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EFFECTS OF HYDROPHOBICITY AND CONFORMATION ON THE RETENTION BEHAVIOUR OF PEPTIDES IN REVERSED-PHASE CHROMATOGRAPHY

by

TERRANCE J. SEREDA

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

FALL 1994

ISBN 0-612-01787-7

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Colin T. Mant
5 July 1995

CHAPTER II

Effect of the α-amino group on peptide retention behaviour in reversed-phase chromatography: Determination of the pK_a values of the α-amino group of 19 different N-terminal amino acid residues

This paper has been submitted to the Journal of Chromatography, has been accepted and appears as the following publication:

(1) T.J. Sereda, C.T. Mant, A.M. Quinn and R.S. Hodges, *J. Chromatogr.*, 646 (1993) 17-30.

INTRODUCTION

It was initially recognized by several research groups, using a wide variety of peptides, that the chromatographic behaviour of peptides in reversed-phase chromatography (RPC) could be correlated with amino acid composition [1 - 3]. These groups determined sets of coefficients for predicting peptide retention using computer-calculated regression analysis of retention data. This laboratory has been active for several years in attempting to correlate peptide retention behaviour in RPC with peptide structure through the use of model synthetic peptide analogues. This minimilistic approach is the method of choice since it allows for a systematic reduction in the number of variables that affect retention behaviour. We have shown that the major contributing factor to peptide retention times on RPC is amino acid composition [4 - 7], although factors such as peptide chain length [4, 8] and the presence of preferred binding domains [4, 9] are also pertinent. Such work has practical relevance not only in determining the best approach to the optimization of peptide separations, but also in deducing the presence of amphipathic α-helical structure in peptides based upon their retention data [4, 9]. In addition, we have shown that the

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DEGREE:	MASTER OF SCIENCE
YEAR THIS DEGREE GRANTED:	FALL 1994

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Effects of Hydrophobicity and Conformation on the Retention Behaviour of Peptides in Reversed-Phase Chromatography** submitted by Terrance J. Sereda in partial fulfillment for the degree of Master of Science.

Robert S. Hodges

Charles F. Holmes

Bob Ryan

Paul G. Scott

Date: September 27, 1994

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ABSTRACT

The purpose of this thesis is to expand our knowledge about the retention behaviour of peptides in reversed-phase liquid chromatography (RPLC) by attempting to correlate retention behaviour of peptides to hydrophobicity and conformation. Model synthetic peptides have been used in all studies in order to sytematically reduce the number of variables that may affect retention behaviour. Studies in this report focus on the use of either non-helical peptides or α-helical peptides (amphipathic and non-amphipathic).

We have examined the contribution of the α-amino group to retention behaviour for peptides in RPC using two series of non-helical peptide analogues, either N^{α}-acetylated or non-acetylated , i.e., containing an α-amino group. The effect of the α-amino group, at pH 2, on the hydrophobicity of the side-chain of the N-terminal residue was obtained by referencing the retention time of the acetylated or non-acetylated peptide to the retention time of a glycine analogue. It was shown that the presence of an α-amino group could decrease or increase the hydrophobicity of the side-chain of the N-terminal residue with respect to the hydrophobicity of the side-chain in the absence of an α-amino group. Increasing pH was shown to increase retention time dramatically for the non-acetylated analogues, through the deprotonation of the α-amino group. By separating pairs of acetylated/non-acetylated analogues over the pH range 2-9, it was possible to determine the pK_a of the α-amino group, where it was shown that the pK_a was dependent on two probable factors: (1) the inherent hydrophobicity of the stationary phase; and (2) the amino acid substituted in the N-terminal position.

Two series of potentially amphipathic α-helical peptides, a native Ala peptide (AA9) and a native Leu peptide (LL9), were designed so that its non-polar face, which may interact specifically with the stationary phase, contains 7 residues of either Ala or Leu, respectively. This design results in an overall hydrophobicity of the non-polar face of the Leu peptide that is greater than that of the non-polar face of the native Ala peptide. Mutants of the native Ala-face peptide, AX9, and the native Leu-face peptide, LX9, were designed

by replacing one residue in the centre of the non-polar face in both series of peptides. Therefore, by changing the hydrophobicity of the environment surrounding the mutated amino acid side-chain, the effect on the hydrophilicity /hydrophobicity of each amino acid side-chain could be determined. Using all 20 amino acid substitutions it was shown that the maximum hydrophilicity of these amino acid side-chains could be determined when the environment surrounding the mutation is maximally hydrophobic; whereas, its maximum hydrophobicity can be determined when the environment surrounding the mutation is minimally hydrophobic.

The reversed-phase behaviour of 2 different series of peptides, one non-helical and the other α-helical, were studied under 4 different linear AB gradients in order to determine the effect of conformation on the selectivity of the separation. We have shown that plots of $\log \bar{k}$ *vs* $\bar{\phi}$ are very similar for any one peptide conformation, i.e., peptides from either the non-helical or amphipathic α-helical series exhibit similar S values. It has also been shown that if mixtures of peptides from the two different series are separated using either increasing or decreasing gradient rates, large increases in resolution occur due to selectivity (which may be attributed to the difference in S values). In addition, the S value of a polymer from a peptide in the non-helical series and the S value of a non-amphipathic α-helical peptide further suggest that the difference in selectivity between the two series of peptides is due to the difference in conformation.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. R.S. Hodges, in 2 respects; firstly for facilitating my entrance into the Department of Biochemistry to start a Master's program and secondly for his direction in the topics chosen and for his enthusiastic and knowledgeable discussions on the many and varied aspects of the biochemistry directly relating to the topics in my project.

I would also like to thank Dr. C.T. Mant for his involvement in my work throughout the program, specifically, for his involvment in completion of the projects, in terms of manuscript preparation. It is without doubt that his command of the English language allowed for rapid acceptance of manuscripts for publication. As well, access to his vast knowledge of chromatographic techniques made it easy to learn various concepts in the field and his willingness to listen to practice seminars is greatly appreciated. In addition, I am grateful for the extensive amount of time devoted to the development of the ligand-receptor model described in Chapter III.

I would also like to thank Dr. F. Sönnichsen for his work on the molecular modeling, which was an integral part of the publication, done in Chapter III.

I would also like to thank my committee members, Dr. C.F.B. Holmes, Dr. R.O. Ryan and Dr. P.G. Scott for their critical analysis and evaluation of the thesis.

The number of peptides synthesized, purified and characterized in the studies throughout the program was enormous and thus only in a department which has the technical expertise could this be accomplished. I therefore thank, in no particular order, the people responsible for completing this task: Paul Semchuk, Ian Wilson, Christi Andrin, Len Daniels and Cindy Shaughnessy for peptide synthesis, purification and mass spectral data; Terri Keown and Mike Carpenter for amino acid analysis and Kim Oikawa for CD spectra and finally T.W.L. Burke for his technical skills and willingness to help with any problem that arose in the lab.

Lastly, I would like to thank those who helped me deal with the daily trials and tribulations of working in such a competitive atmosphere: Jennifer Van Eyk, Rose Caday, Oscar Monera, Morris Aarbo, Dawn Lockwood and Janet Wright.

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LIST OF ABBREVIATIONS AND SYMBOLS

a	parameter showing the effect of the α-amino group on retention behaviour
Ala (A)	alanine
Asn (N)	asparagine
Asp (D)	aspartic acid
Arg (R)	arginine
β	ratio of gradient times = t_{G2}/t_{G1} or a carbon atom one displaced from the α-carbon
b	gradient steepnees parameter
Boc	tert-butyloxycarbonyl
Br-Z	2-bromobenzyloxycarbonyl
Bzl	benzyl
CD	circular dichroism
Cl-Z	2-chlorobenzyloxycarbonyl
CHO	formyl
4-CH_3Bzl	4-methylbenzyl
CN	cyano
CVFF	consistent valence force field
Cys (C)	cysteine
Δt or Δt_R	difference in retention times
ΔNPASA	change in non-polar surface area between mutant and a Gly mutant peptide
ΔΔNPASA	difference between the ΔNPASA of the Ala-face and the ΔNPASA of the Leu-face
DCM	dichloromethane
deg	degrees
DIEA	N,N-diisopropylethylamine

LIST OF ABBREVIATIONS AND SYMBOLS: continued

dmol	decimole
DMF	dimethylformamide
Dnp	dinitrophenyl
ϕ	backbone dihedral angle = -67°, amide nitrogen to α-carbon
$\bar{\phi}$	median volume fraction, i.e., the volume fraction of organic when the peptide is at the midpoint of the column
Gly (G)	glycine
Gln (Q)	glutamine
Glu (E)	glutamic acid
h	hydrophobicity of amino acid side-chain in the presence of an α-amino group
H	hydrophobicity of amino acid side-chain in the absence of an α-amino group
H^1 - NMR	proton nuclear magnetic resonance
HPLC	high performance liquid chromatography
His (H)	histidine
HOBT	hydroxybenzotriazole
$\bar{k}$	median capacity factor, i.e., the capacity factor when the peptide is at the midpoint of the column
k_0	capacity factor of peptide in the absence of organic modifier
Ile (I)	isoleucine
Lys (K)	lysine
Leu (L)	leucine
M or MW	molecular weight
Met (M)	methionine
$NaClO_4$	sodium perchlorate
NPASA	non-polar accessible surface area
OBzl	o-benzyl

LIST OF ABBREVIATIONS AND SYMBOLS: continued

OcHex	o-cyclohexyl
Pro (P)	proline
Phe (F)	phenylalanine
PSDV	polystyrene-divinylbenzene
RPC	reversed-phase chromatography
RPLC	reversed-phase liquid chromatography
ψ	backbone dihedral angle = -44°, α-carbon to carbonyl carbon
Ser (S)	serine
SPPS	solid phase peptide synthesis
S	slope for the graph of $\log\bar{k}$ *vs* $\bar{\phi}$
t_g	retention time of peptide under gradient conditions
t_0	column dead time
t_0'	column dead time plus column dwell time
t_G	time for gradient to reach 100% B
t_R	retention time in min
TEA	triethylamine
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
Thr (T)	threonine
Tos	4-toluenesulfonyl
TEAP	triethylammonium phosphate
Trp (W)	tyrptophan
Tyr (Y)	tyrosine
Val (V)	valine
Z	benzyloxycarbonyl

CHAPTER I

General Introduction

INTRODUCTION

High resolution separations and analysis techniques, through the use of high performance liquid chromatography (HPLC), are essential to biochemical studies in the life sciences [1] and more specifically reversed-phase chromatography (RPC) has been an important tool in the study of peptides and proteins [2]. This fact is evidenced by recent textbooks on this subject: (1) HPLC of Peptides and Proteins [3] (2) HPLC of Proteins, Peptides and Polynucleotides [4] and recent reviews: (1) Separation and Analysis of Peptides and Proteins [2] and (2) Analysis of Peptides [5].

The purpose of this thesis is to gain a better understanding of the reversed-phase chromatographic behaviour of peptides through the use of model synthetic peptides. Each chapter in this thesis contains a brief introduction on the topic being studied; therefore, a brief description of reversed-phase chromatography including the types of reversed-phase supports (e.g., silica based and polymer based) and their characteristics, mobile phases and mobile phase additives and monitoring techniques will be discussed here. A brief description of the characteristics of the retention process in RP separations of peptides/proteins will also be discussed.

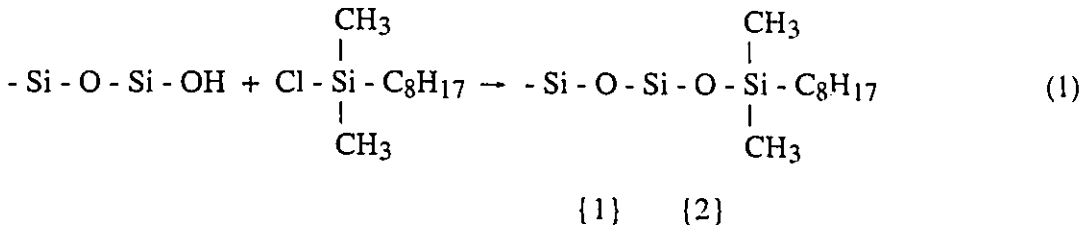
REVERSED-PHASE CHROMATOGRAPHY

Silica based columns

Silica-based supports are most widely used in HPLC applications [6,7] and specifically the vast majority of peptide separations are performed on reversed-phase supports, in which a non-polar ligand is covalently bound to the silica [5]. The composition of the silica that makes up these supports is of the general formula $[SiO_2 \cdot mH_2O]_n$ [8] and

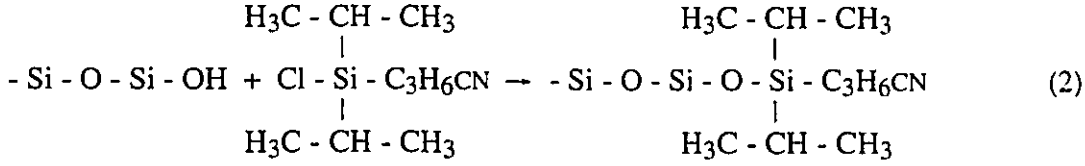
the water that is bound to the silica is responsible for forming the silanol groups [7], i.e., Si-OH which are important sites in the chromatographic process, e.g., the site for covalent linkage of the non-polar ligands that characterize a particular RP support or a site which may cause unwanted chromatographic effects. These silanols exist in 3 different forms, single (or free silanols [9]) geminal or vicinal [10] and depending on their pK_a, these residual silanols [10] may form negatively charged sites, i.e., $Si\text{-}O^-$ that are named siloxanyl sites [8]. It is these residual silanols that are responsible for the undesirable chromatographic effects, e.g., peak tailing and increased retention times, seen with peptide solutes containing positively charged amino acid residues [11]. It has also been suggested that in addition to these ionic type interactions with basic solutes, hydrogen bonding of the silanol group may also occur with amines [10]. A non-polar ligand may be covalently linked to the silica using alkylchlorosilanes, e.g., octyldimethylchlorosilane $\{ClSi(CH_3)_2C_8H_{17}\}$ [6], through the following reaction: $-SiOH + Cl\text{-}SiR_3 \rightleftharpoons Si\text{-}O\text{-}SiR_3 + HCl$ [7] which is shown in Figure I-1. There are a variety of bonded phases including groups as C_3, C_4, C_8, C_{18}, 3-cyanopropyl ($-C_3H_6CN$) and the benzyl group ($-C_6H_5$) [8]. Silica supports are normally used in the pH range 2 to 8 [5], this being due to two different types of silicon-oxygen-silicon siloxane bonds, i.e., Si-O-Si in bonded reversed-phase supports. The first type of siloxane bond, labeled {1} in Figure I-1, first equation, which comprises the majority of the silica particle, is hydrolysed at high pH, e.g., pH >7.5 [12] and the second type of siloxane bond, labeled {2} in Figure I-1, first equation, that which links the silane to the silica support is hydrolysed at low pH typical of peptide/protein separations, e.g., use of aqueous TFA [13]; whereas, the siloxane bond labeled {2} is apparently not significantly attacked at higher pH [12]. Recently, stable bonded phases have been developed, in which the dimethyl groups of the silane are replaced by the larger and bulkier diisopropyl groups, i.e., (3-cyanopropyl)-diisopropylchlorosilane [14] which is shown in Figure I-1, second equation, or diisobutyl groups, i.e, diisobutyloctadecyl-

silane [12] which results in greater stability of the siloxane connecting the silane to the support at low pH conditions (0.1% TFA in buffers at 50° C and 1% TFA at 90° C



(1)

{1} {2}



(2)

Figure I-1 Derivatization of silica with monofunctional silanes. First equation: the monofunctional silane, octyldimethylchlorosilane, is covalently linked to the silica support. After derivatization of the silica, two types of silicon-oxygen-silicon siloxane bonds will be present in the structure, labeled here as {1} and {2}. The first siloxane bond {1} represents the types of bonds that will be present in the basic structure of the silica particle; whereas, the second siloxane bond {2} represents the covalent linkage of the silane to the support. Second equation: depicts the covalent linkage of (3-cyanopropyl)-diisopropylchlorosilane to the silica support.

respectively). It has also been suggested that these diisobutyl stable bond columns may be used up to pH 9 for short periods of time (approx. 300 hrs) [12]. The reversed-phase supports that are produced by the reaction described above, using monofunctional silanes, result in the formation of a monolayer that covers the silica support [14]. Other RP-supports can be made using polyfunctional silanes which can result in the formation of a polymerized layer on the support surface, with the major disadvantage being the reproduction of the polymerized surface (therefore resulting in differences in chromatographic behaviour from batch to batch). It should also be noted that silicas may contain trace amounts of metals [10] resulting in undesirable effects, e.g., iron and nickel may cause peak tailing with solutes that form complexes with these ions [9] or peak tailing may be observed with basic solutes due to the presence of Al, which increases the acidity of the silanol group [9,10].

