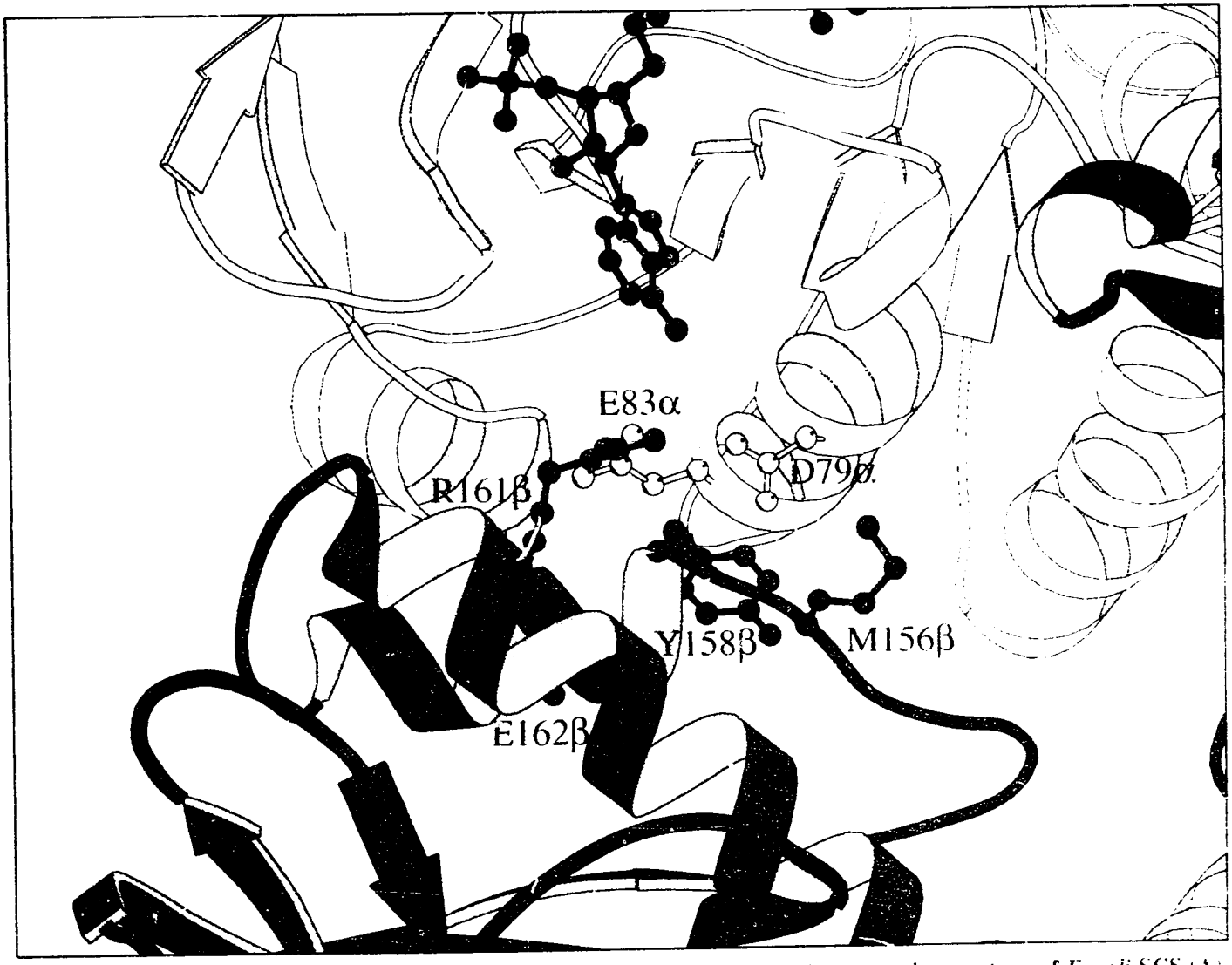


Figure 8.4 Structural representation of the interactions between dimers in the 'alternative' tetrameric structure of *E. coli* SCS (A). This close up view of the region of interaction between the αβ dimers in the 'alternative' tetramer is the same as that shown in **Figure 8.2**. The interactions of a single loop and helix of the β2 (aqua) subunit with three helices of the α1 (yellow) subunit in the adjacent dimer are responsible for inter-dimer contacts within the tetramer. The symmetrical interactions between β1 (green) and α2 (gold) are identical. Subunit residues are depicted in their respective subunit colours while CoA is represented as a red ball-and-stick model. M156β and R161β interact with D79α and E83α, and Y158β interacts with D86α (not shown). This graphic was generated by M. Fraser for [36].

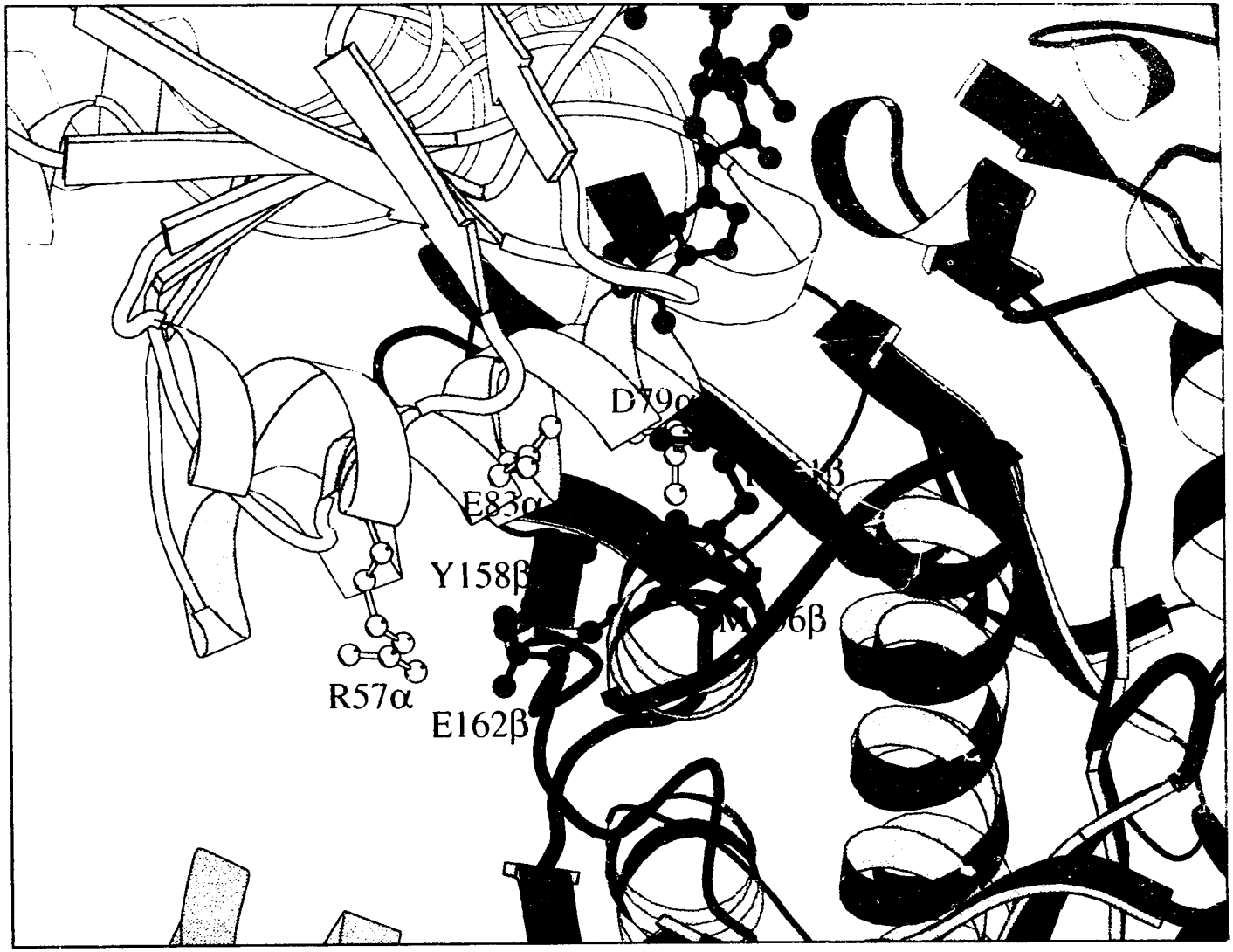


Figure 8.5 Structural representation of the interactions between dimers in the 'alternative' tetrameric structure of *E. coli* SCS (B). This close up view of inter-dimer associations in the 'alternative' tetrameric structure proposed for *E. coli* SCS is related to that shown in **Figure 8.4** by a 90°rotation to the left, along a vertical axis in the page. This perspective emphasizes the interactions between R161ß (ß2, aqua) and both D79α and E83α (α2, yellow). In addition, E162ß interacts with R57α contributing to the salt bridging network formed between dimers. This graphic was generated by M. Fraser for [36].

Table 8.1

Close contacts between αß dimers in the alternative quaternary structure of E. coli *SCS and comparison of these interacting residues with those at equivalent position in pig heart SCS*

Interacting *E. coli* ß subunit residue	Interacting *E. coli* α subunit residue	Equivalent pig heart ß subunit residue	Equivalent pig heart α subunit residue
D150ß	K110α	D157ß	R118α
L152ß	V109α	I159ß	H117α
T153ß	L82α	E160ß	N90α
M156ß	D79α	K163ß	A87α
P157ß	D79α	D164ß	A87α
Y158ß	D79α	S165ß	A87α
Y158ß	D86α	S165ß	D94α
R161ß	D79α	Q168ß	A87α
R161ß	E83α	Q168ß	E91α
E162ß	R57α	R169ß	K65α
F165ß	T55α	E172ß	T83α

conserved when compared to the appropriate sequence in the dimeric pig heart ß subunit (see **Table 8.1**).