In addition to the type of bonded phases, silica supports are usually identified based on 2 additional characteristics: (1) pore diameter (p_d) and (2) particle size (d_p). Typical commercial packings have a pore diameter of 150 or 300 Å, but larger sizes may also be obtained including 500, 1000 and 4000 Å [8]. Views differ when it comes to the appropriate pore size that should be used for a particular separation; but, *generally* speaking for a peptide/protein that is greater than 15 000 MW [15] or in the range 10 000 to 100 000 [16], a 300 Å silica should be used. For example, a 200 Å silica was used to separate a very large range of peptides and proteins resulting in reasonable separations: (1) a mixture of 5 peptides including oxytocin (1007) to angiotensen I (1673) (2) a mixture of 5 proteins including ribonuclease (12 640) to ovalbumin (43 500) and (3) a mixture of 8 proteins including aprotinin (6 500), carbonic anhydrase (29 000) and hexokinase (100 000) [17]. Two other types of silicas have been shown to be useful in the separation of peptides: the purification of melittin (26 amino acids) on a micropellicullar stationary phase consisting of a thin C_{18} layer on 2 µm solid silica microspheres [18] and the separation of ovalbumin and carbonic anhydrase, lysozyme and cytochrome C on a superficially porous silica microsphere, i.e., a particle that consists of a 5 µm solid core and a 1 µm thick outer shell of 300 Å pore size [19]. As with pore size, a large range of particle sizes may be obtained commercially with typical sizes being 3, 5, 7, 10, 15, 20, and 40 µm [8] where the 5 to 10 µm particle size is generally used for analytical separations [5], supports of 3 µm are used for high efficiency separations and the 10 µm or greater may be used for preparative separations [7].

Polystyrene based columns

As previously mentioned, silica based supports may be degraded by high pH buffers, e.g., pH > 7.5; therefore, in order to circumvent this problem polystyrene based supports were developed with improved mechanical stability so that they could be used for HPLC applications. A very common polymer based support is polystyrene-divinylbenzene (PSDV) which is produced by polymerizing styrene, i.e., CH_2 = CH-phenyl in the

presence of divinylbenzene, i.e., CH_2 = CH-C_6H_4-CH = CH_2 which crosslinks the polystyrene thereby adding additional mechanical strength to the polymer [20]. In addition to an extended range of pH operation, i.e., pH 1 to 14 [16], it is also been suggested that these supports have an advantage over silica based supports because at high pH, the polymeric supports will not contain residual silanols. As with silica based supports, the polymeric supports are available in the same distribution of pore size and the same general rules for solute /pore size apply as for silica based supports [20]. When the PSDV supports are used for a RP separation, one may expect selectivity differences, as compared to a silica based column containing non-polar ligands, due to the possibility of π - π interactions or charge transfer interactions [20] (double bonds may function as a Lewis base, i.e., electron pair donors, and therefore may be involved in Lewis acid-base interactions which are termed π-complexes). An example of selectivity differences between a C_8 and a PSDV column has been shown [5] where, relative to the same analog, a Tyr and Trp analog are 1.3 and 2.2 min more retentive on PSDV.

Mobile phase and mobile phase additives

Elution of peptides/proteins from a reversed-phase column is typically achieved under linear AB gradient conditions where the mobile phase is composed of an aqueous eluent A and a non-polar eluent B. These eluents can be divided into 2 different categories: (1) organic modifier (2) ion-pairing reagents and buffer systems.

Organic modifiers. Peptides/proteins are usually loaded onto a reversed-phase column in a mobile phase that is typically aqueous and elution of the solute is then achieved by introduction of a second eluent which is mainly composed of an organic eluent. The most typically used organic modifier is acetonitrile (CH_3CN); however, the more non-polar solvent isopropanol (2-propanol) may be used to elute peptides which are very hydrophobic or the more polar solvent methanol may be used to elute very hydrophilic peptides [21]. Using a series of 5 different standard peptides of increasing hydrophobicity,

it has been shown that the order of elution is of increasing hydrophobicity and using the organic modifiers mentioned above, isopropanol results in the shortest retention times of the standards; whereas, the use of methanol results in the longest retention times indicating that increased non-polarity of the organic modifier results in increased effectiveness in

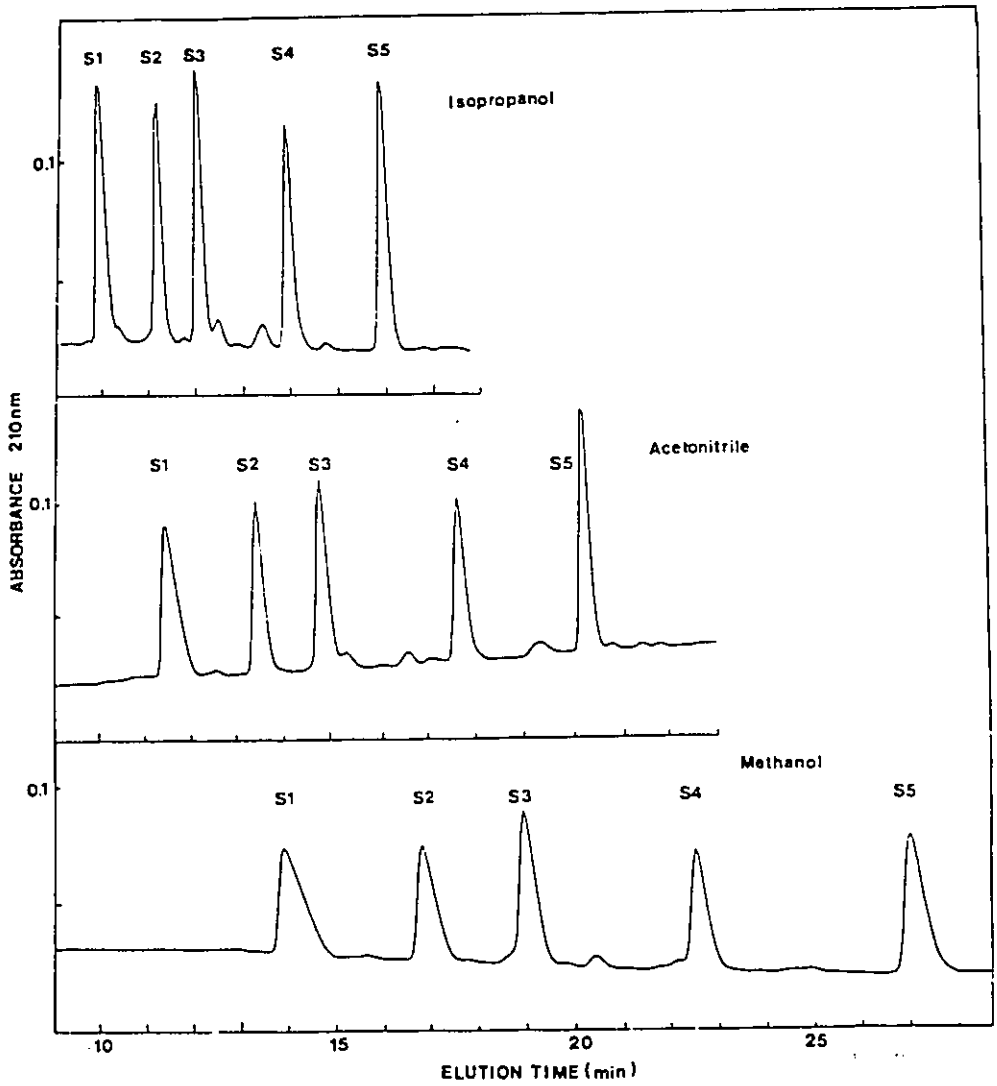


Figure I-2 Effect of organic modifier on the reversed-phase separation of a mixture of 5 synthetic peptide standards. Column: SynChropak RP-P C_{18}, 6.5-μm particle size, 300 Å pore size. Mobile phase: linear AB gradient (1%B/min) where eluent A is 0.1% TFA and eluent B is 0.1% TFA in either isopropanol, acetonitrile or methanol at a flowrate of 1ml/min. S2-S5 are 10 residue peptides which are N-terminal acetylated and C-terminal amides which differ by only one amino acid: S2 = Gly-Gly, S3 = Ala-Gly, S4 = Val-Gly and S5 = Val-Val and S1 is S3 with a free α-amino group. Taken from [21].

eluting a solute from the stationary phase [21]. The increasing retention time with increasing polarity of the organic modifier may be observed in Figure I-2, e.g., the peptide S5 has a retention time of approximately 16, 20.5 and 27 min in isopropanol, acetonitrile and methanol respectively. Since the peptides in this figure differ only by one amino acid, the resolving power of RPC is well demonstrated. Other organic modifiers that have been used for the elution of peptides/proteins from reversed-phase columns include (1) 1-butanol (2) 1-propanol and (3) ethanol [22].

Ion-pairing reagents and buffer systems. The eluents used in RPC may consist of unbuffered solutions (e.g., at pH 2) or buffered solutions at higher pH values. At low pH (pH 2) the additives essentially act as ion-pairing reagents, i.e., a charged component of the reagent ion-pairs with a charged component of the peptide/protein; whereas, at higher pH, the additive may ion-pair with the peptide in addition to maintaining the pH of the eluent. In terms of ion-pairing capabilities, these reagents may be classified as either anionic or cationic.

Anionic ion-pairing reagents have a negatively charged component that may ion-pair with the positively charged groups in the peptide, e.g., a free α-amino group or the positively charged groups of the side-chains of Lys, Arg or His. Typical anionic ion-pairing reagents used at low pH and unbuffered are (1) phosphoric acid, H_3PO_4 (2) trifluoroacetic acid, TFA and (3) heptafluorobutyric acid, HFBA; where it has been shown that the increasing hydrophobicity of the ion-pair (i.e., H_3PO_4 < TFA < HFBA) results in increased retention time of positively charged peptides [23]. The increasing retention time with increasing hydrophobicity of ion-pairing reagent may be observed in Figure I-3, where it can be seen that, e.g., the peptide C3 elutes at progressively longer retention times in the presence of H_3PO_4, TFA and HFBA respectively. The reagents TFA and HFBA are considered to be hydrophobic anionic ion-pair reagents; whereas, $H_2PO_4^-$ from phosphoric acid is considered to be a hydrophilic anionic ion-pair reagent [23]. These anionic pairing reagents are typically used at low concentrations, e.g., 0.05 to 0.1% v/v;

but it has been shown that if a mixture of peptides of varying charge is separated, the selectivity of the separation may be altered by increasing the concentration of the ion-

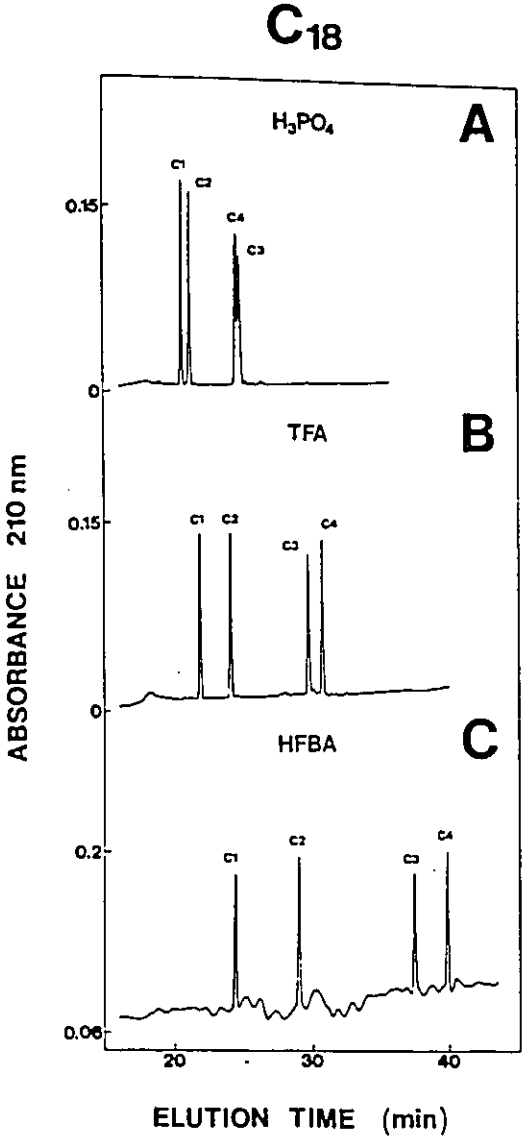


Figure I-3 Effect of ion-pairing reagents on the separation of a mixture of synthetic peptide standards in RPC. Column: SynChropak RP-P C_{18}, 6.5-μm particle size, 300 Å pore size. Mobile phase: linear AB gradient (1%B/min) where eluent A is water and eluent B is acetonitrile, both eluents containing 0.1% H_3PO_4, TFA or HFBA at a flowrate of 1ml/min. C1 to C4 represent a series of synthetic peptides which vary in net charge from +1 to +4 respectively. Taken from ref [23].

pairing reagent (e.g., to 0.8%) [23]. This observation can be made from Figure I-4, where it may be observed that peptides 4 and 6 exhibit dramatic increases in retention time at 0.8% TFA as compared to 0.05% TFA. TFA and HFBA are volatile reagents [24] and therefore preferred to phosphoric acid, which is non-volatile [23], in situations where removal of the eluent by lyophilization is required. The sodium salt of the organic acids from a group of alkylsulphonates (e.g., 1-hexanesulphonic acid) may also be used as an anionic ion-pairing reagent, at a concentration of 0.01 M, for the separation of polar peptides [25].

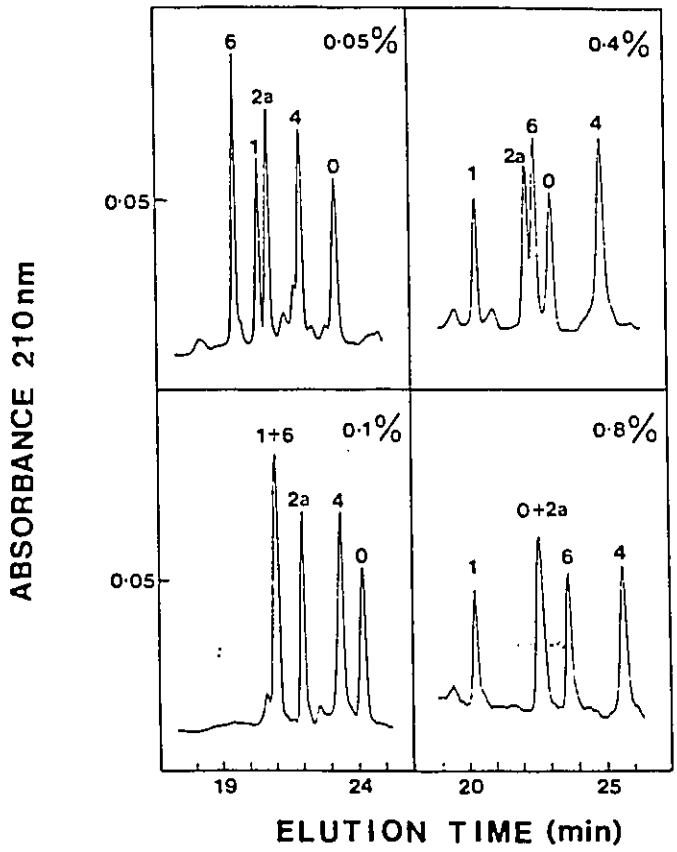


Figure I-4 Effect of concentration of anionic pairing reagent in RPC on the elution profile of a mixture of peptides with varying numbers of positively charged groups. Column: as in Figure I-3. Conditions: linear AB gradient (1%B/min) where eluent A is water and eluent B is acetonitrile, both eluents containing 0.05, 0.1, 0.4 or 0.8% TFA, at a flowrate of 1ml/min. Designation of peptide refers to the net positive charge on the peptide. Taken from [23].

Cationic ion-pairing reagents have a positively charged component that can ion-pair with negatively charged groups in peptides/proteins (e.g., an ionized carboxyl group). Generally these reagents are used over a wide range of pH values [24,25] where they act to maintain pH or ion-pair with the α-carboxyl group and the side-chain carboxyl groups of Asp and Glu. Typical tertiary alkylamines used as ion-pairing reagents are (1) triethylammonium phosphate, TEAP [23] or triethylammonium acetate, TEAA, which is volatile [24]. Quaternary ammonium ions may be used as cationic ion-pairing reagents for the separation of peptides in RPC. For example, tetraalkylammonium salts may be used for this purpose at a pH of 7, e.g., tetrabutlyammonium phosphate (0.01M) [25] and similarily the salt $(NH_4)_2HPO_4$ may also be used at pH 7 [11].

Retention behaviour

As indicated in the previous discussion, peptides may be eluted from a reversed-phase column by the introduction of an organic modifier such as acetonitrile. From plots of log capacity factor *vs* acetonitrile concentration, it has been shown that the process of elution for small organic molecules e.g., ketones and for a series of small peptides (10 amino acid residues) is not the same [26]. From Figure I-5, it may be observed that the slope for the organic solute propiophenone (filled circles; $CH_3CH_2C(O)C_6H_5$) is smaller than the slope for either the peptide (open circles; Ac-RGVVGLGLGK-amide) or the protein Apomyoglobin (open squares), with ApoMb exhibiting the largest slope. It is assumed that a slope that approaches a vertical line indicates a 100% adsorption/desorption mechanism. The authors therefore suggested that the organic solute partitioned with the stationary phase to a greater extent than the peptide or protein. More recently; this effect has been further supported using proteins eluted under linear gradient RP conditions, where it was suggested that proteins move very slowly initially, partition with the stationary phase once an appropiate concentration of organic modifier is attained and finally interacts only to a small extent once a certain concentration of modifier is exceeded [27]. Due to this effect, it was further suggested in this report that at high gradient rates, partitioning with the

stationary phase would be less and therefore the column length becomes independent of elution profile and retention time, i.e., with increasing column length, selectivity does not improve and the retention time becomes dependent on the void volumn of the column. Since the slope, from plots of log capacity factor *vs* acetonitrile concentration, is the only factor that is different between peptides and proteins, this would suggest that the only

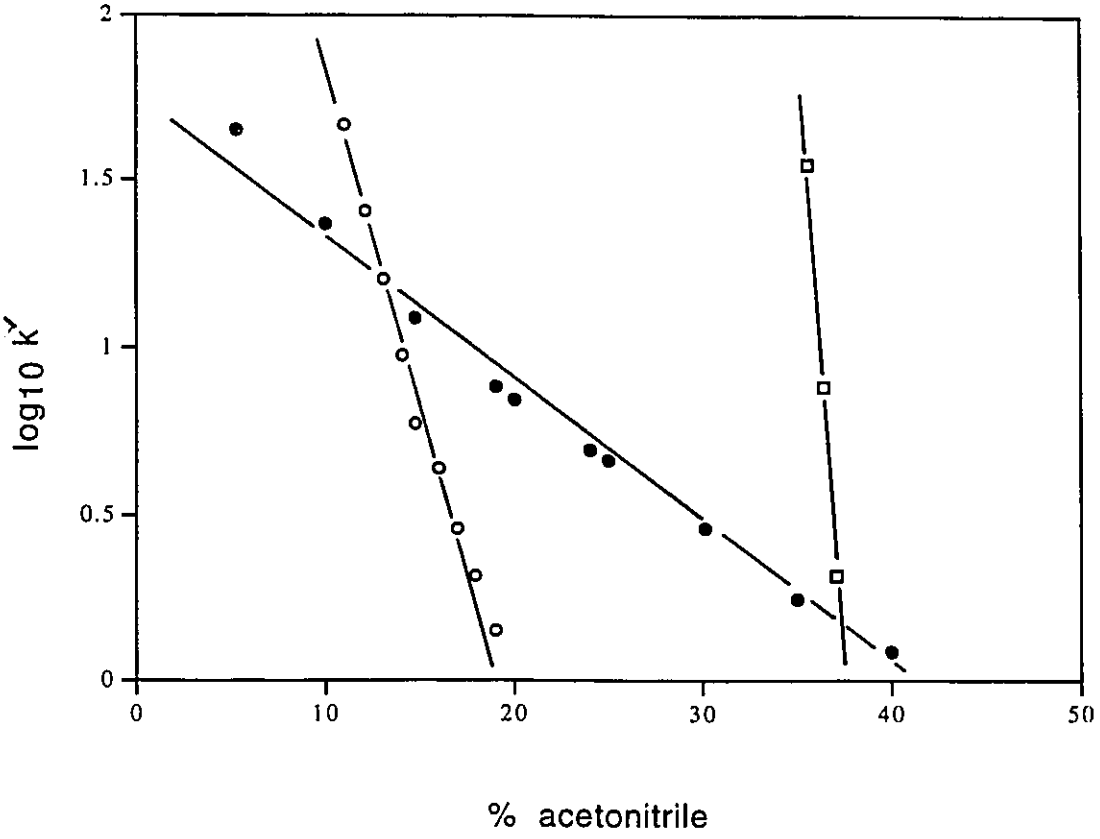


Figure I-5 Effect of acetonitrile concentration on the $\log_{10} k'$ (capacity factor) values of alkylphenones, peptides and proteins. Column: Aquapore C_8 (7-µm particle size, 300-Å pore size). Conditions: isocratic elution with 0.05% aqueous TFA containing various percentage concentrations (v/v) of acetonitrile at a flowrate of 1ml/min. Capacity factor is calculated as: $k' = (t_r - t_0)/t_0$, where t_r is the retention time of the solute and t_0 is the retention time of a unretained solute. The closed circles represent propiophenone, a open circle represents a 10 residue peptide and the squares represent apomyoglobin. Adapted from [26].

difference between peptides and proteins would be the degree to which one would expect peptides to partition with the stationary phase as compared to proteins. It has also been

demonstrated that elution of the peptides neurotensin (13 amino acid residues) and a neurotensin fragment (residues 1-8) and the protein myoglobin on a PSDV column exhibit a similar elution isotherm (retention time *vs* % organic) to that obtained for silica columns, suggesting that the elution mechanism is similar for both types of supports [20].

Monitoring a separation

The most commonly used detection system for monitoring the elution of peptides/proteins is to monitor the ultraviolet (UV) absorption of the peptide bond at a wavelength between 200 and 220 nm with 210 being most commonly used [28,29]. Even though the absorption maximum is approximately 185 nm [28], the use of organic solvents for elution prevents use of wavelengths below the UV cutoff value of these eluents, e.g., the cutoff for acetonitrile is 188 nm; whereas, it is 205 nm for methanol [29]. In peptides, other chromophores may absorb in the UV region, e.g., 280 nm, are Trp and Tyr [28] as well as Phe [29]. Their absorption maxima at neutral pH are 280, 274 and 257 nm for Trp, Tyr and Phe respectively [30]. For peptides/proteins, there is an approximate 4 fold decrease in sensitivity with a 10 nm increase in wavelength [29]. Therefore, this allows one to use a particular wavelength for a particular application, e.g., for analytical separations where only small quantities of material are used, low wavelengths would add sensitivity to the separation; whereas, if large quantities of sample are being used, as in preparative situations, a higher wavelength with the lowered sensitivity may be used to follow the separation.

Since several different types of ion-pairing reagents are used in RPC, it is important to know their absorption characteristics in the wavelength regions used for following an elution profile. For TFA, an optimum wavelength, i.e., for the smallest change in baseline, is 215 nm [31,25]; whereas, for HFBA the optimum is 219 nm [25]. TEAP buffers exhibit varying degrees of absorption which is dependent on pH [25]; e.g., if a pH of 7 is required, the optimum wavelength to monitor would be 220 nm, because at pH > 6 absorption at 210 and 215 increases dramatically. Other cationic ion-pairing reagents such

as tetrabutylammonium phosphate also has an optimum pH of about 5 and absorption increases to pH 8, but not as drastically as for TEAP [25].

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CHAPTER II

Effect of the α-amino group on peptide retention behaviour in reversed-phase chromatography: Determination of the pK_a values of the α-amino group of 19 different N-terminal amino acid residues

This paper has been submitted to the Journal of Chromatography, has been accepted and appears as the following publication:

(1) T.J. Sereda, C.T. Mant, A.M. Quinn and R.S. Hodges, *J. Chromatogr.*, 646 (1993) 17-30.

INTRODUCTION

It was initially recognized by several research groups, using a wide variety of peptides, that the chromatographic behaviour of peptides in reversed-phase chromatography (RPC) could be correlated with amino acid composition [1 - 3]. These groups determined sets of coefficients for predicting peptide retention using computer-calculated regression analysis of retention data. This laboratory has been active for several years in attempting to correlate peptide retention behaviour in RPC with peptide structure through the use of model synthetic peptide analogues. This minimilistic approach is the method of choice since it allows for a systematic reduction in the number of variables that affect retention behaviour. We have shown that the major contributing factor to peptide retention times on RPC is amino acid composition [4 - 7], although factors such as peptide chain length [4, 8] and the presence of preferred binding domains [4, 9] are also pertinent. Such work has practical relevance not only in determining the best approach to the optimization of peptide separations, but also in deducing the presence of amphipathic α-helical structure in peptides based upon their retention data [4, 9]. In addition, we have shown that the

effects on peptide retention of ion-pairing reagents and their concentration of these reagents in the mobile phase, are predictable [10]. Indeed the research in this area, carried out in this laboratory, has lead to the development of a commercially-available computer-based teaching and research program, ProDigest-LC [4, 11, 12], which simulates peptide elution profiles on the major modes of high-performance liquid chromatography (HPLC) employed for peptide separations (size-exclusion, ion-exchange and reversed-phase chromatography).

Under the run conditions employed by researchers for peptide separations, i.e., aqueous trifluoroacetic acid/acetonitrile gradients at pH 2 [4, 13, 14], an α-amino group will exhibit a full positive charge, whilst an α-carboxyl group will be fully protonated, i.e., neutral. Guo et al. [6] demonstrated that, in contrast to a protonated α-carboxyl group which contributed little (either negatively or positively) to peptide retention times in RPC, a positively charged α-amino group made a significant hydrophilic contribution to peptide retention behaviour, i.e., peptide retention times decreased relative to a blocked (acetylated) N-terminal residue. In addition, it was noted that there was a possibility that the magnitude of this contribution by an α-amino group may be dependent on the particular N-terminal residue. When one considers that peptides obtained from such sources as proteolytic or chemical digests of proteins contain N-terminal α-amino (and less importantly, C-terminal α-carboxyl) groups, the importance of taking into account such factors when attempting to correlate peptide structure with peptide retention behaviour in RPC becomes clear.

In the present study, we prepared a series of 40 decapeptide amide analogues (α-amino or N-acetylated), where the N-terminal position was substituted by the 20 amino acids found in proteins. From the retention behaviour of these peptide analogues during RPC, we set out to determine how the presence of a α-amino group affects the retention behaviour of a peptide during RPC and to what extent this effect is dependent on the N-terminal residue.

EXPERIMENTAL

Materials

HPLC-grade water and acetonitrile were obtained from J.T. Baker (Phillipsberg, NJ, USA). ACS-grade orthophosphoric acid and triethylamine (TEA, redistilled before use) was obtained from Anachemia (Toronto, Ontario, Canada). Trifluoroacetic acid (TFA), 99+%, was obtained from Aldrich Chemical Company (Millwaukee, WI, USA). Sodium perchlorate ($NaClO_4$) was obtained from BDH Chemicals Ltd. (Poole, England).

Instrumentation

The high-performance liquid chromatography (HPLC) system consisted of either a Varian Vista Series 5000 chromatograph (Varian, Walnut Creek, CA, USA) or an HP1090 liquid chromatograph (Hewlett-Packard, Avondale, PA, USA), coupled to an HP1040A detection system, HP9000 series 300 computer, HP9133 disc drive, HP2225A Thinkjet printer and HP7460A plotter.

Columns

Peptides were separated on two columns: (1) a silica-based Pep-S C_{18}/C_2 column (250 x 4 mm I.D., 5-µm particle size, 100-Å pore size, plus a 10 x 4 mm I.D. guard cartridge) from Pharmacia LKB Biotechnology (Baie d' Urfé, Quebec, Canada); and (2) a non-silica-based (PSDV) PLRP-S column (250 x 4.6 mm I.D., 5 µm, 100 Å) from Polymer Laboratories Ltd. (Church Stretton, Shropshire, UK).

Peptide Synthesis

Peptides were synthesized using an Applied Biosystems (Foster City, CA, USA) 430A peptide synthesizer using the following protocol. The peptides were synthesized on co-poly (styrene-1% divinyl-benzene) benzhydrylamine-hydrochloride resin (0.92 mmole/g resin). All amino acids were protected at the α-amino position with the Boc-group and the following side-chain protecting groups were used: [Asp(OcHex), Glu(OBzl), Ser(Bzl), Thr(Bzl), Tyr(Br-Z), Lys(Cl-Z), Arg(Tos), Cys(4-CH_3Bzl), His(DNP), Trp(CHO)]. All

amino acids were single coupled as preformed symmetrical anhydrides (with the exception of Arg, Asn and Gln which were coupled as the HOBT active ester) in dichloromethane. Boc-groups were removed at each cycle with an 80 sec reaction with 33% TFA/dichloromethane (v/v), followed by a second reaction with 50% TFA/dichloromethane (v/v) for 18.5 min. Neutralizations were carried out using 10% DIEA/DMF (v/v). N-terminal residues were acetylated using 25% acetic anhydride/dichloromethane (v/v) for 10 min. Prior to cleavage the DNP group of His was removed by treating the peptide resin with a solution of 2-mercaptoethanol (20%) and diisopropylethylamine (10%) in dimethylformamide for 2 hours. The peptides were cleaved from the resin by treatment with anhydrous hydrogen fluoride (20ml/g resin) containing 10% anisole and 2% 1, 2-ethanedithiol for 1 hour at -4° C. After cleavage from the resin, the formyl group of Trp was removed by treating the peptide with 2% piperidine/water (v/v). The resulting deprotected peptide solution was freeze dried. Peptides were determined to be pure by HPLC and mass determination (using a BioIon 20 plasma desorption time of flight mass spectrometer).

RESULTS AND DISCUSSION

Model Synthetic Peptides

Two series of 20 decapeptide analogues (one series of peptides containing an α-amino group and a second series of peptides with an acetylated N-terminal) were synthesized, where the N-terminal residue was substituted with the 20 amino acids commonly found in proteins. The analogues were based on the following sequence:

Ac-Xxx-(Leu-Gly-Ala-Lys-Gly-Ala-Gly-Val-Gly)-amide	Series 1
H-Xxx-(Leu-Gly-Ala-Lys-Gly-Ala-Gly-Val-Gly)-amide	Series 2
Ac-(Leu-Gly-Ala-Lys-Gly-Ala-Gly-Val-Gly)-amide	Core sequence

where Xxx denotes the point of substitution of each of the 20 amino acids found in proteins. In Series 1, the N-terminal residue of the peptides are N^{α}-acetylated and will be referred to in this report as either the acetylated peptides or Ac-Xxx peptides [representing an N^{α}-acetylated peptide analogue substituted at the N-terminal position with residue Xxx, e.g., Ac-Ser represents the acetylated serine analogue]. In Series 2, the peptides contain an α-amino group and will be referred to in this report as either the non-acetylated peptides or H-Xxx peptides [representing a peptide analogue with an α-amino group substituted at the N-terminal position with residue Xxx, e.g., H-Ser represents a N-terminal serine with an α-amino group]. The acetylated core sequence was also synthesized.

The core sequence Ac-(Leu-Gly-Ala-Lys-Gly-Ala-Gly-Val-Gly)-amide was chosen since it lacks any ability to form an amphipathic helix, a preferred binding domain, known to cause deviations from predicted retention behavior [9]. A 10-residue length for the peptide analogues was chosen for two reasons: (1) this size of peptide represents an average sized fragment that may be obtained from a proteolytic digest of a protein; and (2) the effect of chain length on the retention behaviour of a peptide of this size will be small [8]. Residues in the core sequence represent an overall hydrophobicity, as defined by Guo et al. [6], which results in a peptide that is eluted within, or near to, a 15 - 40% acetonitrile range during linear AB gradient elution, the region where the best resolution may generally be obtained [15]. The presence of a lysine residue at position five of the peptide analogues ensures sufficient peptide solubility.

Effect of the α-amino group on the retention behaviour of peptides in RPC

In order to determine the effect of the α-amino group on the retention behaviour of the two series of peptide analogues, peptide pairs (acetylated and the corresponding non-acetylated analogues) were separated on a silica-based C_8 column by linear AB gradient elution (1% acetonitrile/min at a flow-rate of 1 ml/min) over a pH range of 2 to 6.8. Figure II-1 summarizes the retention time data obtained from these preliminary chromatographic separations (only 15 peptide pairs were available).

From Figure II-1, which plots retention time *versus* pH for a number of analogues, it can be seen that over the pH range tested, pH has a very small effect on the retention behaviour for the majority of acetylated analogues (left panel), as would be expected. The

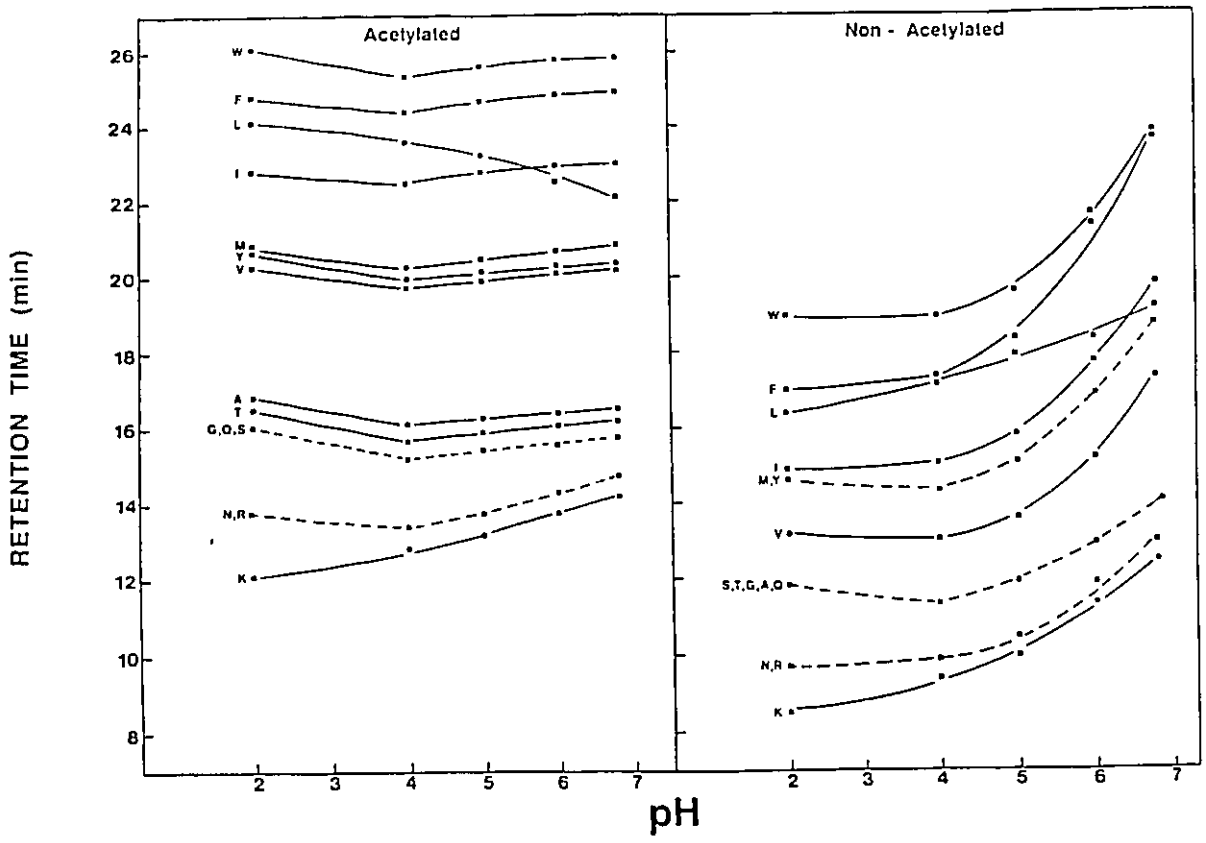

Figure II-1 Plot of retention time of acetylated (left) and non-acetylated (right) peptide analogues *versus* pH. Column: Pep-S C_{18}/C_2 , 250 x 4 mm I.D., 5-µm particle size, 100-Å pore size; Mobile phase: linear AB gradient elution (2% B/min, equivalent to 1% acetonitrile/min) at a flow-rate of 1ml/min. In the *pH 2 system,* A is 100 mM aqueous H_3PO_4 and B is 100mM H_3PO_4 in acetonitrile/H_2O (1:1); In the *pH 4-7 system,* A is 100 mM aqueous triethylammonium phosphate (TEAP) and B is 100 mM TEAP in acetonitrile/H_2O (1:1). Dotted lines denote the general retention time *versus* pH profile of peptide analogues whose profiles matched very closely. The one letter amino acid code (presented in Table II-1) denotes the substitution made at the N-terminal position in each peptide analogue as described under Model Synthetic Peptides.

interesting exception is the Leu analogue, which shows a distinct drop in the retention time as the pH is raised. In the pH range of 2 to 6.8, the elution order of the Leu and Ile analogues (the side-chains of these residues have the same number of carbon atoms, but differ in that Ile is β-branched) is reversed.