Considering the ß sequence at residue 150-165, two approaches were taken in the development of disruption mutants. First, complete replacement of the interacting region in the *E. coli* ß subunit was made with non-conserved residues of the equivalent sequence from the pig heart ß subunit. Second, in the case where this large substitution would disrupt the local packing and therefore the proper conformation of the ß subunit, mutations were directed to residues only on the exterior face of the coil and α-helix. Since R161ß and E162ß ion paired with D79α and R57α, respectively (see **Figures 8.4, 8.5**), it was hypothesized that simply changing the order of these residues in the helix of ß subunit would develop enough electrostatic repulsion in this area to prevent local interactions. These mutations (R161ß→E and E162ß→R) were constructed in combination with the following: 1) The substitution from residues 151 to 158 in the *E. coli* ß subunit sequence with non-conserved pig heart sequence (PLTGPMPY→IIEGIKDS); the resulting mutant was named Ph/ER (see **Table 8.2**). 2) The creation of two surface mutations of the loop and helix, M156ß→D and Y158ß→D; this resulting mutant was named DDER. In the crystal structure of *E. coli* SCS, M156ß and Y158ß hydrogen bond with D79α and D86α of the neighboring α subunit, respectively (see **Figure 8.4**). Therefore, the DDER mutant would have been expected to effectively disrupt any interactions between the αß dimers in the alternative structure due to the combined effect of the introduction of electrostatic repulsion and the removal of most hydrogen bonding interactions.

It has been suggested that electrostatic bonds between complementary surfaces in multimeric proteins provide the specificity required for proper subunit interactions [26]. Although small compared to van der Waals' and hydrophobic forces [27], the contributions of salt bridges exert a strong 'passive' influence [26]. If unpaired charges at interacting surfaces are not compensated for, they can prevent the stable association of

Table 8.2

E. coli ß *subunit mutations designed to disrupt the alternative tetrameric structure - substitution of nonconserved pig heart sequence and the introduction of local electrostatic repulsion at the αß dimer-dimer interface*

Wild type *E. coli* ß subunit sequence (151 - 162)	PMPYQGRE
Equivalent pig heart ß subunit sequence	[illegible]SQAQR

Disruption mutants of the alternative quaternary structure

Ph/ER mutant sequence (151 - 162) - replacement of *E. coli* ß sequence (151-158) with equivalent pig heart sequence and R161ß→E / E162ß→R	[illegible]**IEGIKDS**QG**ER**
DDER mutant sequence (151 - 162) - electrostatic repulsion introduced between αß dimers by mutation of surface residues: M156ß→D / Y158ß→D / R161ß→E / E162ß→R	PLTGP**D**P**D**QG**ER**

such proteins. This principle has been effectively used to disrupt the quaternary structure of several proteins. Through single point mutations at subunit interfaces, both charge repulsion and unpaired charges have been observed to alter the oligomeric structure of tyrosyl-tRNA synthetase (F164→D) [2], insulin (S9B→D, P28B→D, etc.) [3], triosephosphate isomerase (N78→D) [28], and hemoglobin (D99→K) [6].

The mutants were constructed in M13mp18.202 and expressed from the pGS202 in the *E. coli* SCS null strain, TK3(D18), according to previously discussed methods (see **Chapter 7**).

B. Characterization of an interface disruption mutant of the alternative quaternary structure

1. ***Mutant activity and solubility*** - The cellular extracts of the expressed mutant proteins were assayed for SCS activity. The cleared lysates expressing the Ph/ER mutant demonstrated no detectable enzymatic activity, whereas the TK3 cells expressing the DDER mutant exhibited low activities of approximately 10 units/mg (**Table 8.3**). Wild type *E. coli* SCS expressed under similar conditions gave activities of approximately 15 units/mg in crude extracts. The absence of SCS activity observed for the Ph/ER mutant was confirmed in three independent clones which were expressed and analyzed. SDS-PAGE analysis of the soluble and insoluble protein fractions that were separated after sonication, revealed that, in fact, the Ph/ER mutant was completely insoluble and associated with the pelletable fraction (Panel A, **Figure 8.6**). Although the expressed DDER mutant more closely resembled the wild type enzyme with respect to its solubility, approximately twice as much of these mutant proteins were found associated with the pellet (Panel B, **Figure 8.6**).

The treatment of the aggregated Ph/ER mutant with denaturants followed by renaturation and refolding failed to reconstitute an active mutant of SCS (see **Table 8.3**). These unsuccessful attempts prevented further structural analysis of this mutant. Its

Table 8.3

Enzymatic activities of E. coli *SCS disruption mutants based on the alternative tetrameric structure*

Purification stage[1]	SCS Activity[2] (unit		
	Ph/ER	**DDER**	Wild type *E. coli* SCS (pGS202 / TK3)
TK3 lysate supernatant	0 (3)	11.5 (5)	15.3 (2)
TK3 lysate pellet solubilized / refolded[3]			
- 24 hours	0 (1)	12.0 (1)	
- 48 hours	0 (1)	11.4 (1)	
- 72 hours	0 (1)	9.0 (1)	
- concentrated	0 (1)	9.9 (1)	
FPLC peak fractions[4]	n/a[6]	38 (6)	41 (3)
Homogeneous preparation[5]	n/a[6]	53 (1)	50-55 (>1)

1 The SCS proteins were produced from the expression plasmid, pGS202, in TK3(D18) cells.

2 The enzymatic activities listed represent averages of several independent measurements, numbers in brackets.

3 The pellets from the cellular lysates were solubilized with 6 M guanidine-HCl (pH 7.4) and the proteins were renatured by dialysis against a refolding buffer composed of 50 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM KCl and 1 mM ATP (pH 7.4). Aliquots were measured for SCS activity at the time points indicated and after the concentration of the dialysate.

4 The lysate samples were applied to Superose™12 sizing column, and the eluted peak fractions containing SCS proteins were assayed for enzymatic activity.

5 The DDER mutant was purified to homogeneity through treatment of the TK3 expression lysate with protamine sulfate, ATP phosphorylation, and DEAE Sephacel affinity chromatography followed by SS300 HR molecular sizing chromatography. See text for details.

6 n/a - not available due to insolubility

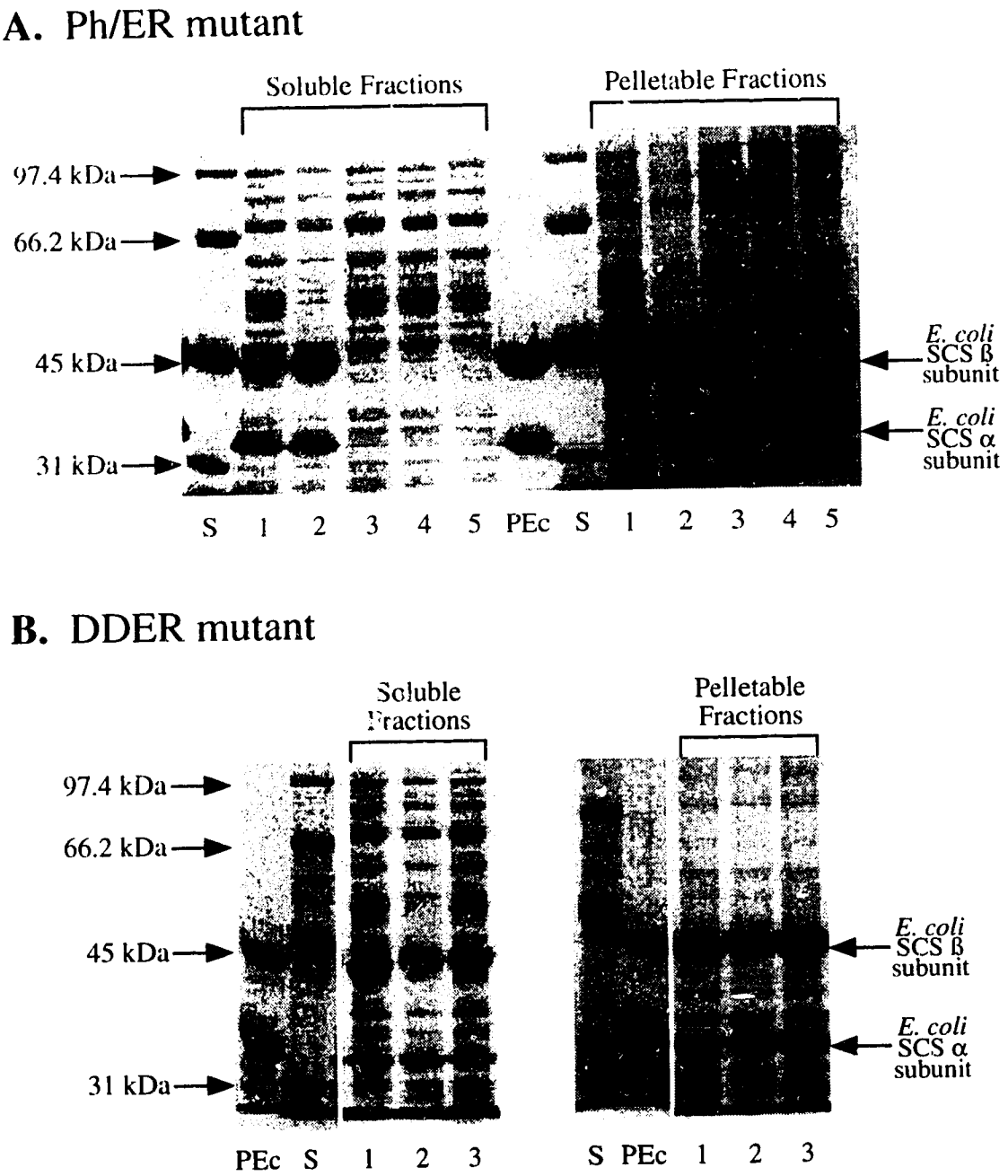


Figure 8.6 Solubility analysis of *E.coli* SCS tetramer disruption mutants based on the alternative quaternary structure. TK3 (D18) cells expressing either wild type *E.coli* SCS or the Ph/ER or DDER disruption mutants were lysed by sonication and divided into soluble and pelletable fractions after centrifugation. Aliquots of each fraction were analyzed by SDS-PAGE. S - molecular weight standards (listed left of each gel). PEc - purified *E. coli* SCS (positions of corresponding ß and α subunits are indicated at right of each gel). **A. Ph/ER mutant** - Lanes: 1, 2 - wild type *E. coli* SCS expressed from pGS202. Lanes 3-5 -independent clones of Ph/ER. **B. DDER mutant** - Lanes: 1- wild type *E. coli* SCS. Lanes 2,3 - independent clones of DDER.

altered properties were consistent with a structural change in the enzyme induced by the substitution of pig heart sequence at the proposed αß dimer-dimer interface.