With respect to the non-acetylated peptide analogues (right panel), there is a sharp increase in retention time with increasing pH, suggesting that the hydrophobicity of the analogues is increasing through the deprotonation ($NH_3^+ \rightarrow NH_2$) of the hydrophilic, positively charged α-amino group as the pH is raised. In the same manner as the acetylated Leu analogue, the non-acetylated Leu analogue is an interesting anomaly.

If one were to assume that the only effect of the deprotonation of the α-amino group would be to increase the overall hydrophobicity of each peptide analogue (i.e., decrease its hydrophilicity, resulting in an increase in its retention time), one would expect that all analogues would exhibit the same profile in the plots shown in Figure II-1 (right panel). However, this is not the case, since each analogue or group of analogues exhibits a unique profile in these plots, i.e., the α-amino group not only affects the hydrophobicity of the non-acetylated analogues but is also sequence dependent with respect to the N-terminal residue. This is further illustrated in Figure II-2, where a mixture of 5 different analogues, either acetylated (upper profile) or non-acetylated (lower profile) were separated on a C_8 column at pH 2. One might expect, if the only effect of the α-amino group were to decrease (equally) the retention times of the non-acetylated analogues, then the elution profiles for the non-acetylated analogues would look exactly the same as the profile for the acetylated analogues, except that they would be displaced to shorter retention times (i.e., the selectivity of the separation would remain the same). In fact, the retention times of the non-acetylated analogues are indeed decreased, but the relative elution positions have changed, e.g., the Ac-Ser and Ac-Ala (upper profile) analogues are baseline resolved at pH 2, whereas the H-Ser and H-Ala analogues are coeluted (lower profile). The other acetylated/non-acetylated peptide pairs exhibit a similar effect, but to different extents, e.g., the retention time difference between Ac-Ser and Ac-Ala is smaller than that seen for the Ac-Tyr and Ac-Ile pair, upper profile; therefore, one might expect that the H-Tyr and H-Ile (lower profile) would be baseline resolved at pH 2, but this is not the case. This effect appears to be dependent on the substitution in the N-terminal position, again suggesting

that the α-amino group affects the retention behaviour of the non-acetylated analogues through its effect on the hydrophobicity of the N-terminal residue.

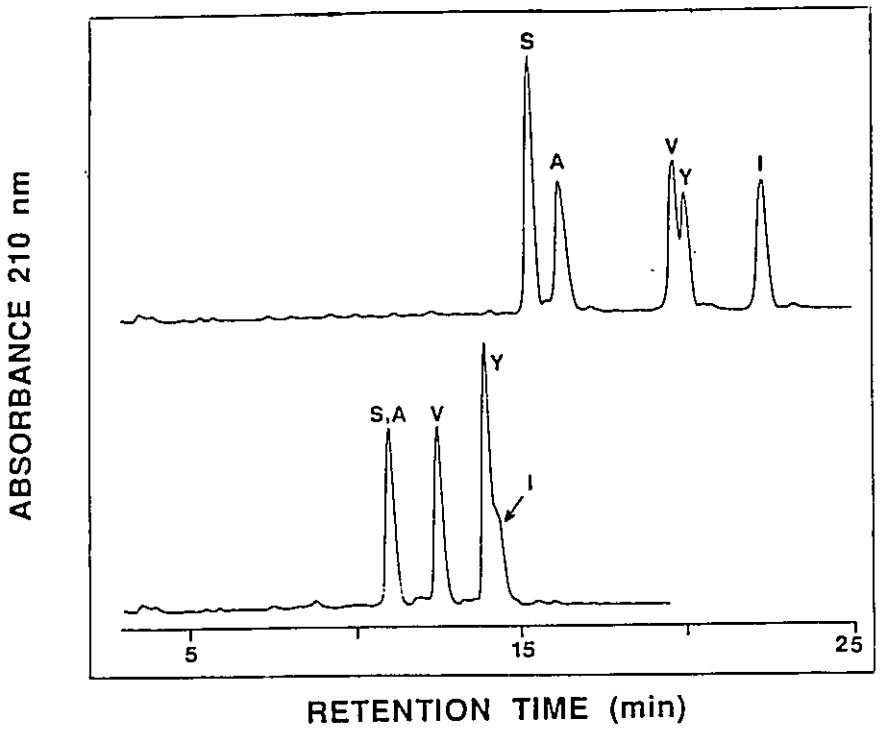

Figure II-2 Effect of the α-amino group on the elution profile of a mixture of 5 peptide analogues, either acetylated (upper) or non-acetylated (lower), at pH 2. Column: same as in Figure II-1. Mobile phase: linear AB gradient elution (2% B/min, equivalent to 1% acetonitrile/min) at a flow-rate of 1 ml/min, using the pH 2 system as in Figure II-1.

The results shown in Figure II-1 suggest that pH may be used to effect a chromatographic separation of the non-acetylated analogues using alternate pH conditions. Specific examples of this are shown in Figure II-3, where groups of non-acetylated peptides were separated at pH 2 and pH 6.8. It can be seen that these peptide analogues show different selectivities under different pH conditions. Figure II-3A and II-3B show examples of profiles where specific peptide pairs are better separated at pH 2 than at pH 6.8. Thus in Figure II-3A, the H-Leu and H-Met analogues are eluted as a doublet at pH 6.8; whereas, at pH 2, these analogues are well separated ($\Delta t = 2$ min). As well, in Figure II-3B, the H-Trp and H-Phe analogues are coeluted at pH 6.8; whereas, at pH 2 they are

baseline resolved (Δt = 2 min). In Figure II-3D, an example is shown where a mixture of non-acetylated analogues is better resolved at pH 6.8 than at pH 2. At pH 2, the H-Met and H-Ile analogues are not baseline resolved; whereas, this is achieved at pH 6.8. Also from

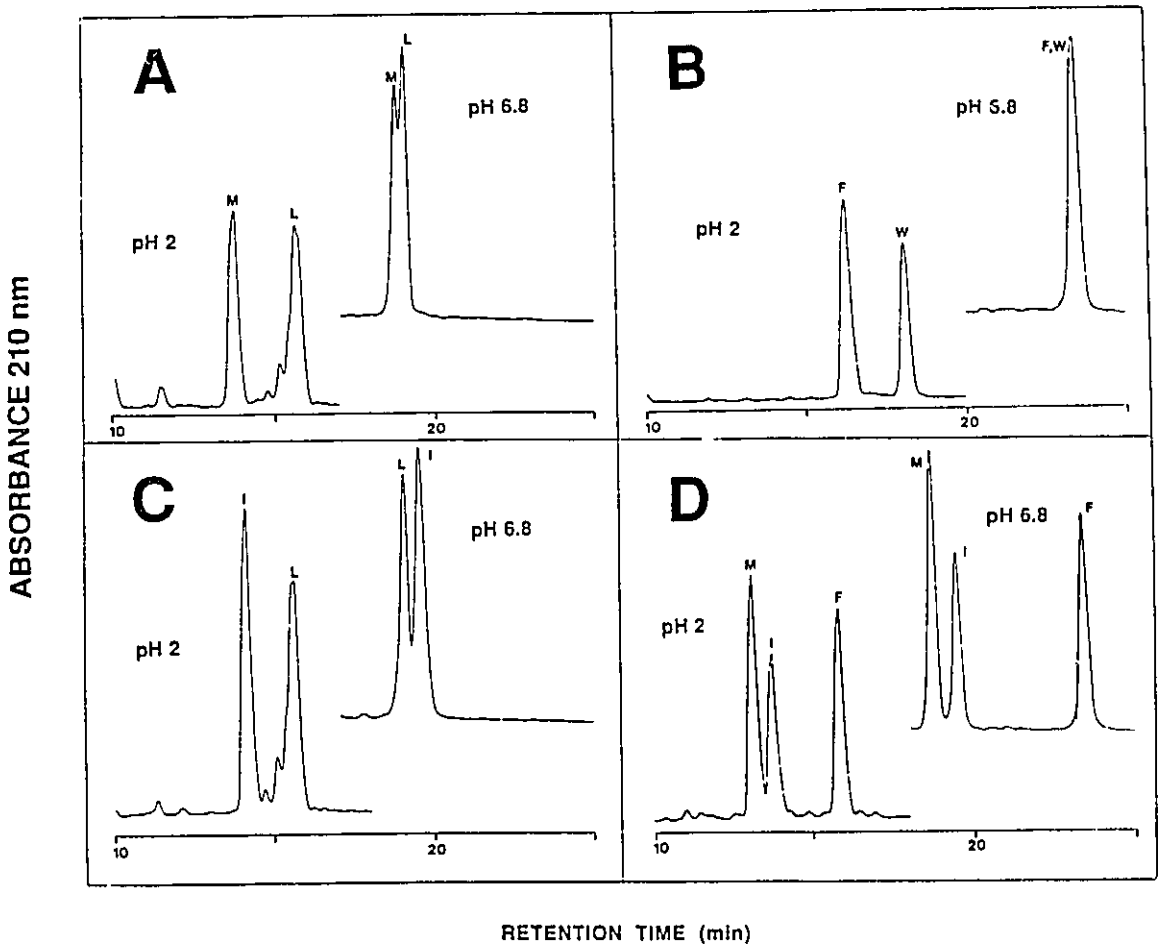

Figure II-3 Effect of pH on reversed-phase chromatographic elution profiles of specific pairs of non-acetylated peptide analogues. Column and mobile phase: same as in Figure II-1 using pH 2 and pH 4-7 systems.

Figure II-3D, the H-Phe analogue is much better separated from the other two analogues at pH 6.8 (Δt for the Phe/Ile pair = 3.9 min) than at pH2 (Δt for the Phe/Ile pair = 2.1 min).

Figure II-3C shows the inversion of elution order of the non-acetylated analogue Ile/Leu pair at pH 6.8 and pH 2 (it should be noted that this effect is also seen with the acetylated Ile/Leu pair; Figure II-1, left panel).

Effect of α-amino group on the hydrophobicity of the N-terminal side-chain

In order to quantitate the effect of the α-amino group on the hydrophobicity of the N-terminal residue, pairs of acetylated and non-acetylated peptide analogues were separated on a polystyrene column. The polystyrene column was chosen over the silica based column to allow for the repeated use of high pH buffers to determine the pK_a values of both the α-amino group and the basic side-chains. Retention time data from these chromatographic separations, on the polystyrene column (using linear AB elution at 1% acetonitrile/min at a flow-rate of 1ml/min at pH 2) were analysed using the following equations:

$$a = t_R\text{H-Gly} - t_R\text{Ac-Gly} \quad \text{(i)}$$

$$H = t_R\text{Ac-Xxx} - t_R\text{Ac-Gly} \quad \text{(ii)}$$

$$h = [t_R\text{H-Xxx} - t_R\text{Ac-Gly}] - a \quad \text{(iii)}$$

$$s = h - H \quad \text{(iv)}$$

The effect of the α-amino group on the retention behaviour of each of the peptide analogues at pH 2 was determined using equation (i), where "a" is the difference between the retention time of the α-amino glycine analogue, t_RH-Gly, and that of the acetylated glycine analogue, t_RAc-Gly. From equation (ii), the retention time of the acetylated analogue, t_RAc-Xxx, minus the retention time of the acetylated glycine analogue, t_RAc-Gly, defines the hydrophobicity, H, of the side-chain in the absence of an α-amino group at pH 2. The retention time difference between the α-amino analogue, t_RH-Xxx, and the acetylated glycine analogue, t_RAc-Gly, is a combination of two effects: the α-amino group contribution, "a", as defined by equation (i), plus the hydrophobicity of the side-chain of the N-terminal residue, h, in the presence of an α-amino group. As a result, the hydrophobicity of the side-chain of the N-terminal residue in the presence of a free α-amino

group may be calculated from equation (iii). It should be noted that, in subtracting the retention time of the acetylated glycine analogue from either the acetylated or non-acetylated analogues, the effect seen will be due to the side-chain of each substituted amino acid. The effect of the α-amino group on the side-chain of the N-terminal residue, s, may therefore be calculated from the difference between the values of h and H (equation iv), i.e., the hydrophobicity, h, of the side-chain in the presence of an α-amino group minus the hydrophobicity, H, of the side-chain in the absence of an α-amino group will provide a value for the effect of the α-amino group on the hydrophobicity of the side-chain. The calculated values obtained from retention time data are summarized in Table II-1.

From Table II-1, the side-chain of the N-terminal residue of each of the peptide analogues exhibits a positive or negative value for the hydrophobicity in the absence of an α-amino group (H). Predominantly hydrophobic residues (e.g., Trp, Phe, Leu, Ile, etc.) show a positive value, suggesting that the side-chains of these residues would contribute positively to the retention times of these peptide analogues at pH 2, relative to the glycine analogue. Predominantly hydrophilic residues (e.g., Lys, His, Arg, etc.) show a negative value, suggesting that the side-chains of these residues would contribute negatively to the retention times of these peptide analogues at pH 2, relative to the glycine analogue. These effects are generally consistent with Guo et al. [6], who used model synthetic peptides (acetylated peptide amides) to develop a set of retention coefficients for each of the 20 commonly occuring amino acids found in proteins.

The hydrophobicities of the side-chains in the presence of an α-amino group (h) are also shown in Table II-1. For example, in the presence of an α-amino group, the hydrophobicity of the Trp side-chain is decreased from 10.1 min to 9.3 min, suggesting that the presence of the fully positively charged α-amino group (at pH 2) caused the reduction in hydrophobicity of the Trp side-chain, resulting in the 0.8 min decrease in the retention time for this peptide sequence. The presence of an α-amino group also results in

decreased hydrophobicities for other hydrophobic residues, e.g., Phe was reduced from 8.8 to 5.5 min and Leu was reduced from 7.5 to 4.6 min. Alternatively, the presence of a charged α-amino group causes the hydrophilicity of the side-chain of several hydrophilic

TABLE II-1 Effect of the α-amino group on hydrophobicity of the side-chain of the N-terminal residue.

Peptide [a] analogue	H [b] (min)	h [c] (min)	s [d] (min)
Trp (W)	10.1	9.3	-0.8
Phe (F)	8.8	5.5	-3.3
Leu (L)	7.5	4.6	-2.9
Ile (I)	5.8	3.0	-2.8
Met (M)	4.8	3.0	-1.8
Tyr (Y)	4.5	3.1	-1.4
Val (V)	3.5	1.3	-2.2
Cys (C)	3.4	2.9	-0.5
Pro (P)	2.7	0.7	-2.0
Glu (E)	0.3	0.5	0.2
Ala (A)	0.2	0.1	-0.1
Asp (D)	0.0	0.6	0.6
Gly (G)	0.0	0.0	0.0
Thr (T)	-0.1	1.0	1.1
Ser (S)	-0.8	-0.1	0.7
Gln (Q)	-0.9	0.0	0.9
Asn (N)	-3.0	-2.1	0.9
Arg (R)	-3.1	-2.1	1.0
His (H)	-3.3	-1.5	1.8
Lys (K)	-3.5	-1.6	1.9

[a] amino acid represents the substitution made in the N-terminal position of each peptide analogue, as described under Model Synthetic Peptides.

[b] H is defined as the hydrophobicity of the N-terminal amino acid side-chain in the absence of an α-amino group and is calculated from retention time data as described in the text.

[c] h is defined as the hydrophobicity of the N-terminal amino acid side-chain in the presence of an α-amino group and is calculated from retention time data as described in the text.

[d] s is defined as the difference between h and H, indicating the effect of the α-amino group on the hydrophobicity of the side-chain of the N-terminal amino acid.

[e] Column and mobile phase as in Figure II-5, using the pH 2 system.

residues (e.g., Lys, His, Arg) to decrease, e.g., a decrease in side-chain hydrophilicity is seen for Lys, where the negative contribution to the retention time of this peptide sequence results in a decrease from -3.5 min to -1.6 min. It should be noted that similar values of H and h were obtained when these series of peptide analogues were separated by linear AB gradient elution using a C_8 silica based column (data not shown).

If the α-amino group did not affect the hydrophobicity of the side-chain of the N-terminal residue, then it would be expected that the value of s (which defines the effect of the α-amino group on the hydrophobicity of the side-chain of the N-terminal residue) would be zero, since the hydrophobicity of the side-chain in the absence of an α-amino group (H) and the hydrophobicity of the side-chain in the presence of an α-amino group (h) would be the same; thus h - H, would be equal to zero. However, from Table II-1, each peptide analogue exhibits an unique value of s; a negative value indicating that the hydrophobicity of the side-chain has decreased or a positive value indicating that the hydrophobicity has increased (decreased hydrophilicity) due to the presence of a charged α-amino group. Therefore, these results confirm that not only does the α-amino group affect the hydrophobicity of the side-chain of the N-terminal residue but that this effect varies depending on the N-terminal residue.

The results shown in Figure II-4 and II-5 are further evidence that the α-amino group does affect the hydrophobicity of the side-chain of the N-terminal residue. If, at pH 2, one assumes that the α-amino group did not affect the hydrophobicity of the side-chain, then one would expect that the retention time difference between the acetylated analogue and the non-acetylated analogue [i.e., t_RAc-Xxx - t_RH-Xxx)] would be the same for all peptide analogues, essentially, this difference would be equal to the contribution of the α-amino group. From Figure II-5A, it can be seen that the difference between each acetylated/non-acetylated analogue pair exhibits a unique value. For example, t_RAc-Ile minus t_RH-Ile has a value that is slightly greater than 8; whereas, t_RAc-Asn minus t_RH-Asn has a value that is slightly greater than 4. This further supports the concept that the α-

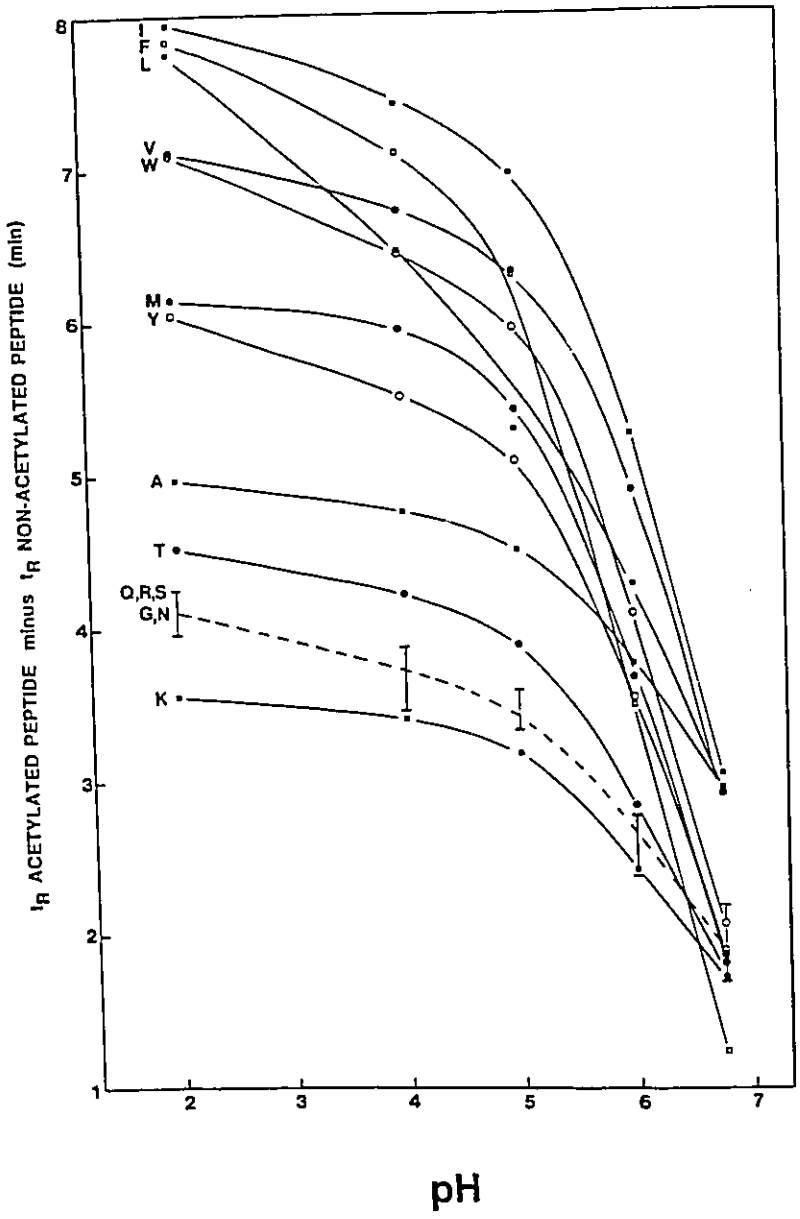

Figure II-4 Plot of retention time of acetylated peptide analogue minus retention time of non-acetylated peptide analogue *versus* pH. Column and mobile phase: same as in Figure II-1, using pH 2 and pH 4-7 systems. Dotted lines denote an average retention time difference *versus* pH profile of peptide analogues whose profiles matched very closely (the bars indicate the retention time range of these peptides at specific pH values).

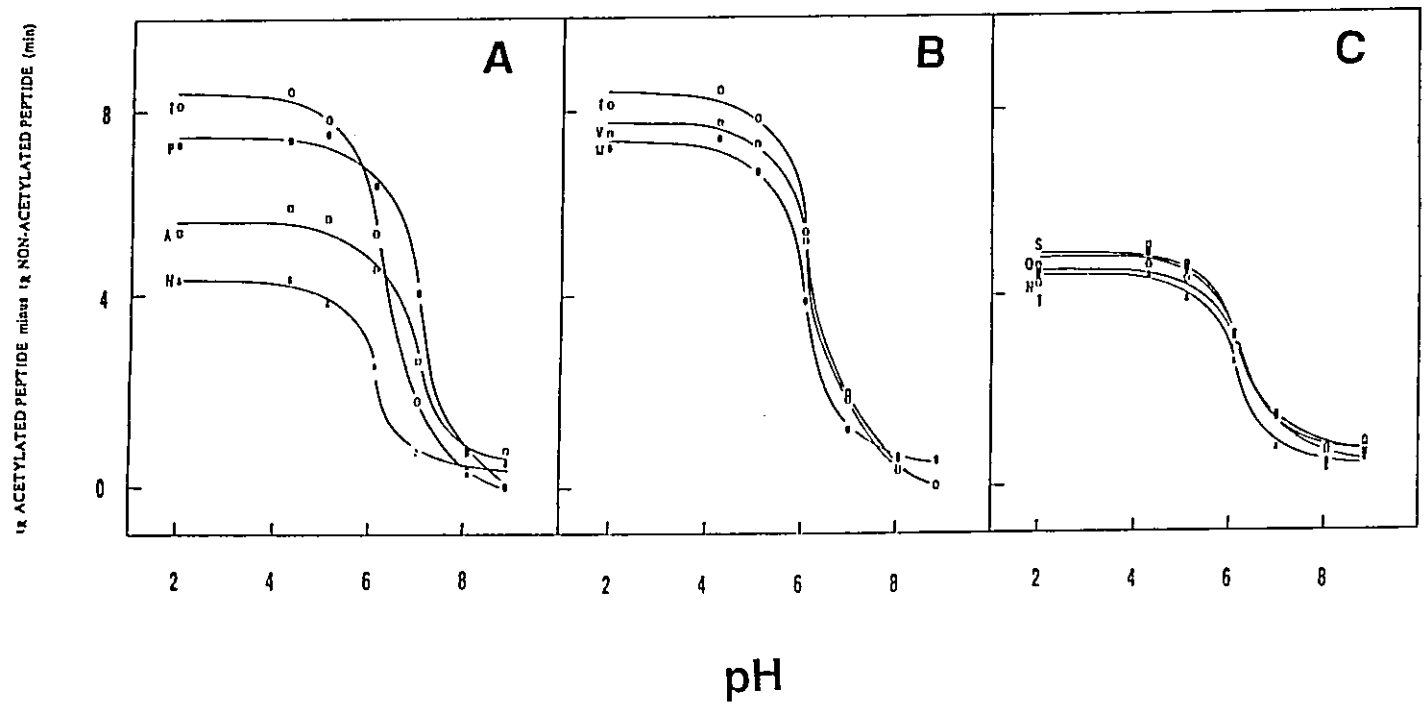

Figure II-5 Plot of retention time of acetylated peptide analogue minus the retention time of the non-acetylated peptide analogue versus pH. Column: PLRP-S (250 x 4.6 mm I.D., 5 µm, 100 Å). Mobile phase: linear AB gradient elution (2% B/min equivalent to 1% acetonitrile/min) at a flow-rate of 1ml/min. In the pH 2 system, A is 20 mM aqueous H_3PO_4 containing 2% acetonitrile and B is 20mM H_3PO_4 in acetonitrile/H_2O (1:1); In the pH 4-7 system, A is 20 mM aqueous triethylammonium phosphate (TEAP) containing 2% acetonitrile and B is 20 mM TEAP in acetonitrile/H_2O (1:1). In the pH 7-9 system:, linear AB gradient elution (2% B/min, equivalent to 1% acetonitrile/min), A is 10 mM aqueous $(NH_4)_2HPO_4$ containing 2% acetonitrile and B is 10 mM $(NH_4)_2HPO_4$ in acetonitrile/H_2O (1:1), both eluents containing 100 mM sodium perchlorate. Panels A-C represent 11 examples of the peptide analogues used in this study.

amino group affects the hydrophobicity of the N-terminal residue and that it does so in a residue dependent manner.

Titration of the α-amino group of the peptide analogues

Initial studies of the non-acetylated peptide analogues suggested that increasing the pH resulted in the deprotonation of the α-amino group as evidenced by the increasing retention times seen in Figure II-1, right panel. This suggested that it might be possible to titrate the α-amino group of these analogues.

Retention time data for the acetylated and the non-acetylated analogues generated for producing Figure II-1, were used to produce Figure II-4, which plots retention times of acetylated analogues minus the retention times of the corresponding non-acetylated analogues *versus* pH. These plots represent partial titration curves for the α-amino group. From Figure II-4, it can be seen that each analogue, or group of analogues, exhibits an unique profile, suggesting that the α-amino group is being deprotonated at different rates depending on the N-terminal substitution. For example, the Leu analogue shows a very dramatic retention time difference compared to the Lys analogue, where the retention time difference is considerably smaller. This plot suggested that, if the pH range of the plot could be extended to higher pH values, the pK_a of the α-amino group for each analogue could be determined. In order to extend this range, acetylated and non-acetylated analogues were chromatographed on a poylstyrene column, PLRP-S, using linear AB gradient elution (1% acetonitrile/min at a flow-rate of 1 ml/min) over a pH range of 2 to 9. The retention time data from these runs is summarized in Figure II-5, which shows the α-amino group titration curves for selected analogues and the pK_a values obtained from these data are presented in Table II-2. Figure II-5A illustrates the large range of pK_a values exhibited by the peptide analogues, e.g., Asn has a pK_a value of 6.1; in contrast, Pro has a pK_a of 7.1. Figure II-5B and II-5C show that for some analogues, the pK_a values may be very similar, e.g., the values for Ile and Val are 6.4 and 6.5, respectively, and those of Ser and Gln are 6.3 and 6.4, respectively. It should also be noted that, at pH 9, all analogues approach a

similar retention time difference [i.e., t_RAc-Xxx - t_RH-Xxx], suggesting that the deprotonated α-amino group has a similar contribution to retention behaviour as that of an N^{α}-acetylated terminal.

From Table II-2, it can be seen that the range of pK_a values of the α-amino groups

TABLE II-2 pK_a of the α-amino groups of peptide analogues containing 19 different N-terminal amino acid residues.

Peptide [a] analogue	this study [b]	free amino acid [c]
Pro (P)	7.1	10.6
Gly (G)	7.0	9.8
Asp (D)	6.8	9.6
Ala (A)	6.8	9.7
Glu (E)	6.6	9.7
Val (V)	6.5	9.6
Ile (I)	6.4	9.7
Gln (Q)	6.4	9.1
Trp (W)	6.3	9.4
Ser (S)	6.3	9.2
Thr (T)	6.3	9.1
Leu (L)	6.3	9.6
His (H)	6.3	9.2
Lys (K)	6.2	9.2
Asn (N)	6.1	8.8
Arg (R)	6.1	9.0
Tyr (Y)	6.1	9.1
Met (M)	6.1	9.2
Phe (F)	6.0	9.2
Cys (C)	-----	10.5

[a] amino acid denotes the substitution made in the N-terminal position of each peptide analogue as described under Model Synthetic Peptides.

[b] pK_a of α-amino group as determined from analysis of retention times of peptide analogues as shown in Figure 5. Column and mobile phase: same as in Figure 5. Data was fitted to a sigmoidal type curve and subsequently pK_a values were obtained using the program Table Curve (Jandel Scientific, version 3.1).

[c] pK_a of α-amino groups of amino acids [16].

obtained in this study varies from 7.1 (Pro) to 6.0 (Phe); in contrast, the pK_a values for

the free amino acids vary from 8.8 to 10.6 [16], i.e., the pK_a values obtained in this study are considerably lower than those for free amino acids in solution. The pK_a for the α-amino group in some proteins may be much lower than that found for free amino acids in solution, e.g., in human CO-hemoglobin (α-chain), the value is 6.72 [17] and in bovine pancreatic ribonuclease A the value is 8.14 [18] representing pK_a values that are on the order of 1 - 2 pH units lower than that found for the free amino acids. It has been previously reported that an increasingly hydrophobic environment may affect the ionization of a potentially ionizable group as evidenced by the decreased dissociation of the α-carboxyl group of glycine (e.g., increased pK_a from 2.35 to 3.96) and the increased dissociation of the α-amino group of glycine (e.g., decreased pK_a from 9.78 to 7.42) with increasing percentage of organic solvent in aqueous solutions of this amino acid [19]. These examples taken together suggest that the environment can have a major effect on the dissociation of an ionizable group. From Table II-2, it can be seen that the pK_a of the α-amino group determined in this study is reasonably similar to the pK_a values found in some proteins (i.e., 6.0 to 7.1 for this study as opposed to 6.72 to 8.14 found in proteins). Due to the inherently hydrophobic nature of the stationary phase that was used to separate these peptide analogues and the fact that a hydrophobic environment could affect the pK_a of an α-amino group, this suggests that the hydrophobic stationary phase may be a reasonable mimic for the hydrophobic environment created by proteins. It has also been previously reported, with simple organic molecules, that substitutions on the carbon atom adjacent to an ionizable group (e.g., COOH) may alter the ionization of that group, e.g., the carboxyl group of ethanoic acid, $H_3C\text{-}CO_2H$, where the α-carbon contains three hydrogen atoms, has a pK_a of 5.55; whereas that of 2, 2-diethylbutanoic acid, $(CH_3CH_2)_3\text{-}C\text{-}CO_2H$, where the α-carbon has three ethyl groups attached to it, has a pK_a of 6.44 [19]. Creighton [19], refers to this effect as a steric effect. In the present study, the pK_a values for the Ala and Leu substituted analogues were 6.8 and 6.3, respectively. This decrease in pK_a could be explained either by a steric effect or simply an increase in the hydrophobicity of the amino

acid side-chain between Ala and Leu. It has been previously reported by Cantor and Schimmel [20] that electrostatic interactions can also have a significant effect on the pK_a of the α-amino group. For example, when titrating the α-amino group of the amino acid Ala (where there are favourable electrostatic interactions between the α-amino group and the α-carboxyl group) the pK_a value is 9.69; in contrast when titrating $(Ala)_4$ (where this same electrostatic interaction is no longer significant), the pK_a value is 7.94, suggesting that a favourable interaction results in a decreased dissociation of the α-amino group [20]. In the present study, the same effect is seen when comparing the N-terminal residue of the peptide analogues containing an acidic side-chain as opposed to those containing a basic side-chain. Basic residues result in a lower pK_a (e.g., Arg = 6.1, Lys = 6.2) than acidic residues (e.g., Glu = 6.6, Asp = 6.8), possibly through unfavourable (repulsive) electrostatic interactions between the positively charged side-chain of the basic residue and the α-amino group, resulting in increased dissociation of the α-amino group (i.e., a lower pK_a); in contrast, there is the potential for favourable (attractive) electrostatic interactions between the negatively charged side-chain of an acidic residue and the α-amino group, resulting in decreased dissociation of the α-amino group (i.e., a higher pK_a). From Table II-2, it can be seen that a range of pK_a values (6.0 to 7.1) was obtained for the analogues tested, suggesting that the pK_a is dependent on the N-terminal residue. This dependency is consistent with the pK_a of the α-amino group of substituted dipeptides of glycine (e.g., the pK_a for Asp-Gly = 9.07, Pro-Gly = 8.97, Gly-Gly = 8.13, Ser-Gly = 7.33 and Asn-Gly = 7.25) [21] which suggests that the pK_a is dependent on the N-terminal residue. These data taken together suggest that the pK_a values obtained in this study are dependent on three factors: (1) the hydrophobicity of the environment which stems from the hydrophobicity of the stationary phase (2) the hydrophobicity of the N-terminal residue and (3) the charge on the side-chain of the N-terminal residue.