2. ***FPLC analyses*** - The quaternary structure of the DDER mutant was analyzed by gel filtration through a Superose™12 sizing column. Standardization of this column with respect to SCS was accomplished using tetrameric and dimeric species. The wild type *E. coli* SCS ($\alpha_2ß_2$) was found to elute from the column at 12.2 ml, whereas the pig heart SCS (αß) eluted at a position corresponding to 13.7 ml. When applied to the column, the DDER mutant eluted in a volume fraction which was larger than that of the wild type *E. coli* enzyme and equal to that of dimeric pig heart SCS (13.7 ml). This result suggested that the mutations encoded by DDER were successful in modifying the quaternary structure. Further FPLC analysis confirmed this difference when wild type *E. coli* enzyme was mixed with the DDER mutant (**Figure 8.7**): the two proteins were clearly separated and, therefore, different in molecular size. When the DDER mutant was combined together with native pig heart SCS, the elution of the two proteins coincided to one fraction. Given that the dimeric pig heart enzyme is 75 kDa and a potential dimer of *E. coli* SCS would be 71 kDa, this result was consistent with the possibility that the DDER mutant was dimeric in structure.

It was also noted that the SCS activity measured in the FPLC purified peak fractions of the DDER mutant reached levels of 35-40 units/mg, closely approximating that of the wild type enzyme (see **Table 8.3**).

3. ***Non-dissociating PAGE and Western analysis*** - One method of testing the apparent quaternary structure resulting from FPLC analysis, was the use of non-dissociating polyacrylamide gel electrophoresis. However, migration of proteins in unmodified non-denaturing (or native) gel systems is highly dependent on the charge state of the molecules. This effect can confound the interpretation of results obtained from such experiments. The method of 'Blue Native PAGE' [23] was adopted to counteract this problem. Here, the binding of Coomassie blue G (Serva) to proteins prior

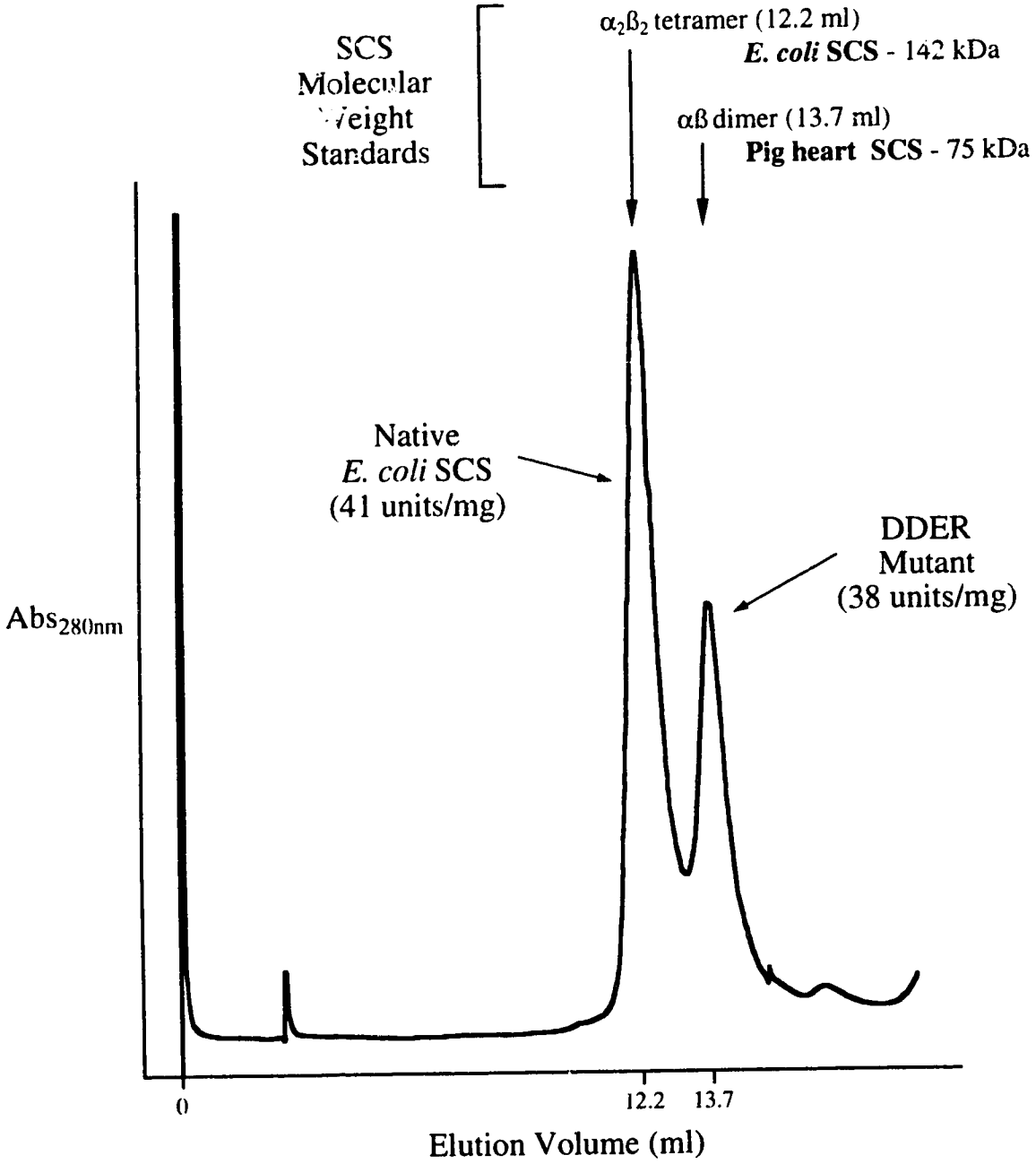


Figure 8.7 Superose™12 FPLC separation of native and DDER mutant *E. coli* SCS. Samples of wild type *E. coli* SCS and the disruption mutant, DDER expressed in TK3(D18), were mixed and subjected to FPLC using a Superose™12 HR 10/30 molecular sizing column. Proteins were prepared and eluted from the column using KPCEP buffer (50 mM KH_2PO_4, 50 mM KCl, 1 mM EDTA and 0.1 mM PMSF (pH 7.4)) with a flow rate of 0.2 ml/min.

to electrophoresis imparts a uniform negative charge without disrupting the native conformation. Therefore, proteins can be separated electrophoretically based on their charge to size ratio under non-dissociating conditions. Samples of SCS purified independently and FPLC peak fractions were separated by 'Blue Native PAGE' and in some cases subjected further to Western analysis. As shown in Panel A of **Figure 8.8**, the DDER mutant migrated further than tetrameric wild type *E. coli* SCS and corresponded to a position near that of dimeric pig heart SCS. These results corroborated those of gel filtration and supported the suggestion that the DDER mutant was dimeric.

The results of the Western analysis of the DDER mutant are presented in Panel B of **Figure 8.8**. Blots of the proteins separated by 'Blue Native PAGE' and transferred subsequently to Immobilon membranes were probed with antibodies directed against either native *E. coli* or pig heart SCS. The antibodies specific to tetrameric wild type *E. coli* SCS recognized and bound equally well to the DDER mutant. This evidence suggested that although the quaternary structure was perturbed by the mutations at the αß dimer-dimer interface, the remaining structure stayed intact. That the DDER mutant retained a high level of catalytic competency, supported this conclusion.

4. ***Sedimentation velocity determinations*** - Analysis of the quaternary structure differences existing between pig heart and *E. coli* SCS was carried out by Wolodko *et al.* [18] using sedimentation velocity centrifugation. This work demonstrated unequivocally that the *E. coli* SCS functioned as a non-dissociating tetramer, whereas the pig heart SCS existed as a non-associating dimer. In addition to the indication that both enzymes behaved as typical, non-interacting globular proteins, the sedimentation coefficients determined for these two species were different, clearly representative of their structurally distinct shape and size. Therefore, to categorize the quaternary structure of the DDER mutant unequivocally, samples of this enzyme in parallel with *E. coli* and pig heart SCS were subjected to ultracentrifugational analyses. The results of these investigations are presented in **Figure 8.9**. The sedimentation coefficients ($s_{20,w}$ at 1.1