Titration of ionizable side-chains of N-terminal residue of peptide analogues

Figure II-6 plots retention times of acetylated analogues minus the retention time of the acetylated core peptide *versus* pH. This plot is effectively a titration curve of residues with ionizable side-chains. From Figure II-6A, it can be seen that, for these analogues, with neutral amino acids at the N-terminal, increasing pH has little effect on retention behaviour. This would be expected, since these substitutions do not contain ionizable side-chains (with the exception of Tyr) and the data presented in this plot is consistent with the

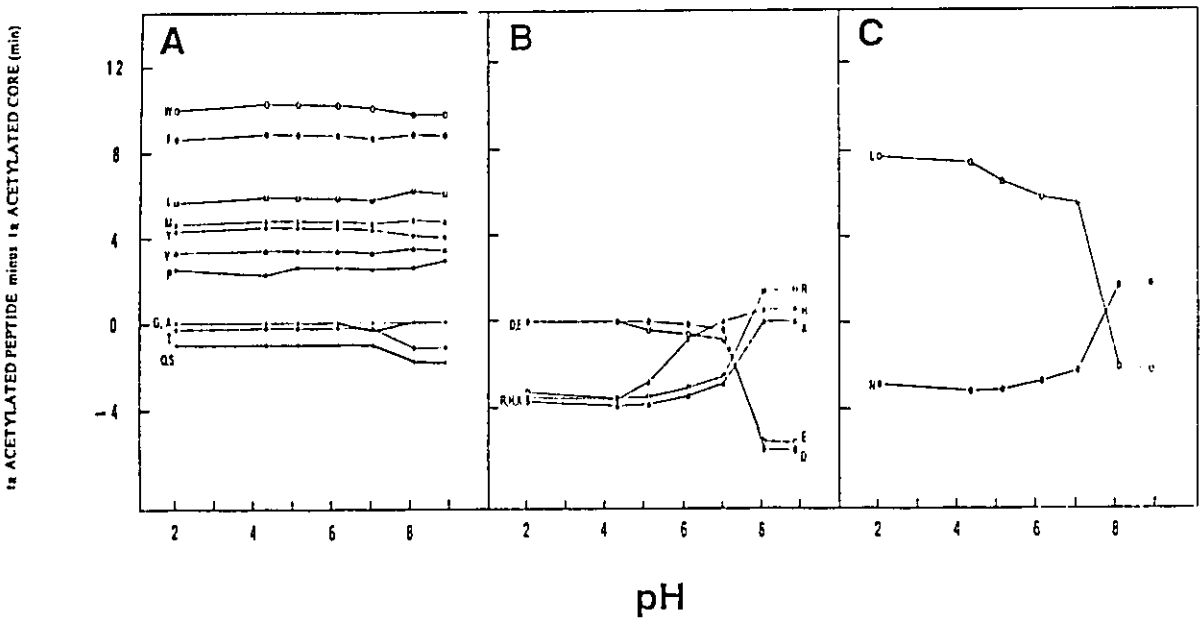

Figure II-6 Plot of retention time of acetylated peptide analogue minus the retention time of the acetylated core (Ac-Leu-Gly-Ala-Lys-Gly-Ala-Gly-Val-Gly-amide) *versus* pH. Column and mobile phase: same as in Figure II-5.

plot in Figure II-1, i.e., the plot in Figure II-6 is an alternate way of representing the effect of pH on the acetylated analogues, with the exception that the pH range has been extended to pH 9.

Figure II-6B allows one to observe the titration of ionizable side-chains of the N-terminal residue. The pK_a values obtained from this plot are presented in Table II-3. From this table, it can be seen that the pK_a value for the side-chain of an acidic residue can be significantly higher in proteins than for the pK_a in free amino acids. For example, the pK_a

TABLE II-3 pK_a values of the functional side-chains of five N-terminal amino acid residues.

Peptide [a] analogue	this study [b]	amino acid [c]	proteins
Asp (D)	7.5	3.65	10 [d]
Glu (E)	7.4	4.25	6.0, 6.5, 8.0-8.5 [e]
Arg (R)	7.3	12.48	11.6 - 12.6 [f]
Lys (K)	7.4	10.79	9.11/5.9 [g]
His (H)	5.8	6.0	5.0 - 8.0 [h]

[a] amino acid denotes the substitution made in the N-terminal position of each peptide analogue as described under Model Synthetic Peptides.

[b] pK_a of side-chain of N-terminal amino acid as determined from analysis of retention times of peptide analogues as shown in Figure 6. Column and mobile phase: same as in Figure 5.

[c] pK_a of side-chain of amino acid [16].

[d] Asp (D): Asp 96 = 10, Asp in the photosynthetic protein bacteriorhodopsin [22].

[e] Glu (E): Glu 35 in lysozyme; native, enzyme-inhibitor (NAG_3) complex and enzyme-substrate (glycol-chitin) complex respectively in 0.15M KCl [25, 27].

[f] Arg (R): Arg = 11.6 - 12.6, p.120 [28].

[g] Lys (K): Lys 41 = 9.11 in bovine pancreatic ribonuclease A [18]; Lys = 5.9, Lys at active site of acetoacetate decarboxylase [29].

[h] His (H): His = 5.0 (trypsin, pig, β form) and His 146β = 8.0 (human hemoglobin, deoxy) [16].

of Asp is 3.65 for the free amino acid; in contrast, in some proteins this value may be as high as 10 (Asp 96 in the membrane bound photosynthetic protein bacteriorhodopsin from the bacterium *Halobacterium halobium*) [22]. From the crystal structure of bacterio-rhodopsin (determined by electron cryomicroscopy), Asp 96 is known to be in a very hydrophobic channel above a Schiff base [23, 24]. Also, the pK_a of Glu is 4.25 for the

free amino acid; in contrast, for example, it may be as high as 6.0 in lysozyme (e.g., Glu 35). From the crystal structure of lysozyme, it is known that Glu 35 is in a hydrophobic environment and in addition, the ionization of this Glu is thought to be affected by the ionization of Asp 52, resulting in a pK_a that is much higher than that of the free amino acid [25, 26]. It has been shown, using mutant lysozymes, that if Trp 108 (W108) is replaced with less hydrophobic residues Tyr (W108Y) or Gln (W108Q), the pK_a of Glu 35 also decreases suggesting that the hydrophobicity of the W108 plays a role in determining the pK_a of this residue [26]. In addition, the pK_a of Glu 35 may be significantly altered when either inhibitor or substrate is bound at the active site (e.g., pK_a is 6.5 and 8.0 to 8.5 for bound inhibitor or substrate, respectively) [27]. In comparison, the pK_a values determined in this study were found to be significantly higher than that for the free amino acid (e.g., Asp = 7.5 and Glu = 7.4) but comparable to those observed in proteins. In the same manner that the stationary phase (or hydrophobic environment of a protein) could potentially cause the value to decrease for the α-amino group, the stationary phase could be responsible for the values obtained for these acidic residues (i.e., a more hydrophobic environment results in decreased dissociation, i.e., a higher pK_a value) and the high values obtained in this study could be a reflection of the very hydrophobic nature of the stationary phase. The high values obtained for the side-chains of the acidic residues could have important implications in the use of cationic pairing agents, where it has been suggested that 0.01 M triethylamine acetate at pH 5.5 or 0.01 M tetrabutylammonium phosphate at pH 7 [28], be used to effect a separation of peptides/proteins containing acidic residues. With the pK_a of the acidic residues being as high as 7.5/7.4 in RPC (as determined in this study), there may be no ion-pairing at the lower pH (5.5). From Table II-3, it can also be seen that, for residues with basic side-chains, the pK_a in proteins can be lower than that for the free amino acids. For example, the pK_a for Arg is 12.48 in the amino acid; whereas, in proteins this value may be decreased to 11.6. The pK_a value for Lys is 10.49 in the free amino acid; whereas, in proteins this value may be decreased to 9.11 (Lys 41 in bovine

pancreatic ribonuclease A, where this reduced value appears to be the result of a neighboring charged Arg residue) [18]. In addition, the pK_a of the ε-amino group of a Lys residue in the active site of the enzyme acetoacetate decarboxylase (the pK_a is only 5.9), may be significantly different than that of the free amino acid [29]. It has also been shown that when Val 66 (V66) is replaced with a Lys (V66K) in a mutant of Staphylococcal nuclease, the pK_a of the Lys side-chain is 6.4 [31]. The x-ray crystal structure of the mutant shows the Lys side-chain to be in a fully hydrophobic core of the nuclease, i.e., all atoms in van der Waals contact are hydrophobic and there is no salt bridges or hydrogen bonding to the Lys. As with the α-amino group, the environment of these side-chains can significantly alter the values for the pK_a and the values obtained in this study may reflect the very hydrophobic nature of the stationary phase. No value for the pKa was obtained for Tyr and this is probably due to the fact that the pK_a values for this residue in proteins can range from 9.5 to >12 [16], which is out of range for this study.

Panel C, Figure II-6, illustrates once again the anomolous behaviour of the Leu analogues that was seen in Figure II-1, with the exception that this effect is more pronounced at the higher pH. In addition, the Asn analogue also exhibits an unexpected profile.

CONCLUSIONS

This study showed that the α-amino group exhibits a large contribution to the retention behaviour of peptides in RPC and as well, that the α-amino group affects the hydrophobicity of the side-chain of the N-terminal residue. These two observations have important implications in work involving the prediction of retention times of peptides (generated from proteolytic digests) in RPC. This study also showed that the pK_a of the α-amino group may be dependent on two factors: (1) the hydrophobicity of the stationary phase and, (2) the substitution in the N-terminal position. The pK_a values determined in this study for the α-amino group and ionizable side-chains of the peptide analogues were very similar to that found in proteins, suggesting that the stationary phase may perhaps be a

reasonable mimic for the hydrophobic environment created by a protein. This environment is dramatically different from the environment encountered by individual amino acids in solution.

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CHAPTER III

Reversed-phase chromatography of synthetic amphipathic α-helical peptides as a model for ligand-receptor interactions: Effect of changing hydrophobic environment on the relative hydrophilicity/hydrophobicity of amino acid side-chains.

This work has been submitted to the Journal of Chromatography, has been accepted and is currently in press and would appear as:
(1) T.J. Sereda, C.T. Mant, F.D. Sönnechesin and R.S. Hodges, *J. Chromatography*, 676 (1994) 139-153.

INTRODUCTION

One of the most interesting developments of liquid chromatography analysis lies in the employment of reversed-phase chromatography (RPC) as a physicochemical model of biological systems. Studies in this area have generally centred on attempting to correlate the retention behaviour of peptides [1] during RPC or proteins [2-6] with their conformational stability; the rationale behind this approach lies in the assumption that the hydrophobic interactions between peptides and proteins with the non-polar stationary phase characteristic of RPC [7] reflects the interactions between non-polar residues which are the major driving force for protein folding and stability. A recent report [8] also suggested that the hydrophobic stationary phase of RPC may be a reasonable mimic for the hydrophobic environment created internally by proteins, e.g., as a probe of how the pKa values of potentially ionizable side-chains in the hydrophobic interior of a protein, frequently important in catalytic groups, are influenced by their environment. Indeed, RPC provides

an excellent example of the way the original purpose for method development of a particular chromatographic mode may be transcended by its employment in a different field.

Another area of profound biological importance where RPC is likely to be a good model is that of ligand-receptor interactions. A ligand binding domain may be defined as the region on the surface of a receptor protein that has a preference or specificity to interact with a complementary surface. In addition, this region may be a protrusion, depression or groove that is surface exposed. The complementary surface to such a receptor binding domain may be another protein, peptide, macromolecule or other non-protein surface. In a similar manner to their importance in folding and stabilization of proteins, hydrophobic interactions also play a key role in the binding of such ligands to their receptors. Although the concept of employing RPC as a mimic of such ligand-receptor interactions is not new, little has been reported to date to verify the potential of this approach mainly due, in the authors view, to the lack of a flexible and well defined model system.

Horváth et al. [9] postulated 18 years ago that the hydrophobic surface characteristic of the stationary phase of reversed-phase packings may be a useful probe of amphipathic helices induced or stabilized in hydrophobic environments. Indeed, this structural motif has much to recommend it as a part of a ligand-receptor model system, in terms of practical considerations and biological relevance. From the latter perspective, amphipathic α-helical structures are an important determinant of the biochemical and/or pharmacological properties of peptide hormones and neurotransmitters [10-13]; a whole class of cytotoxic peptides, including bee or wasp venom peptides such as melittin or one of the mastoparans, are capable of forming amphipathic α-helices upon binding to hydrophobic surfaces [14-18]; amphipathic helices putatively have a role in the activation of G proteins (trimeric GTP-binding regulatory proteins) by membrane receptors and peptides, including mastoparan [19,20]; A family of peptides known as magainins which are potentially amphipathic α-helices exhibit antimicrobial activity (e.g, bacteria and fungi) [21,22]; finally, other functions of amphipathic helices in ligand-receptor interaction

include their involvment in T-cell recognition [23], lipid-associating domains of apolipoproteins [24,25] and the hydrophobic domains of coiled-coil proteins that bind to DNA (the so-called leucine-zipper proteins) [26, 27]. From a practical point of view, model single-stranded amphipathic α-helices have much to offer in terms of both stable three-dimensional structure capable of tolerating sequence changes, as well as relatively straightforward chemical synthesis of analogues [28,29]. In addition, since the hydrophobic domain of these model amphipathic helices will bind preferentially to a hydrophobic stationary phase, even subtle environmental variations within this domain may well be expressed as a variation in RPC retention behaviour.

In the present study, we describe a simple model ligand-receptor system based on observing the retention behavior during RPC of *de novo* designed single-stranded amphipathic α-helical peptides. In addition, as an initial evaluation of this system, we set out to determine whether, and to what extent, the relative hydrophilicity/hydrophobicity of a centrally-located side-chain in the hydrophobic domain of the amphipathic helix was determined by its environment.

EXPERIMENTAL

Materials

HPLC-grade water and acetonitrile were obtained from BDH Chemicals Ltd. (Poole, England). Trifluoroacetic acid (TFA) was obtained from Aldrich Chemical Company (Millwaukee, WI, USA). Trifluoroethanol (TFE) was obtained from Sigma (St. Louis, MO, USA).

Instrumentation

Peptide synthesis was carried out on an Applied Biosystems peptide synthesizer Model 430 (Foster City, CA, U.S.A.). Crude peptides were purified by an Applied Biosystems 400 solvent delivery system connected to a 783A programmable absorbance detector.

Amino acid analyses of purified peptides were carried out on a Beckman Model 6300 amino acid analyser (Beckman Instruments, Fullerton, CA, U.S.A.).

The correct primary ion molecular weights of peptides were confirmed by time of flight mass spectroscopy on a BIOION-20 Nordic (Uppsala, Sweden).

Circular dichroism spectra were recorded on a JASCO J-500C Spectropolarimeter (Easton, MD, U.S.A.) attached to a JASCO DP-500N data processor and a Lauda (Model RMS) water bath (Brinkman Instruments, Rexdale, Ontario, Canada) used to control the temperature of the cell. The instrument was routinely calibrated with an aqueous solution of recrystallized d-camphorsulphonic acid at 290 nm. Constant N_2 flushing was employed.

Peptide Synthesis

Peptides were synthesized by the solid-phase technique (SPPS) on co-poly (styrene- 1% divinyl-benzene) benzhydrylamine-hydrochloride resin (0.92 mmole/g resin). All amino acids were protected at the α-amino position with the Boc-group and the following side-chain protecting groups as described in Chapter II. All amino acids were single coupled as preformed symmetrical anhydrides (with the exception of Arg, Asn and Gln which were coupled as the HOBT active ester) in dichloromethane. Boc-groups were removed at each cycle with an 80-sec reaction with TFA/dichloromethane (33:67, v/v), followed by a second reaction with TFA/dichloromethane (50:50, v/v) for 18.5 min. Neutralizations were carried out using (10:90, v/v), diisopropylethylamine in dimethylformamide (DIEA/DMF). N-terminal residues were acetylated using acetic anhydride/dichloromethane (25:75, v/v) for 10 min. Prior to cleavage the DNP protecting group of His was removed by treating the peptide resin with a solution of 2-mercaptoethanol (20%) and diisopropylethylamine (10%) in dimethylformamide for 2 h. The peptides were cleaved from the resin by treatment with anhydrous hydrogen fluoride (20ml/g resin) containing 10% (v/v) anisole and 2% (v/v) 1, 2-ethanedithiol for 1 h at -4° C. After cleavage from the resin, the formyl group of Trp was removed by treating the peptide with piperidine/water (2:98, v/v).