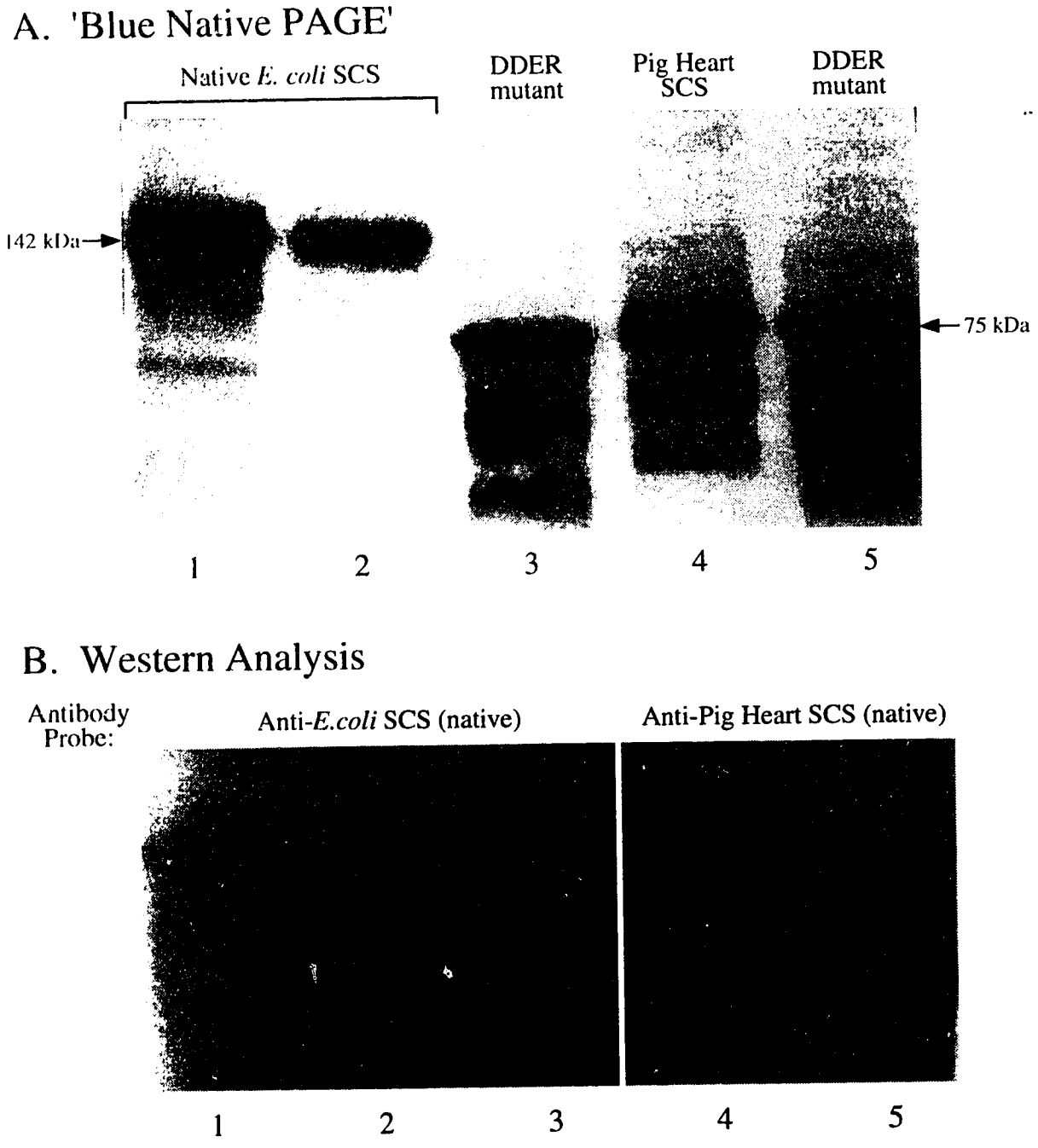
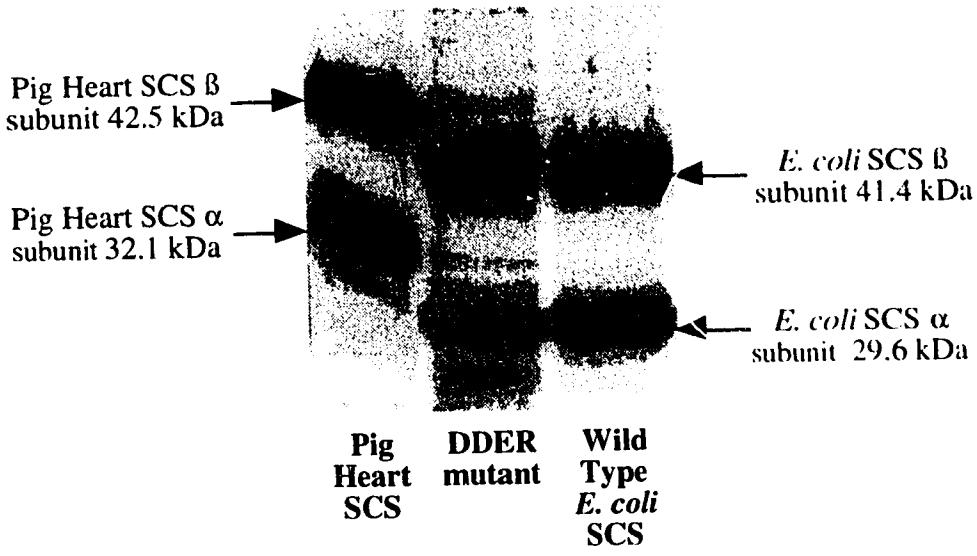


Figure 8.8 'Blue Native PAGE' and Western analysis of the DDER disruption mutant, native *E. coli* and pig heart SCS. Samples of SCS complexed with the dye Coomassie Blue R (Serva) [21] were subjected to PAGE under non-denaturing conditions using gradient gels. The separated proteins were either stained further with Coomassie Blue R-250 (**A**), or transferred to Immobilon-P membranes for subsequent Western analysis (**B**). Lane 1, Superose-12 purified, wild type *E. coli* SCS expressed in TK3 cells; Lane 2, native *E. coli* SCS purified to homogeneity; Lane 3 and Lane 5, DDER mutant *E. coli* SCS (Superose-12 purified); Lane 4, purified pig heart SCS. The blots of lanes 1-3 were probed with antibodies raised against native *E. coli* SCS. The blots of lanes 4 and 5 were probed with anti-pig heart SCS antibodies. The visualization of the primary antibody-blot complexes was achieved using alkaline phosphatase-conjugated secondary antibodies and Lumi-Phos 530 as a chemiluminescent substrate (see text for details).

A. *SDS-PAGE of samples used in sedimentation experiments*



B. *Sedimentation coefficients*

SCS sample	$s_{20,w}$ (present study)		$s_{20,w}$ (Wolodko *et al.*, 1986)	
	1.1 mg/ml	2 mg/ml	1.1 mg/ml	2 mg/ml
E. coli ($\alpha_2\beta_2$)	6.88 S	6.26 S	6.93 S	6.84 S
Pig heart ($\alpha\beta$)	4.58 S	-	4.51 S	-
DDER mutant	**4.08 S** **4.03 S**	**4.33 S** **-**	- -	- -

Figure 8.9 Sedimentation velocity coefficients of native *E. coli* SCS, pig heart SCS and the tetramer disruption mutant, DDER. The SCS enzymes were dissolved in 50 mM KH_2PO_4, 50 mM KCl and 1mM EDTA (pH 7.4) prior to analysis. **A**. The homogeneity of each source was demonstrated by subjecting aliquots of these proteins to SDS-PAGE. **B**. Sedimentation velocity determinations were conducted at 60 x 10^3 rpm (20 °C) using a Beckman Spinco Model E analytical centrifuge and a schlieren optical system. Protein concentrations were either 1.1 or 2 mg/ml.

mg/ml) calculated for both controls, the pig heart and the wild type *E. coli* SCS, 4.58 S and 6.88 S, respectively, were in close agreement with those determined previously at the similar concentrations [18]. The $s_{20,w}$ values of 4.08 S and 4.03 S at 1.1 mg/ml and 4.33 S at 2 mg/ml determined independently for the DDER mutant were consistent with the quaternary structure of this protein being smaller than that of a tetramer and closely approximating that of the dimeric pig heart enzyme.

Considering the results obtained from these experiments with the others described previously, the following conclusions could be made. The mutations within the DDER protein, which were directed against the αß dimer-dimer interface of the alternative quaternary structure of *E. coli* SCS, had effectively disrupted the tetramer resulting in a dimeric form of the enzyme. In contrast to the failed attempts of the disruption mutagenesis using the published tetrameric structure, the outcome of the experiments discussed here indicated that the true quaternary structure of *E. coli* SCS was that of the alternative model. This approach of disruption mutagenesis may have a wider applicability to ambiguous cases where quaternary structure assignments in multimeric proteins cannot be made due to equally credible crystal-packing contacts among component subunits.

This alternative arrangement of dimers inferred a number of different aspects for the quaternary structure of this enzyme. The active site was not formed from the contributions of an α and ß subunit from one dimer and the ß subunit from the neighboring dimer, as was suggested by the published tetrameric structure. Therefore, the possibility that two dimers were required to create each active site was eliminated as a rationale for the tetrameric structure. Instead, the active sites in the alternative model were more independent. This was in keeping with the observation that the mutant dimeric form of the enzyme retained catalytic activity. The extended, open configuration of the overall alternative arrangement may lend itself to interact with other proteins. To

this point, specific interactions have been observed between SCS and the α-ketoglutarate dehydrogenase complex of the citric acid cycle [29].

C. Purified dimeric *E. coli* SCS exhibits wild type levels of activity

The DDER-dimer mutant of *E. coli* SCS exhibited a high degree of catalytic competency. As indicated in **Table 8.3**, crude SCS activities demonstrated by this altered form nearly approximated that of the wild type enzyme. To determine the specific activity of this dimeric form of *E. coli* SCS, a homogeneous protein sample was prepared (see **MATERIALS AND METHODS**). Using DEAE affinity and gel filtration chromatography and two precipitating reagents (ammonium sulfate and protamime sulfate), the dimeric form of the enzyme was purified and found to exhibit a specific activity of 53 units/mg (**Table 8.3**). Since specific activities of 50-55 units/mg were routinely obtained for native *E. coli* SCS prepared under similar conditions [30], the dimer form was wild type in regard to its catalytic efficacy.

To ensure that this high activity was not due to contaminating wild type enzyme, the purified, expressed mutant protein was reassessed. Using gel filtration chromatography, a sample of the purified protein was applied to a column of Bio-Gel A resin, previously standardized with *E. coli* and pig heart SCS. The protein eluted in a volume which was consistent with a dimeric species of SCS (data not shown). Furthermore, samples of these proteins (2 mg/ml) were analyzed by sedimentation velocity centrifugation. The sedimentations coefficients determined from these experiments were in agreement with those found previously (**Figure 8.9**). Therefore, it can be concluded that the purified enzyme was dimeric in structure and exemplified a wild type level of specific activity.

Since the dimer mutant retained full catalytic competency, quaternary structure was not a prerequisite for function in the native *E. coli* enzyme. Rather, it appeared that two independent dimeric enzymes were complexed as a tetramer for other purposes.

Since the dimeric form was found to be slightly less soluble and stable when compared to wild type *E. coli* SCS, these observations offered suitable rationales for the tetrameric structure.

D. Thiophosphorylation studies of dimeric and native *E. coli* SCS

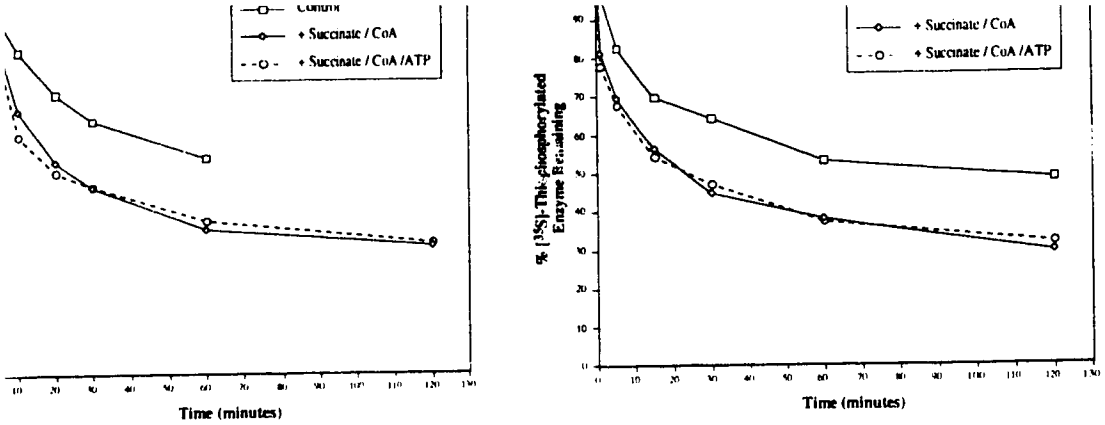
The isolation of an active dimer of *E. coli* SCS now enabled further investigation of the rationale for the tetrameric subunit structure of the wild type enzyme. As discussed earlier (**Chapter 1**), several studies [10-17] had suggested that a mechanism of alternating sites cooperativity operated through the αß dimers of *E. coli* SCS. One line of evidence in support of the alternating sites cooperativity model was the facilitating action of phosphorylation by ATP, in the presence of succinate and CoA, to drive the slow release of [^{35}S]-thiophosphate from thiophosphorylated enzyme [12]. Since, in the studies, *E. coli* SCS was determined to incorporate one mol of thiophosphate per tetramer, phosphorylation by ATP was believed to occur at an unphosphorylated site. This action was suggested to promote reciprocal changes in the conformation of the two active sites to enhance the de-thiophosphorylation rate at the first site. However, rapid de-thiophosphorylation in the presence of succinate, CoA and ATP was also observed in dimeric pig heart SCS [31]. To determine whether this facilitated de-thiophosphorylation effect in *E. coli* SCS required two alternating and cooperative active sites (dimers) or simply one, as in the case of pig heart SCS, similar experiments were repeated using the DDER dimer mutant.

First to obtain enzymes which were thiophosphorylated to a high degree, purified and highly active tetrameric and dimeric SCS were de-phosphorylated in the presence of succinate and CoA. Thiophosphorylation of the de-phosphorylated *E. coli* enzymes with ATPγS resulted in levels of 1.45 and 1.1 mole thiophosphate (-PSO_2) per mole SCS, for the native and dimer mutant forms, respectively. In agreement with earlier reports [24, 32-34], these near maximal levels of thiophosphate incorporation (two -PSO_2 per $\alpha_2\beta_2$ for the native tetramer, and one -PSO_2 per αß for the dimer mutant) were inconsistent

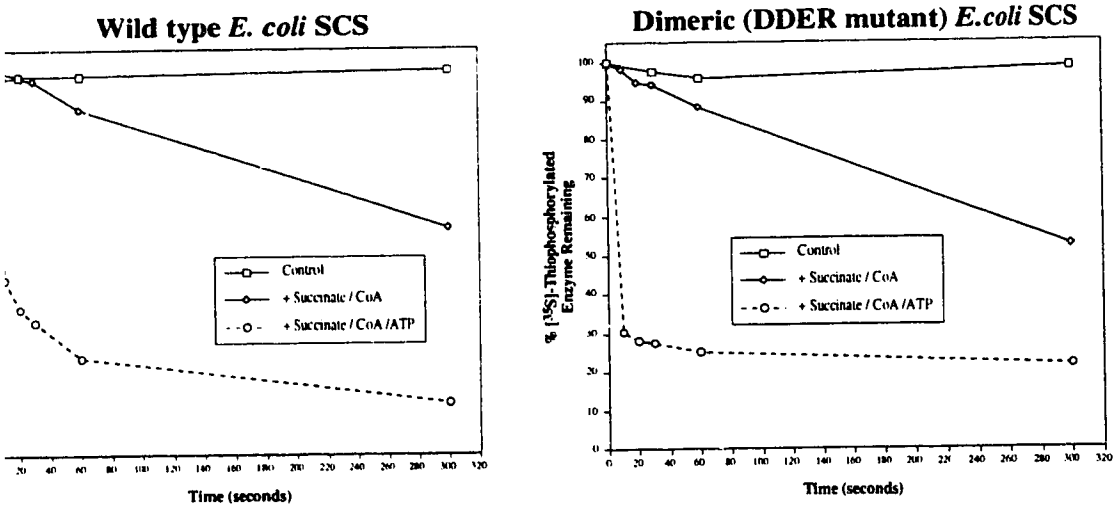
with the concept of 'half-of-the-sites' phosphorylation required for alternating sites cooperativity.

Thiophosphorylated wild type and dimer mutant enzymes of *E. coli* SCS were examined for the rate of de-thiophosphorylation in the presence of substrates. [illegible]riments performed on both enzymes in the presence of phosphate (44 mM - native; [illegible]M - DDER dimer mutant) revealed only a slow release of [^{35}S]-thiophosphate with time (see Panel A, **Figure 8.10**). Both the addition of succinate and CoA, or of succinate, CoA and ATP were found to elicit only a small increase in the level of de-thiophosphorylation over that of the control. Interestingly, contrary to prior observations [12, 35], the preser[illegible] of phosphate appeared to inhibit the rapid de-thiophosphorylation caused by succinate and CoA (≤ 5 minutes), and the nearly instantaneous discharge by ATP (acting 'synergistically' with succinate and CoA). The removal of phosphate by dialysis or preparation of thiophosphorylated SCS in its absence alleviated this inhibitory effect on de-thiophosphorylation for both enzymes (Panel B, **Figure 8.10**). The mass action effect by phosphate may be responsible only for a small proportion of this inhibition. Since the increased rate of de-thiophosphorylation was the same for succinate and CoA as for succinate, CoA and ATP, excess phosphate appeared to specifically block the facilitating effects of ATP.

The residual levels of thiophosphorylated enzyme remaining after treatment with substrates was higher than those previously indicated for *E. coli* SCS [12]. This may have been a manifestation of the experimental procedure that was used to remove free [^{35}S]-thiophosphate. Using a phenol extraction method [25] to measure the de-thiophosphorylation of pig heart SCS [31], residual levels of 10-15% of initially labeled material remained after substrates were allowed to react to completion. Nevertheless, as shown in **Figure 8.10** (Panel B), the addition of ATP greatly enhanced the rate by which succinate and CoA de-thiophosphorylated both the native and the dimeric forms of *E. coli* SCS. Since this action occured as effectively in the DDER dimer as in the wild type



)e-thiophosphorylation in the absence of phosphate



·e 8.10 De-thiophosphorylation of wild type and mutant dimeric (DDER) *E. coli* in the presence and absence of phosphate. [^{35}S]-labeled thiophosphorylated *E. coli* enzymes (native and DDER dimer mutant forms) were de-thiophosphorylated according : following modification to the method developed by Wolodko *et al.* [17]. **Control** - hosphorylated enzyme with 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 0.5 mM DTT; **ccinate / CoA** - control with 10 mM sodium succinate and 0.5 mM CoA; **+ Succinate / / ATP** - + Succinate / CoA with 1.0 mM ATP. **A.** De-thiophosphorylation (minutes) in resence of either 44 mM (wild type *E. coli* SCS) or 25 mM (DDER dimer mutant) $^{3}O_4$. **B.** De-thiophosphorylation (seconds) in the absence of phosphate (< 50 µM).

enzyme, the ATP-facilitated de-thiophosphorylation was inferred to be a single site effect in *E. coli* SCS. Since the dimer mutant exhibits wild type activity and contains only one active site, it can be concluded from these results that an alternating sites cooperative mechanism is not required by *E. coli* SCS.

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Chapter 9

OVERALL CONCLUSIONS AND FUTURE PERSPECTIVES

Several differences exist between succinyl-CoA synthestase from *E. coli* and pig heart (see **Figure 1.2**, page 7). While the enzyme from the bacterium has retained a tetrameric subunit structure [1] with a broad specificity for nucleotide substrates [2], mammalian SCS has evolved into a dimer [1] with restricted nucleotide specificity [3]. Why then has nature maintained a tetrameric structure for SCS in *E. coli* when a dimeric form is catalytically effective? To address the nature of the structure and function relationships existing in SCS, the work presented in this thesis was initiated to investigate some of the distinctive features of the pig heart and *E. coli* enzymes, and to provide a rationale for the variance between them.

A comparison of the available amino acid sequences of the α and ß subunits of SCS from various tetrameric and dimeric sources showed the α subunit to be more highly conserved than the ß subunit. The α subunits were 70% identical and 90% similar when conservative replacements were included [4], whereas the ß subunits from tetrameric sources were 45% identical and 65% similar. From these primary structure comparisons, the α subunit appeared to provide very little insight for the differences in properties between bacterial and mammalian forms of SCS. Rather, it suggested that this diversity could be attributable to differences in the ß subunits. **Chapter 3** described the identification and characterization of the first mammalian ß subunit clone. This cDNA encoded the ß subunit of SCS from newborn pig heart tissue. When compared to the ß subunit sequence of bacterial sources, the deduced amino acid sequence of the pig heart clone was found to have a level of similarity (65%) equal to that observed when the bacterial forms were compared among themselves. However, a codon for tryptophan was notably absent in the original cDNA and in all of the further characterized, independent clones. This was surprising considering that others have reported that adult pig heart SCS contained one Trp residue in the ß subunit, and that this residue may be a conserved marker for active site function [5-7].