Columns and HPLC Conditions

Crude peptides were purified on a semi-preparative Synchropak RP-P C_{18} reversed-phase column (250 x 10 mm I.D., 6.5-μm particle size, 300-Å pore size) from Synchrom, Lafayette, IN, U.S.A. The peptides were purified at pH 2 by linear AB gradient elution (0.5% B/min) at a flow-rate of 5 ml/min, where Eluent A is 0.1% aqueous TFA and Eluent B is 0.1% TFA in acetonitrile.

Analytical runs were carried out on an Aquapore RP-300 C_8 reversed-phase column (220 x 4.6 mm I.D., 7-μm particle size, 300-Å pore size) from Applied Biosystems, by employing linear AB gradient elution (1% B/min) at a flow-rate of 1 ml/min, using the same eluents as above.

Calculation of accessible surface areas and hydrophobic moment

All peptide structures were generated in an idealized conformation using equilibrium bond lengths, and angles and dihedral angles (Insight II, Biosym Technologies Inc., San Diego, CA, U.S.A.). Backbone dihedral angles were set to ideal α-helical values of -67 for ϕ and -44 for ψ [30]. The structures were subsequently relaxed by conducting 100 steps of steepest descent and 2000 steps of conjugate gradient minimization in vacuum using a distance dependent dielectric model [31]. The minimizations were performed with Discover (Biosym) and the CVFF force field on a Silicon Graphics Crimson Elan workstation. The solvent accessible surface areas of the minimized peptides were calculated using a 1.4-Å solvent probe in the program Anarea [32]. Individual surface areas per atom were summed to yield hydrophobic, hydrophilic and charged surface areas according to the definition by Eisenberg [33].

The hydrophobic moment is calculated, as described by Eisenberg et al. [39] and is the vector sum of the hydrophobicities of the side chain of a helix. The length of the vector is equivalent to the hydrophobicity, as described by the normalized concensus scale of Eisenberg et al. [57], of each side chain and the direction of the vector is determined by the orientation of the side-chain around the helix, i.e., 1 residue every 100 degrees. In order to

compare the hydrophobic moment of peptides of differing length, the hydrophobic moment, as described, is divided by the number of residues in the peptide, which results in the mean helical hydrophobic moment which is generally referred to as the hydrophobic moment [39].

RESULTS AND DISCUSSION

Design of ligand/receptor model system

We wished to pursue an incremental approach to assessing factors involved in ligand/receptor interactions. By reducing the number of variables in a defined model system, it was felt that both interpretation of results and their extrapolation to biological systems would be simplified. Since a minimum of two hydrophobic surfaces are involved in ligand/receptor interactions, the basic requirements for a flexible model system are: (1) the hydrophobic surface representing the protein receptor may remain constant, whilst that representing the ligand is varied; (2) conversely, the surface representing the protein receptor is varied, whilst that representing the ligand remains constant; (3) finally, the relative hydrophobicity of the surfaces representing both the ligand and receptor are varied concomitantly.

For this initial study, only one surface was varied. Thus, option (1) was selected, i.e., it was decided to vary the hydrophobic surface of the ligand, represented by the hydrophobic face of the synthetic amphipatic α-helical peptide analogues; the non-variable hydrophobic surface of the receptor was represented by the stationary phase of the reversed-phase column.

As noted by Opella et al. [34], relatively short polypeptide sequences perform functional roles as isolated molecules, as oligomers and as domains of large proteins. Indeed, many of the physical (and chemical) properties of large proteins are retained by synthetic oligomeric analogues. Thus, the results of working with a defined model peptide representing a ligand binding to a protein receptor may potentially be directly applied to naturally occuring ligands of similar size; alternatively, such results may be extrapolated to amphipathic sequences within larger polypeptides and proteins responsible for binding to a protein receptor.

Design of model "native" synthetic amphipatic α-helical peptide. We have designed and synthesized an 18-residue peptide ligand for our model ligand/receptor model system. The amino acid sequence is Ac-Glu-Leu-Glu-Lys-Leu-Leu-Lys-Glu-Leu-Glu-Lys-Leu-Leu-Lys-Glu-Leu-Glu-Lys-amide, which has a high potential to form an amphipathic helix (Figure III-1, right). In the design of this peptide, leucine, glutamic acid and lysine residues were selected in light of their highly intrinsic helical propensities [35-37]; leucine as an apolar aliphatic residue and glutamic acid and lysine as, respectively, potentially negatively charged and positively charged residues, depending on pH.

The amino acid sequences of amphipathic α-helices tend to have a strong periodic distribution of hydrophobic amino acids along the chain with three to four residue repeats [38-40] and this is reflected in the design of the "native" model peptide ligand. In addition, the glutamic acid/lysine pairs located in i and i + 3 or i and i + 4 positions along the sequence could provide additional stability to the α-helical structure by intra-chain side-chain electrostatic interactions [41, 42] at neutral pH values.

Figure III-1 (right profile) represents this "native" sequence as an α-helical net, with the hydrophobic face of the helix consisting of leucine residues and the opposite hydrophilic face of the helix consisting of lysine and glutamic acid residues. It should be noted that the width of the hydrophobic face, involving 7 hydrophobic residues at positions 2, 5, 6, 9, 12, 13, and 16 (between the solid lines), as expressed in this helical net representation is wider than the relatively narrow hydrophobic face (between the dotted line and the right-hand solid line) of amphipathic α-helices making up two-stranded α-helical coiled-coil structures in which there is a 3-4 hydrophobic repeat [38, 43-45], involving 5 hydrophobic residues at positions 2, 5, 9, 12 and 16. It was felt that the wider hydrophobic face of our model peptide would have more validity as a general mimic of the non-polar face of ligands from a wide variety of sources than the relatively narrow hydrophobic face characteristic of amphipathic α-helices present in coiled-coil systems. Other advantages of this wide hydrophobic face will become apparent later in this manuscript.

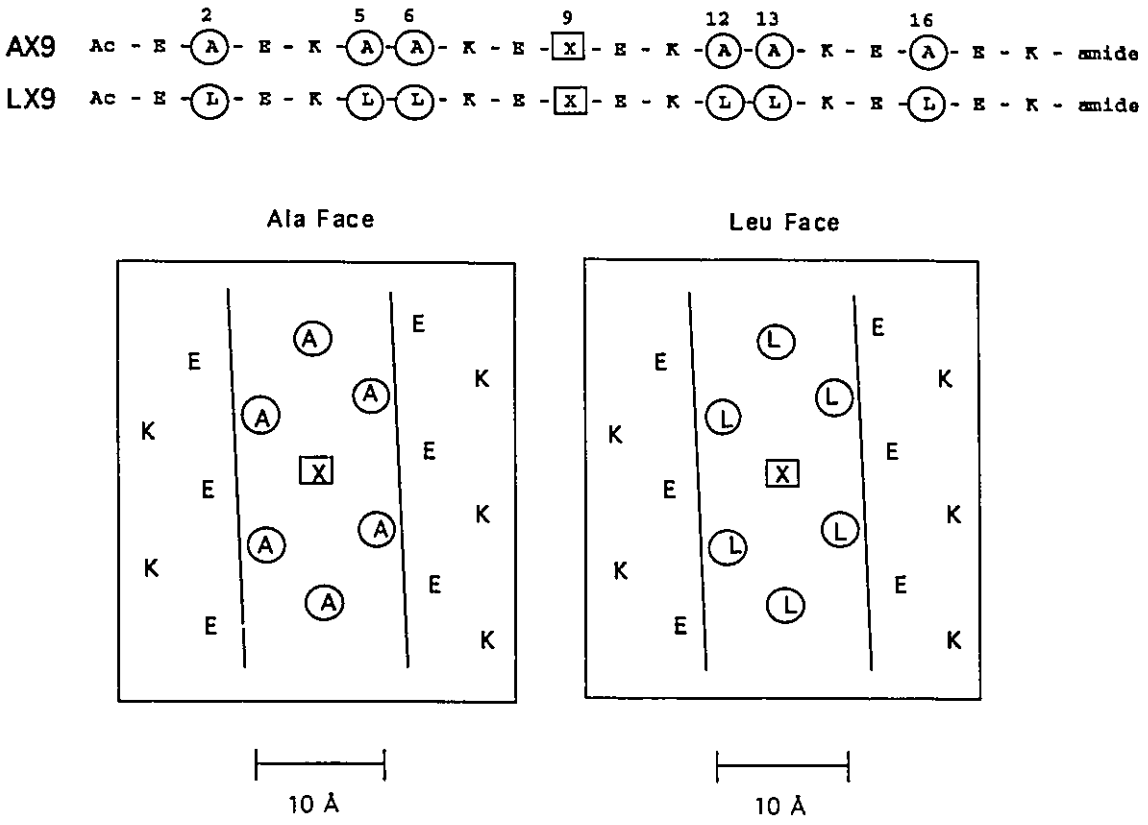

Figure III-1 Design of model synthetic peptides. Top: Sequence of mutant peptides, AX9 and LX9, where the first letter represents amino acid residues used in the hydrophobic face of the peptide, the X represents each of the 20 amino acids (boxed) (single letter code given in Table III-1) substituted at position 9. The residues that are circled or boxed and labelled 2, 5, 6, 9, 12, 13 and 16 are in the hydrophobic face of the amphipathic α-helical peptides. Lysine and glutamic acid residues make up the hydrophilic face of the amphipathic helix. Bottom: "Native" Ala-face (AA9, left) and Leu-face (LL9, right) model peptides represented as α-helical nets. The radius of the α-helix is taken as 5 Å with 3.6 residues per turn, a residue translation of 1.5 Å and thus a pitch of 5.4 Å. The area between the solid lines on the α-helical nets represents the wide hydrophobic face of the peptides. The area between the dotted line and the right-hand solid line in the α-helical net representations of the "native" peptides represents the narrower hydrophobic face (made up of a 3-4 or 4-3 hydrophobic repeat) characteristic of coiled-coil peptides (see text for details).

Conformation and helicity of model peptide ligands. The α-helicity of the "native" peptide ligands was determined by circular dichroism (CD), with the CD spectrum measured in 0.1 M KCl, 50 mM potassium phosphate buffer (pH 5.2) containing trifluoroethanol (TFE), 1:1 (v/v), a solvent that induces helicity in single-chain potentially

α-helical peptides [46, 47]. Studies [48, 49] have shown that the presence of 50% TFE will ensure that the high amphipathicity of a peptide, such as our model ligands, does not lead to aggregation in aqueous solution through intermolecular hydrophobic interactions. The observed ellipticity of -26 640 deg cm^2/dmol for the model peptide (LL9) in 50% TFE yielded an estimate of 86% α-helix in solution, based on a value of -31 060 deg cm^2/dmol calculated for a 100% α-helical 18-residue peptide [50].

Choice of hydrophobic stationary phase. As noted above, in this initial study, the non-polar stationary phase of a reversed-phase packing represents the hydrophobic binding region of a protein receptor. However, although the hydrophobicity of a specific reversed-phase packing is constant, as required for the present study, we still wished there to be a scope for a variation in overall stationary phase hydrophobicity for future investigations, i.e., it was deemed important to retain flexibility in the characteristics of the second component of our ligand/receptor model. A silica-based stationary phase was chosen for the following reasons: (1) the stability (particularly at low pH) and efficiency of such columns makes them particularly advantageous for peptide separations [7]; (2) the nature of the functional group attached to the silica matrix (e.g., C_1, C_3, C_8, C_{18}, CN, phenyl) offers a wide choice of stationary phase hydrophobicity, and (3) the ligand density may be varied, also offering a range of stationary phase hydrophobicity. Concerning points (2) and (3), it is possible to prepare and pack silica-based stationary phases of varying functional group and/or ligand density in the laboratory [51]; thus, the potential for tailored stationary phases then becomes an option, considerably enhancing the flexibility of the ligand/receptor model.

For this initial study, a C_8 packing was used. In addition to the common usage of such columns for peptide separations [7], the specific column employed has, in our hands, proved to be reliably stable and efficient.

Retention behaviour of amphipathic α-helices during RPC. On binding to a reversed-phase column, the high hydrophobicity of the stationary phase stabilizes

secondary (α-helical) structure, mimicking, in fact, the effect of TFE when the peptide is in solution. Indeed, Zhou et al. [49] demonstrated that amphipathic peptides remain α-helical when bound to a reversed-phase column and, due to the preferred binding domain created by the non-polar face of the α-helix, are considerably more retentive than peptides of the same composition but lacking the preferred binding domain.

Effect of environment on relative hydrophobicity/hydrophilicity of amino acid side-chains.

It is known that amino acid side-chain hydrophobicities are influenced by the proximity of other polar or charged atoms [52]. Thus, it is not unreasonable to assume that the proximity of non-polar groups may have a similar fundamental effect on the relative hydrophilicity/hydrophobicity of amino acid side-chains. Such an effect would have profound implications for side-chains involved in biologically-important hydrophobic interactions such as those which characterize ligand/receptor interactions. Thus, the model ligand/receptor system presented in this study was now applied to the question of whether and how the hydrophilic/hydrophobic characteristics of an amino acid side-chain are affected by a varying local hydrophobic environment of the ligand (non-polar face of an amphipathic α-helix).

Design of model peptide series exhibiting varying hydrophobic environment. Two series of synthetic amphipathic peptide analogues were prepared, with their non-polar faces representing homogeneous hydrophobic domains of very different hydrophobicities (Figure III-1). The most hydrophobic series of analogues was based on the "native" model peptide described above (Figure III-1, right), with leucine at all of the hydrophobic positions along the sequence: the "leucine domain" or "Leu-face". The 20 amino acids found in proteins are substituted at residue 9 (the central boxed residue in the helical net presentation; Figure III-1, right). The second series of analogues was based on a peptide with alanine at all of the hydrophobic positions: the "alanine domain" or "Ala-face" (Figure

III-1, left). In a similar manner to the "Leu-face" series, the central residue at position 9 is substituted by the 20 amino acids found in proteins.

The choice of alanine as the non-polar residue making up the hydrophobic face of an amphipathic helix was based on two major considerations: (1) alanine, like leucine, has a high intrinsic helical propensity [35-37]; (2) alanine is considerably less non-polar than leucine [53], resulting in an excellent contrast between the very hydrophobic environment represented by the "Leu-face" and the much less hydrophobic environment created in the "Ala-face".

From the helical net representation of the peptide analogues shown in Figure III-1, it can be seen that the 18-residue length of the peptides, coupled with the wide-face design of the hydrophobic domains of the helices, allowed a central residue (position 9, boxed) to be completely surrounded by identical hydrophobic residues at positions 2, 5, 6, 12, 13 and 16 (circled residues).

The general denotion of the Ala-face series is AX9 (Figure III-1, top), with X referring to the central residue at position 9; the peptide with alanine at this position, and which can be viewed as the "native" peptide of this series, is thus denoted AA9 (Figure III-1, left); with glycine at this position, it is denoted AG9, etc. The same general terminology was also used for the series of analogues based on leucine (general designation LX9), i.e., LL9 for the "native" peptide (Figure III-1, right), LG9 for the analogue substituted by glycine at mutant position 9, etc. For the sake of brevity, the number "9" is frequently omitted from these designations. e.g., LL9 becomes simply LL, AE9 becomes AE, etc.

Conformation and helicity of model peptide analogues. The α-helicities of the peptide analogues of the Ala-face series were determined by CD (in 50% TFE) as described above. With the exception of the proline-substituted analogue (AP9), all of the peptide analogues were shown to exhibit high and similar α-helicity, e.g., an average ellipticity value of -28 196 ± 510 for the Ala-face series, excluding peptide AP9 [54]. In addition, analogues of the Leu-face have also been shown to exhibit similar high α-helicity [55]. As

well, when Eisenberg's [56,57] mean helical hydrophobic moment was used to express the helical amphipathicity of the "native" Ala- and Leu-face peptides, values of 0.59 and 0.73, respectively, were obtained when calculated using a normalized consensus hydrophobicity scale [57]. Native amphipathic α-helices in peptides/proteins have amphipathicity values over the range: coiled-coil proteins, e.g., myosin c-β, residues 449-465, 0.28; transmembrane proteins, e.g., bacteriorhodopsin helix C, residues 1-17, 0.31; apoliproproteins, e.g., C-III, residues 40-67, 0.39; globular proteins, e.g., worm myohemerythrin helix, residues 20-36, 0.47; lytic polypeptides, e.g., bombolitin I, residues 1-17, 0.55; calmodulin regulated protein kinases, e.g., rabbit smooth muscle myosin light chain kinase, residues 1-16, 0.60; and polypeptide hormones, e.g., pancreatic polypeptide, residues 24-34, 0.84. Thus, these model amphipathic peptides used in this study clearly have considerable amphipathic character. It has also been shown independently by ^{1}H-NMR that the α-helical structure extends along the entire peptide chain, except for the terminal residues, for peptides AG9, AA9, AL9, LG9, LA9 and LL9 [28, 55]. Further, these peptides have been shown, by size-exclusion chromatography, to be monomeric when the TFE concentration in solution is greater than 25% (v/v) [28, 55]. Thus, it can be confidently expected that the peptides will bind to a reversed-phase column as monomers at their preferred hydrophobic binding domains. The substituted residue at position 9 in the centre of the hydrophobic face of the amphipathic α-helices will, thus, be interacting intimately with the stationary phase. As indicated above, the proline-substituted analogues were the exception to the high α-helical character of the peptide series, e.g., AP9 showed an ellipticity of -14 600, about 50% that of the average value for the other analogues. Proline is well-recognized as a helix-disrupting residue, making the relatively low helical character of AP9 and LP9 unsurprising. Though Gly has been considered as a helix perturbing residue, this mutation in the peptide sequence used in this study does not effect the helicity of the peptide in a non-polar environment as shown above. In addition, we have previously shown that α-helical peptides with a Gly substitution every seventh

residue can still be completely α-helical even in benign medium in two-stranded α-helical coiled-coils. The strong interhelical hydrophobic interactions stabalizing the coiled-coil override the destabilizing effect of Gly (due to its intrinsic low helical propensity value [58].