Given the polymorphic nature of the pig heart α subunit, the same possibility existed for the ß subunit. In this regard, the ß subunit clone isolated from a library constructed from newborn pig heart tissue, may have been a distinct isoform which was differentially expressed in fetal or newborn tissues. To test this theory, as detailed in **Chapter 4**, ß subunit clones were isolated from a cDNA library constructed from adult pig heart mRNA. These clones were identical in deduced amino acid sequence to the cDNAs from the newborn source, and therefore, did not contain a tryptophan residue.

To substantiate the results obtained from the well characterized cDNAs, the physico-chemical studies which originally suggested the presence of tryptophan in pig heart SCS were repeated (**Chapter 5**). Both tryptophan-specific fluorescence and amino acid compositional analyses completed on highly purified pig heart SCS indicated the absence of tryptophan in these samples. The use of *E. coli* SCS in parallel analyses as a positive control established that these measurements were well within the limit of detection if one Trp residue existed per αß dimer. However, the investigation of an impure preparation of pig heart SCS demonstrated substantial tryptophan fluorescence with an amino acid composition of approximately one Trp per αß dimer. Although suggestive of a contamination being responsible for the earlier observations of Nishimura *et al.* [7], other possibilities existed for the disparity among the results. A subspecies variation with respect to tryptophan content may have existed between the samples of pig heart SCS characterized in these two studies. It is interesting that no tryptophan was detected either in the protein or encoded in the cDNAs in all the sources examined in these studies. A remote possibility may have been that a ß subunit clone encoding Trp existed in the cDNA libraries that were probed, but this isoform escaped detection. This was highly unlikely considering the sensitive and thorough nature of the screening techniques employed in these studies.

A non-AUG codon for translational initiation appeared to be utilized in the ß subunit cDNA from both newborn and adult pig heart tissue. Repeated sequencing of a

number of indep[illegible]nt clones from both newborn and adult cDNA libraries confirmed that sequencing errors or artifacts caused by the reverse transcriptase were not probable causes for the lack of detection of an AUG initiation codon. Instead, a CTG codon in the leader region fulfilled contextual sequence requirements for the efficient non-AUG initiation [8-10]. In addition, the potential of the GC-rich leader sequence to form secondary structure downstream of this codon was consistent with the concep[illegible] of the 40S ribosome stalling in close proximity of the CTG in order to facilitate initiation at this site [11].

However, it was possible that other libraries, not probed in these studies, contained cDNAs which were full length extensions of a longer ß subunit mRNA which incorporated an AUG codon further upstream. This would imply that the signal sequence would be greater than 40 amino acids since a leader sequence of 117 nucleotides existed upstream of the coding sequence for the present n[illegible]ure ß subunit. The isolation of ß subunit clones from other cDNA libraries constructed from various pig tissues should determine if this is the case. Likewise, primer extension experiments using reverse transcriptase could be carried out on mRNA hybridized with the ß subunit cDNA, in order to define the 5' end of the message.

Nevertheless, the initiation of translation at the CTG codon would have created a ß subunit precursor with a 22 amino acid N-terminal presequence for targeting into the mitochondrial matrix. This signal sequence contains basic and hydroxylated residues, a feature typical of matrix-targeted proteins [12]. However, the ß subunit presequence likely did not form an amphiphilic α-helix due to randomly positioned Arg residues and three helix-breaking proline residues. Perhaps it may perform its targeting role as an amphiphilic ß-sheet. To test the role of this 22 amino acid presequence as a legitimate targeting signal, mitochondrial import studies could be carried out using an artificial precursor constructed from this sequence fused N-terminally to the mammalian α subunit. Since such import conditions for the mammalian α subunit have been

previously defined [13], direct comparisons may be made concerning the import competency of artificial versus native precursors.

The isolation of the ß subunit cDNA permitted the first characterization of a cloned isozyme of pig heart SCS. In **Chapter 6**, the bacterial expression of both the Phß subunit and the Phα57 subunit as fusion proteins with protein A was found to be highly productive. However, subsequent isolation of the individual SCS subunits from these fusion proteins was hindered by incomplete and non-specific cleavage by the endoprotease, factor X_a. These results limited the use of this expression system in the quantitative synthesis of individual SCS subunits required to reconstitute functional pig heart SCS. Nevertheless, the refolding of these fusion proteins with isolated pig heart SCS subunits gave the first indication that the formed complex exhibited GTP-specific activity. The fact that the pig heart α subunit, while fused C-terminally to protein A, could obtain a catalytically competent form when refolded with an isolated ß subunit, was remarkable. This offers a possibility for future purification of cloned pig heart SCS. If a suitable means of liberating the α subunit from its protein A tag could be developed, coexpression of this fusion protein with the pig heart ß subunit could enable a one step purification of the cloned mammalian enzyme from bacterial lysates.

Also described in **Chapter 6** was the coexpression of the individual pig heart SCS subunits (Phß and Phα57) from a T7 RNA polymerase-controlled expression system [14]. The significant conclusion from these studies was that the combination of these two cloned subunits produced an isozyme of pig heart SCS with strict GTP specificity. Through the use of three chromatographic matrices, bacterially-expressed pig heart SCS was purified to homogeneity and exhibited a specific activity of 20 units/mg. This high level of activity demonstrated by the cloned enzyme was, however, not wild type with respect to the observed activity of SCS purified from pig heart tissue (30 units/mg).

Several explanations could account for the suboptimal activity observed for the cloned enzyme. The limited ability of the isolated subunits to reconstitute pig heart SCS

activity *in vitro* [7] demonstrated the complex nature of the folding requirements for this enzyme. The bacterial cytoplasm may not duplicate the environment of the mitochondrial matrix, and therefore the expressed pig heart isozyme may have been prevented from properly folding and assembling into its most active conformation. Another explanation is based on the results obtained from the pulse-chase labeling studies. Here, the Phα57 subunit was observed to be degraded to a higher extent than the Phß subunit when the two proteins were coexpressed. Besides possible Phα subunit excesses (arising from the expression conditions) being responsible for this effect (see **Chapter 6**), an extension of 6 amino acids at the N-terminus of this mature subunit likely plays a destabilizing role. This was consistent with the observations that the rat liver α subunit precursors were unstable relative to the mature forms of the same subunit [15]. Therefore, the polypeptide extension of the Phα subunit could have acted as a signal sequence and mildly perturbed the packing of the N- and C-domains of this subunit [15]. This effect could have been transmitted to the overall structure of the enzyme and decreased its catalytic effectiveness. This also fitted with the observation that rat liver precursors were unable to carry out phosphorylation when alone, or assembled with the ß subunit [15]. To distinguish this possibility as a cause for the decreased specific activity, future expression of the cloned pig heart enzyme should involve subunits without N-terminal extensions. Also, kinetic analyses of expressed pig heart SCS would indicate if the binding affinity for any substrate has been modified relative to the wild type enzyme.

SCS is believed to play a variety of metabolic roles of opposing catabolic and anabolic function (the conversion of succinyl-CoA to succinate in the citric acid cycle, and the generation of succinyl-CoA required for ketone body catabolism and biosynthesis) [16, 17]. It has been suggested that such opposing functional demands could best be accommodated by two distinct enzymes [18]. Several reports of distinct ATP and GTP-specific isoforms in a wide range of animals supports this concept [3, 18]. Further, levels of a GTP-specific, mouse liver isozyme were elevated threefold under

conditions of increased porphyrin synthesis [19]. It was suggested that a GTP-specific SCS, operating under high GTP/GDP ratios in the mitochondria would maintain favourably high levels of succinyl-CoA for ketone body metabolism [20]. In support of this hypothesis, Jenkins and Weitzman reported that the levels of GTP-specific SCS activity were significantly increased in tissues of ketotic animals [21]. These results demonstrate a role for GTP-specific SCS in synthesizing succinyl-CoA required for porphyrin synthesis and ketone body metabolism. Given the considerable importance of ketone body metabolism to mammalian heart tissue, the discovery of a cloned pig heart isozyme of SCS displaying GTP specificity was consistent with this proposal. To test the relevance of the cloned GTP-specific enzyme in ketone body metabolism, diabetic ketoacidosis could be induced in pigs by treatment with streptozotocin [22]. Since diabetes is accompanied by enhanced ketone body production by the liver and utilization by the brain and heart [23, 24], RNA from these tissues could be probed with cDNAs encoding both the Phα57 and Phß subunits. Changes in the levels of mRNA detected compared to that from tissues of untreated animals may indicate a role for the cloned enzyme in ketone body metabolism in the heart and possibly other tissues as well.

As part of the above hypothesis linking SCS isozymes with different metabolic functions, the ATP-specific enzyme has been implicated for a role in the citric acid cycle [21, 22, 25]. To date, unfortunately, an ATP-specific mammalian isoform has not been cloned. Of the SCS subunit clones that have been isolated from the pig heart cDNA libraries, only two distinct messages have been detected for the Phα subunit: Phα57 and Phα108 (which are alternatively spliced isoforms) [26] and one for the Phß subunit [this work]. Both complexes of Phß with Phα57 or Phα108 [27] had been determined to be GTP-specific. These results were unexpected for the following reason. ATP-specific activity has been shown to be nearly equal to GTP activity in mammalian heart tissue: the ratio of GTP-specific to ATP-specific SCS activity was reported to be 1.4 in preparations from rat hearts [3]. Given the thorough and sensitive nature of the screening techniques

employed, it was puzzling that clones of isoform subunits which contribute to ATP-specific activity were not detected. It is possible that the ATP-specific enzyme may arise from GTP-specific SCS through some post-translational modification in pig heart tissue. Therefore, all cloned enzymes that were expressed in bacteria would appear GTP-specific due to the lack of such protein-processing systems. It is equally possible, however, that the expression of mRNAs encoding the component subunits of an ATP-specific, pig heart enzyme may have been limited. Future investigations involving the isolation of clones responsible for the ATP-specific activity should use cDNA libraries constructed from tissues where this isozyme is highly prevalent (brain, for example). In addition, the identification of such clones would be aided by N-terminal and internal amino acid sequence information obtained from a purified form of the ATP-specific enzyme prepared from sources where it is abundant.

Attempts at crystallizing the pig heart enzyme purified from tissue have not been successful in obtaining suitable crystals for x-ray diffraction analyses. It was likely that these trials have been hampered by the heterogeneity due to a variety of SCS isoforms present within the isolated protein samples. The expression and purification of a single pig heart SCS isozyme has provided reproducible and homogeneous preparations facilitating structural studies. To that end, crystals of the recombinant enzyme have been grown by microdialysis and have been found to diffract to 1.8 Å resolution [28]. Using the *E. coli* enzyme as a model, molecular replacement methods indicated that cloned pig heart SCS was similar in overall shape and subunit structure to one αß dimer of the *E. coli* tetramer. Heavy atom derivative data will assist the solution of the structure in regions where that of the *E. coli* and pig heart enzymes differ. The crystal structure of the pig heart SCS will provide an invaluable wealth of information essential to the study of the structural and functional relationships in this enzyme and in its comparison to *E. coli* SCS.

To gain an understanding of the reason(s) why *E. coli* SCS existed as a tetramer whereas pig heart SCS was a dimer, investigations were directed towards the interruption of αß dimer-dimer contacts in the tetrameric enzyme (**Chapter 7**). Comparing the cloned ß subunit sequence from pig heart SCS (**Chapter 3**), it was discovered that several ß subunit residues responsible for inter-dimer associations within the structure of *E. coli* SCS [29] were not conserved between the two sequences. The possibility was tested that the residues in these positions were altered in the pig heart enzyme to prevent dimerization of αß dimers from occurring. Through site-directed mutagenesis, the residues of the *E. coli* ß subunit were substituted with the non-conserved pig heart residues at equivalent positions (R14ß→D, R70ß→G, E74ß→K, E231ß→A and Q247ß→K). Surprisingly, no effect on either the quaternary structure or activity was observed in any of these single point or combination mutants. It was rationalized that other factors in addition to the electrostatic and hydrogen bonds of these close contacts were responsible for the specificity of interactions between dimers in the tetramer.

Within the published structure of the *E. coli* tetramer [29], the ß subunit appeared to fulfill the requirements for tetrameric structure by forming part of the CoA binding site on the neighboring dimer. These ß subunit interactions with CoA were removed through mutagenesis (R29ß→D/A, E33ß→A, S36ß→A and K66ß→A) and all resulting single point and combination mutants were unchanged in both subunit structure and activity. Combination of these mutations with the mutations involving the dimer-dimer interface (pig heart residue substitutions) were also ineffective in altering the structure and function of the enzyme. From these results it was concluded that perhaps forces besides electrostatic and hydrogen bonds were responsible for stabilizing the quaternary structure of the tetramer.

In an effort to sterically disrupt van der Waals' and hydrophobic forces between associating dimers, bulky Trp residues were substituted at positions of close contact between the ß subunits (E231ß→W, Q247ß→W and E249ß→W). Although these

mutations had a disruptive effect on catalysis, when alone or in combination with the pig heart substitutions or the CoA binding site mutations, no perturbation was observed in the quaternary structure.

Together, the ineffectiveness of these mutations to disrupt the quaternary structure was surprising. Since the number of mutations were multiplied twofold, and the introduction of unfavorable charged residues and regions of steric interference failed to alter the dimer-dimer associations, it appeared that these disruptive mutations had no structural relevance. Even in the case of the tryptophan mutants, where catalytic activity had been affected, the pattern of stability of these mutants remained unvaried relative to wild type enzyme (**Chapter 7**). This indicated that these mutants were not destabilized conformationally to a level insufficient for complete tetramer disruption. Rather, these studies suggested that a re-evaluation of the assignment of αß dimer-dimer interactions in the structure of *E. coli* SCS was required.

In fact, ambiguity existed in the choice of the crystallographic αß dimers which constituted the tetramer. Within the unit cell, two tetramers were seen to pack around a crystallographic twofold axis to form an octamer. The octamer has approximate 222 symmetry, with two of the twofold axis being non-crystallographic symmetry axes, and one being a true crystallographic axis. The tetramer arrangement which related αß dimers around the non-crystallographic twofold axis was ultimately chosen for publication [29]. This choice was biased to allow the possibility to observe differences between the two halves of the tetramer, including the active sites, if they existed. This tetrameric structure was consistent with the available information obtained from previous solution work [6, 30]. The results of the studies presented here, however, called into question this quaternary structure assignment and suggested the alternative tetramer relating two αß dimers across the crystallographic twofold axis of symmetry, was the correct one. This was in agreement with the structure of *E. coli* SCS which had been refined with new data to higher resolution [31]. In this refined structure, the contact

surface area between the dimers of the alternative tetramer was determined to be larger than the surface area between the two ß subunits which mediated the dimers' associations in the published tetramer. Moreover, some of the close contact distances between dimers of the published tetrameric structure were observed to be increased.

The possibility that the alternative tetramer represented the true quaternary arrangement was investigated just as the previous choice had been tested: through the mutagenic disruption of the associations which stabilize the dimer-dimer interface in this structure (**Chapter 8**). The Ph/ER mutant had the ß subunit sequence from pig heart SCS substituted at these sites of dimer-dimer contact. However, since this mutant was highly altered with respect to its solubility, its characterization was limited. The other tetramer disruption mutant, DDER, was designed to create a region of charge repulsion by substituting acidic residues at close contact points which were involved in inter-dimer salt bridges (M156ß→D, Y158ß→D, R161ß→E and E162ß→R). Although this *E. coli* SCS mutant was found to be mildly affected in its solubility and in its stability with respect to some buffers, its general properties were unaffected. Through gel filtration chromatography, 'Blue Native PAGE' and sedimentation velocity centrifugation experiments, the DDER mutant was determined to behave as an αß dimer. The mutations, directed to the interface between αß dimers of the alternative quaternary structure arrangement, were successful in disrupting the tetramer of *E. coli* SCS. It has been demonstrated that single point mutations introducing unpaired charged residues or regions of electrostatic repulsion were found to be responsible for altering the oligomeric structure in several proteins [32-35]. Considering these other observations, it would be interesting to determine which of the four mutations were absolutely required for this disruption effect on the *E. coli* enzyme.

The production of a dimeric form of *E. coli* SCS with wild type levels of activity indicated that the dimerization of the αß dimers was not a prerequisite for catalytic competency. Moreover, the properties of this mutant dimer suggested a number of

alternate rationales for the native tetrameric structure. Although the *E. coli* dimer was observed to be thermostable, the DDER mutant was found to be less stable than wild type enzyme in the absence of phosphate buffer. This implied that the formation of the tetramer may impart some stability to the overall structure. Indeed, when considering that the energy of stabilization of oligomeric structures is quite large (approximately 10-27 kcal/mol) [36], intersubunit associations can alone provide a rationale for higher ordered structures. The quaternary interactions in *E. coli* SCS are likely to be entropy driven, noting that the large proportion of buried surface area between dimers was hydrophobic in nature. The slightly decreased solubility of the expressed dimers also suggested that the tetrameric form was required to conceal these normally unexposed faces. In addition, the formation of the SCS tetramer may impart to the *E. coli* cell some advantage by reducing internal osmotic pressure. Generally, oligomerization allows proteins to bury portions of their surfaces thereby reducing both the number of ions required to neutralize exposed charges and the amount of ordered water needed to hydrate the protein surface [37]. Albeit a small effect for a single protein, the additive contributions of many multimeric structures make this possibility a feasible consideration. Therefore, it appeared that the formation of the tetramer simply coordinated two equal and independent dimeric enzymes into one molecule for the purposes of increased stability, increased solubility and decreased osmolarity. To test if the stability is a rationale for the tetrameric structure of *E. coli* SCS, wild type and dimer forms could be treated in parallel with a number of cellular proteinases. If the dimer is more susceptible to degradation than the native enzyme, nature may have developed a higher-ordered quaternary structure to prevent these proteolytic enzymes from accessing these protease sensitive sites.

The existance of an active dimer of *E. coli* SCS, undimished in efficacy, also obviated the hypothesis that alternating sites cooperativity operated in this enzyme. This conclusion is consistent with observations noting that the *E. coli* enzyme did not exhibit

half-of-the-sites reactivity with respect to its phosphorylation [38-40] or that the tetramer was dependent on a cooperative mechanism for its function [39]. The observation of phosphorylation of both active site histidine residues and the lack of asymmetry between dimers constituting the tetramer in the crystallographic structure of *E. coli* SCS [29] was also consistent with this deduction.

As described in **Chapter 8**, ATP facilitated de-thiophosphorylation of *E. coli* SCS (in the presence of succinate and CoA) was taken as support for an alternating sites cooperative model functioning in this enzyme [41]. However, the de-thiophosphorylation of the DDER dimeric mutant was observed to be stimulated by ATP as effectively as the wild type enzyme. Therefore, this effect in *E. coli* SCS did not require two alternating and cooperative sites (dimers) but simply one. This result was consistent with the observation that dimeric pig heart SCS demonstrated the same facilitated de-thiophosphorylation by GTP [42]. Moreover, prior dephosphorylation enabled subsequent thiophosphate incorporation to reach maximal levels in both the native (two -PSO_2 per $\alpha_2\beta_2$ tetramer) and dimer (one -PSO_2 per $\alpha\beta$ dimer muant) forms of *E. coli* SCS. These results were inconsistent with the theory of 'half-of-the-sites' reactivity with respect to enzyme phosphorylation. From this evidence and the fact that the dimeric form contained one active site with wild type activity, it can be concluded that an alternating sites cooperative mechanism was not required by *E. coli* SCS.

These results also corroborated those observed with a hybrid tetramer of *E. coli* SCS which was created with one active site inactivated by mutation of the catalytic histidine residue [39]. This hybrid was catalytically active and displayed typical properties of substrate synergism. This result, along with that from the dimer mutant, suggest that tetrameric *E. coli* SCS was composed of independent $\alpha\beta$ dimers which could bind ATP while existing in a phosphorylated state. Since NTP facilitated de-thiophosphorylation in the dimer of both *E. coli* SCS (**Chapter 8**) and pig heart SCS [42], it is likely that both of these enzymes share a similar 'same-site' displacement

mechanism [6, 42] functioning during catalysis. Here, the incoming γ-phosphate of ATP would be rationalized to displace, in a concerted manner, the phosphate from the enzyme to form succinyl-phosphate at the same active site.

High concentrations of phosphate were observed to prevent the 'synergistic' effects of ATP with succinate and CoA to de-thiophosphorylate both wild type and dimer forms of *E. coli* SCS (**Chapter 8**). This inhibitory effect may be due to the occupation of the ATP or phosphoryl binding site by phosphate. This action would preclude ATP from effectively displacing covalently bound thiophosphate from the enzyme. This proposal is consistent with the above 'same-site' mechanism and additionally suggests that phosphorylation by ATP was responsible for the displacement effect.

Of equal importance, these investigations have helped elucidate the correct quaternary structure of *E. coli* SCS. Unlike the published structure, the alternative tetramer was less globular in nature with a relatively open configuration. In addition, the contributions to the active site were different. Instead of being formed from two ß subunits and one α subunit in the published tetramer, each active site in the alternative quaternary arrangement was composed only from contributions of the α and ß subunits in one dimer. The alternative tetramer precluded a potential rationale for the tetramer structure of *E. coli* SCS which was provided by the published structure. Here, the contributions of the ß subunit to the neighboring CoA binding site implied that both dimers were required to form each active site. However, in the alternative model, these interactions were actually those between different tetramers as they packed together in the crystal defined by the space group $P4_322$. Since this crystal form was dependent on CoA [43], this substrate coordinated tetramer associations within the crystal. A second crystal type was grown from phosphate media in the absence of CoA and determined to a have a different space group of $P4_222$ with half a tetramer in the asymmetric unit. Given the differences, these phosphate crystals will have a unique packing of tetramers within the unit cell. Confirmation that the alternative tetramer is the correct quaternary

arrangement for *E. coli* SCS may come from future diffraction analyses on these phosphate crystals. Likewise, crystallization trials of the *E. coli* SCS enzymes containing ß subunit mutations based on the published tetrameric structure (**Chapter 7**), should also be useful in providing corroborating evidence of the true quaternary structure. Following the alternative model, these mutations would exist at sites of crystal contacts between tetramers which pack together in the crystal (spacegroup $P4_322$). Since these interactions are removed, any crystals formed from such mutants would necessarily have to be of a different packing arrangement and likely from different crystallization conditions. Preliminary experimentation to determine if this is the case is in progress. If suitable crystals of a different form are obtained, diffraction data of a higher resolution may conceivably allow an improvement in the refined structure of *E. coli* SCS.

Along a similar line, the crystal structure of the DDER dimer mutant of *E. coli* SCS should provide valuable structural information. Such studies may reveal if any secondary or tertiary structural changes have accompanied the transition from tetramer to dimer. These are likely to be few since the catalytic efficacy of the dimer form has not been compromised. Interestingly, initial crystallization trials indicate that the dimer form of *E. coli* SCS does not crystallize under the same conditions required by wild type enyzme [44].

With the assumption that no great conformational changes in the dimer mutant have occurred, these future structural investigations could be coupled with kinetic analyses. An assessment of the kinetic parameters may indicate if the binding of any substrate has changed. Such information would complement the available structural data for this mutant.

The extended, 'winged' conformation of the alternative tetramer of SCS may imply that this structure might interact with other proteins. Early fractionation studies revealed that the *E. coli* SCS enzyme was associated with components in the membrane fraction [45]. In addition, SCS has been found to associate specifically with the α-

ketoglutarate dehydrogenase complex (αKDC) which catalyzes the production of the succinyl-CoA (and NADH) in the preceeding step of the citric acid cycle [46]. It is therefore possible that substrate channeling [47] might occur between these members of a putative metabolon [48]. To probe the interactions of these proteins, crystal contact mutants of *E. coli* SCS (**Chapter 7**) could be used to determine if the associations between itself and the αKDC have changed. If so, these mutations may implicate a surface region of SCS responsible for specific contacts with this enzyme complex. It would also be interesting to determine if the mutant dimer could also maintain interactions with the αKDC. Productive associations observed in the latter case, could lead to a more in-depth structural analysis of this complex through crystallography.

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Appendices

Appendix I. Oligonucleotides

Name	Sequence (5'→3')
1. λF (λgt11 Forward)	GGTGGCGACGACTCCTGGAGCCCG
2. λR (λgt11 Forward)	TTGACACCAGACCAACTGGTAATG
3. Universal forward (M13)	GTAAAACGACGGCCAGT
4. Universal reverse (M13)	AACAGCTATGACCATG
5. PHßmatNco	TATGGCCATGGTGAACCTGCAGGAATACCAG

E. coli SCS mutagenesis oligonucleotides

(published structure: 6-16; alternative structure: 17, 18)

6. R14ßD	CAACTTTTTGCCGACTATGGCTTACCAGC
7. R70ßG	GAAGACATCGGTGCTTTTGC
8. E74ßK	GCTTTTGCAAAAAACTGGCTGGGC
9. E231ßA	GATCTGCGCGCAATGCGTGACC
10. Q247ßK	GAAGCACAGGCTGCAAAATGGGAACTGAAC TACG
11. R29ßD	GCCTGTACTACTCCGGACGAAGCAGAAGAA GC
12. K66ßA	GTTGTAAACAGCGCAGAAGACATC
13. E33ßA/S36ßA	CGCGCGAAGCAGAAGCAGCCGCTGCAAAAA TCGGTGCCG
14. R29ßA/E33ßA/S36ßA	GCCTGTACTACTCCGGCCGAAGCAGAAGCAG CCGCTGCAAAAATCGGTGCCG
15. E231ßW	CCAGCCTGATCTGCGCTGGATGCGTGACCAG TCGC
16. Q247ßW/E249ßW	GCGTGAAGCACAGGCTGCATGGTGGTGGCTG AACTACGTTGCGCTGG
17. Ph/ER	CATAAAGTTGCGCTTGATATCATCGATGGCA TCAAGGATTCTCAGGGAGAACGCCTG GCGTTCAAACTGG
18. DDER	CCGCTGACTGGCCCGGATCCGGATCAGGGAG AACGCCTCGCGTTCAAACTGG

Appendix II. Bacterial Strains and Genotypes

Strain	Genotype	Reference
DH5α	*supE*44, Δ(*lac*)U169, (ø80 *lacZ*ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1	[1]
Y1090	Δ(*lac*)U169, Δ(*lon*), *araD*139, *strA*. *supF*,*mcrA*, *trpC*22::Tn10, [pMC9 amp^r tet^r]	[2]
JM103	*endA*1, *hsdR*, *supE*, *sbcBC*, *thi*-1, *strA*, Δ(*lac-pro*), F'[*traD*36, *lacI*^{q}ZΔM15, *proAB*$^+$]	[1]
JM109	*recA*1, *mcrA*, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, (r_{k-}, m_{k+}), *supE*44, *relA*1, *thi*Δ(*lac-proAB*), F'[*traD*36, F'[*traD*36, *lacI*^{q}ZΔM15, *proAB*$^+$]	[3]
N99cI$^+$	*E. coli* K-12 λ lysogen; carries wild type λ cI repressor	[4]
N4830-1	F-, *su*-, *his*-, *ilv*-, *gal*KΔ8, Δ(*chl*D-*pgl*), λ, ΔBam, N+, λcI*ts*$_{857}$, ΔH1	[5]
BL21(DE3)	*hsdS*, *gal* (λcI*ts*$_{857}$), *ind*1, Sam7, *nin*5, *lac*UV5-T7, gene*1*	[6]
TK3(D18)	Δ(*kdp-suc*)D18, Δ(*gal-bio*)	[7]
CJ236	*dut*, *ung*1, *thi*-1, *relA*1/pCJ105(*cam*^{r} F')	[8]

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Appendix III. Media and Buffers

Media	Components
M9-minimal	50 mM $Na_2HPO_4 \cdot 7H_2O$, 35 mM KH_2PO_4, 10 mM NaCl, 20 mM NH_4Cl, 0.4% (w/v) glucose
SOB	2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, pH 7.0
LB	1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 1% (w/v) NaCl, pH 7.0
DLB	1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 1% (w/v) KCl, pH 7.0

Buffers	Components
SM	100 mM NaCl, 6.6 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl, pH 7.5
TNT	10 mM Tris-HCl, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 8.0
TK	50 mM Tris-HCl, 150 mM KCl, pH 7.5
TKN	50 mM Tris-HCl, 150 mM KCl, 0.05% (v/v) Tergitol NP-40, pH 7.5
BTK	100 mM Tris-HCl, 150 mM NaCl, pH 7.5
TE	10 mM Tris-HCl, 1 mM EDTA, pH 8.0
TAE	40 mM Tris-acetate, 1 mM EDTA, pH 8.0
TBE	0.5X = 45 mM Tris-borate, 1 mM EDTA, pH 8.0
TST	50 mM Tris-HCl, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 7.6
KPCEP	50 mM KH_2PO_4, 50 mM KCl, 1 mM EDTA, 0.1 mM PMSF, pH 7.4
KPCE	50 mM KH_2PO_4, 50 mM KCl, 1 mM EDTA, pH 7.4