Reversed-phase chromatography of synthetic peptide analogues. Figure III-2 shows the reversed-phase separation at pH 2 of selected peptide analogues. At this pH value, all of the glutamic acid (and aspartic acid) residues will be protonated, i.e., only the lysine residues in the hydrophilic face (and the arginine and lysine residues substituted at position 9 of the hydrophobic face) of the amphipathic helices will be (positively) charged. From panel A, it can be seen that the native leucine peptide (LL9) is, as expected, more retentive than the native Ala peptide (AA9). In fact, the magnitude of the retention time difference between the two peptides (26.1 min) is further evidence that the peptide is interacting with the stationary phase through preferential binding with their hydrophobic faces. Also from panel A, the hydrophobicity of the leucine side-chain was determined relative to glycine in the Ala-face and Leu-face, where the glycine analogues (LG9 and AG9) represent the situation where there is no side-chain present at position 9. Thus, in the Ala-face, the hydrophobicity of leucine may be expressed as t_RAL9 *minus* t_RAG9, i.e., a retention time difference of 8.5 min; in the Leu-face, this value is t_RLL9 *minus* t_RLG9, i.e., 5.01 min. Hence, there is a substantial decrease in apparent hydrophobicity of the leucine side-chain in the Leu-face compared to the less hydrophobic Ala-face.

Figure III-2, panel B, shows the effect of alanine, leucine, lysine and glutamic acid substitutions relative to the glycine substituted analogues. The bars above each series of peptides represents an increase or decrease in apparent hydrophobicity of the side-chain relative to the glycine mutant. The relative hydrophilicity/hydrophobicity of the side-chains shown is clearly dependent on the hydrophobicity of the environment surrounding the site of mutation. This observation not only applies to non-polar residues such as alanine and leucine, where the hydrophobicities of these side-chains relative to glycine (peptides AG

and LG) are of lesser magnitude in the Leu-face (peptides LA and LL) compared to the less

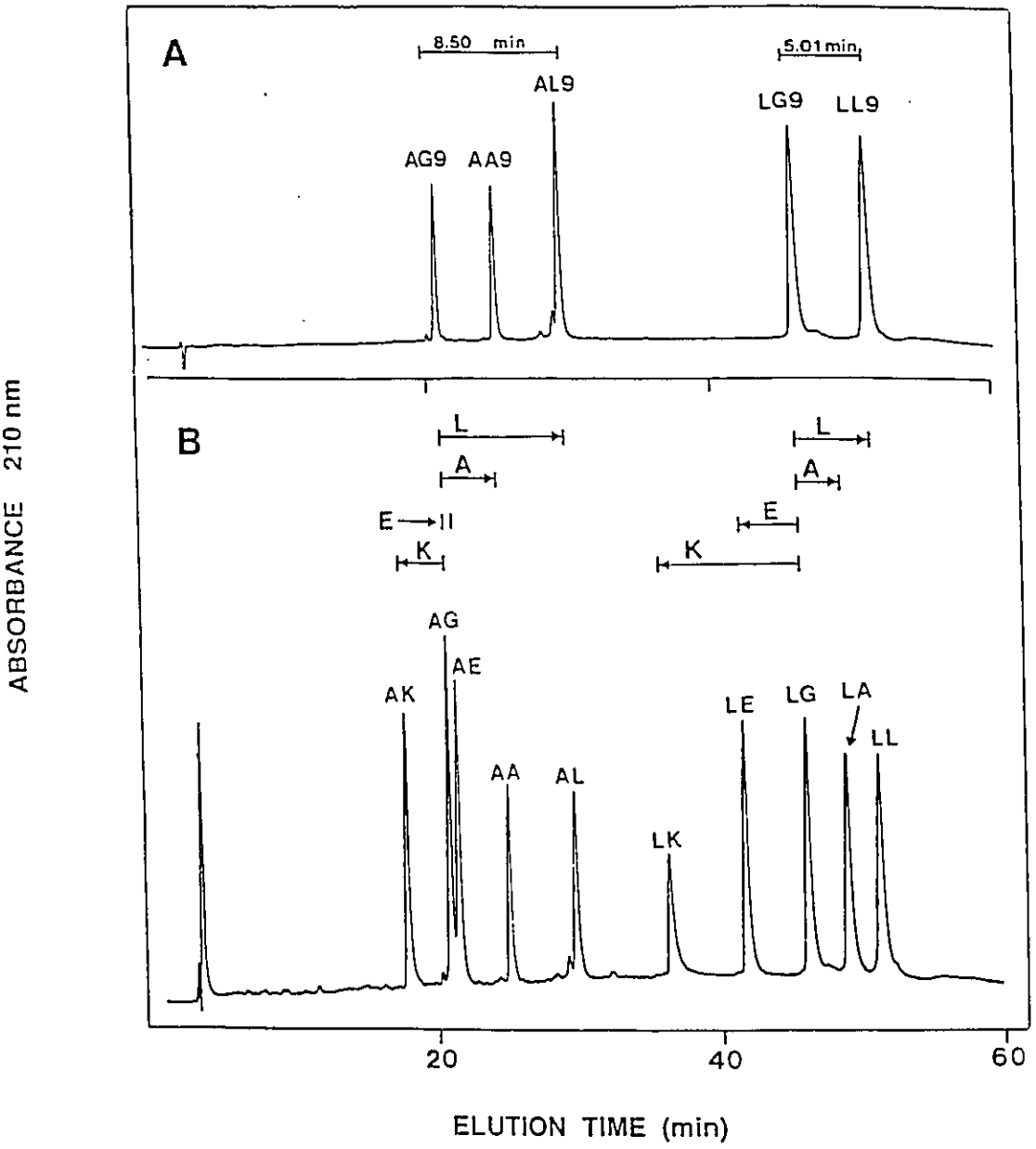

Figure III-2 RPC of model synthetic peptides. Panel A: Separation of the "native" Ala-face peptide (AA9) and the "native" Leu-face peptide (LL9) from mutant peptides AG9, AL9 and LG9. Panel B: Separation of the "native" Ala-face (AA) and the "native" Leu-face peptide (LL) from selected mutant analogues [since all residue substituitions were made at the same position in the peptide sequence (see Figure III-1) the number "9" has been omitted from the peptide designations for the sake of clarity]. The bars above the peptides in panel B represent an increase or decrease in peptide hydrophobicity relative to the glycine-substituted analogue. HPLC column, instrumentation and conditions: see Experimental. The peptide designations are described in the text.

hydrophobic Ala-face (peptides AA and AL), but also to a charged residue such as lysine

which is much more hydrophilic in the Leu-face (peptide LK) compared to the Ala-face (peptide AK). It is interesting to note that, in the Leu-face peptide (LE), the protonated glutamic acid residue is more hydrophilic relative to glycine (peptide LG); in contrast, in the Ala-face peptide (AE), glutamic acid is more hydrophobic compared to glycine (peptide AG).

Table III-1 summarizes the reversed-phase retention behaviour of all 40 peptide

Table III-1 RPC retention times of Ala- and Leu-face mutant peptides.

Amino acid [a] substitution	Ala-face mutants		Leu-face mutants	
	t_R (min) [b] (AX)	Δt_R (min) [c] (AX-AG)	t_R (min) [b] (LX)	Δt_R (min) [c] (LX-LG)
Leu (L)	29.32	8.50	50.83	5.01
Ile (I)	29.32	8.50	51.22	5.40
Phe (F)	28.68	7.86	49.80	3.98
Trp (W)	27.92	7.10	47.37	1.55
Val (V)	27.56	6.74	50.71	4.89
Met (M)	27.15	6.33	48.82	3.00
Cys (C)	25.21	4.39	48.86	3.04
Tyr (Y)	24.98	4.16	44.90	-0.92
Ala (A)	24.78	3.96	48.84	3.02
Thr (T)	21.91	1.09	46.36	0.54
Glu (E)	21.51	0.69	41.89	-3.93
Gly (G)	20.82	0.00	45.82	0.00
Ser (S)	20.23	-0.59	44.67	-1.15
Asp (D)	19.29	-1.53	41.42	-4.40
Gln (Q)	19.29	-1.53	40.06	-5.76
Arg (R)	18.65	-2.17	37.53	-8.29
Lys (K)	17.68	-3.14	36.59	-9.23
Asn (N)	17.36	-3.46	39.99	-5.83
His (H)	17.25	-3.57	37.21	-8.61
Pro (P)	16.95	-3.88	40.84	-4.98

[a] Three letter code and single letter code for the 20 amino acids commonly found in proteins. Amino acid substitutions in either the Ala- or Leu-face at position 9 of the sequence (Figure III-1).

[b] Linear AB gradient, where Eluent A is 0.1% aqueous TFA and Eluent B is 0.1% TFA in acetonitrile with a gradient-rate of 1% acetonitrile/min at a flow-rate of 1ml/min.

[c] Retention time difference between the mutant peptide and the Gly-substituted peptide (i.e., AG or LG).

analogues. The retention times of the Ala-face peptides (column denoted t_RAX in Table III-1) were now plotted against those of the Leu-face peptides (column denoted t_RLX in Table III-1). From Figure III-3, there is a good correlation (r = 0.920) between the two sets of data, suggesting that though the magnitude of the hydrophilicity/hydrophobicity values for the side-chains are different in the Ala- and Leu-face the directional effect on all side-chains is similar when changing the hydrophobicity of the environment surrounding the mutation. Thus, it is the hydrophobic environment surrounding the mutation site that is the major factor in determining the contribution of the mutation to the retention behaviour of the peptide.

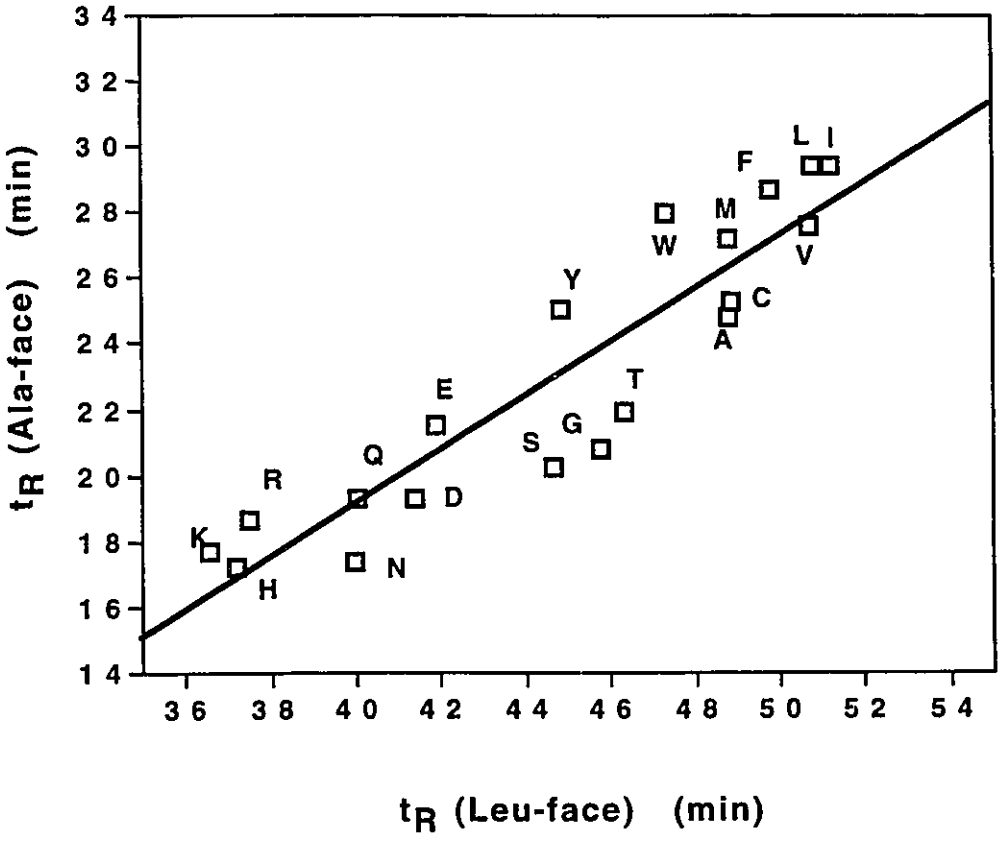

Figure III-3 Plot of t_RAX *vs* t_RLX, where AX and LX represent mutants of either the Ala- or Leu-face peptides. Retention time (t_R) data taken from Table III-1. The single letter code represents the amino acid substitution at position 9 of the peptide sequence (see Figure III-1).

The order of amino acid substitutions shown in Table III-1 was based on decreasing retention time of the Ala-face mutants (t_RAX), starting with the highest retention time for the leucine-substituted analogue (AL; 29.32 min) and ending with the least retained proline-substituted analogue (AP; 16.95 min). When the retention time of the glycine analogue (AG; 20.82 min) has been subtracted from the retention times of the other 19 analogues (AX - AG in Table III-1), the resulting numbers represent a series of coefficients expressing side-chain hydrophobicity (values > 0) or hydrophilicity (values < 0) relative to glycine. Interestingly, the order and magnitude of these values match very closely the side-chain hydrophobicity coefficients derived from the observed reversed-phase retention behaviour of a series of octapeptide analogues reported by Guo et al. [53]. The one exception is the proline-substituted analogue (AP) which these workers reported to have a hydrophobicity similar to that of alanine. In the present study, the proline side-chain is exhibiting the most hydrophilic characteristics relative to glycine (AP - AG = -3.88 min). As noted above, the presence of proline at position 9 of the 18-residue peptide sequence seriously disrupts the α-helical structure of peptide AP compared to the other 19 analogues of the Ala-face. This disruption of the amphipathic α-helix of peptide AP and, hence, modification of the hydrophobic face of this peptide, is presumably affecting the magnitude of interaction of AP with the hydrophobic stationary phase. Thus, it would not be surprising that a value denoting hydrophilicity/hydrophobicity of a proline side-chain relative to other side-chains may be substantially different when calculating this value from the observed retention times of amphipathic α-helical peptides (the present study) compared to the value derived from the retention behaviour of non-amphipathic peptides analogues [53].

From Table III-1, for the Leu-face mutants, there is a decrease in Δt_R (LX - LG) for all 19 amino acids compared to the Ala-face mutants. This suggests that the side-chains of all 19 amino acids decrease in hydrophobicity when surrounded by a more hydrophobic environment. Interesting amino acid side-chains are those of tyrosine and glutamic acid

(also see Table III-1 and Figure III-2) which are hydrophobic relative to glycine in the Ala-face and hydrophilic relative to glycine in the Leu-face. The proline-substituted analogues, AP and LP, have been excluded from the remainder of this study, based on the conviction that HPLC data derived from these mutants would not be directly comparable to the retention behaviour of the other model peptides.

In order to visualize more easily the variation in hydrophobicity of the hydrophobic side-chains between the Ala- and Leu-domains, the positive Δt_R values reported in Table III-1 were normalized, the value for maximum side-chain hydrophobicity [leucine in the Ala-face, where Δt_R (AX - AG) = 8.50 min] being denoted 1.00 and the glycine mutant being assigned a value of 0.0. Table III-2 compares the relative hydrophobicity of the side-chains of hydrophobic residues (i.e., defined as those which are more hydrophobic than glycine) following this normalization procedure. Clearly, these 11 amino acid side-chains vary considerably in hydrophobicity between the two non-polar faces, expressing their maximum hydrophobic characteristics in the Ala-face and their mimimum hydrophobicity in the Leu-face, i.e., when there is an *increase* in *hydrophobicity* of the environment around the mutation, the apparent *hydrophobicity* of the side-chain *decreases* significantly.

The normalization procedure was now applied to comparing the hydrophilicity of the hydrophilic side-chains between the Ala- and Leu-domains. Thus, the negative Δt_R values from Table III-1 were now normalized, the maximum value for side-chain hydrophilicity [lysine in the Leu-face, where Δt_R (LX - LG) = -9.23 min] being denoted -1.00 and the glycine mutant again being assigned a value of 0.0. Table III-3 compares the resulting relative hydrophilicities of these hydrophilic side-chains (i.e., defined as those which are more hydrophilic than glycine). In a similar manner to the observed behaviour of the hydrophobic residues (Table III-2), there is a clear and substantial variation in hydrophilicity of these 9 side-chains between the two non-polar faces. These side-chains express their maximum hydrophilic characteristics in the Leu-face and their minimum hydrophilicity in the Ala-face, i.e., when there is an *increase* in *hydrophobicity* of the

Table III-2 Relative hydrophobicity of hydrophobic amino acid side-chains.

Hydrophobic amino acid side-chains [a]	Maximum relative hydrophobicity (Ala-face) [b]	Minimum relative hydrophobicity (Leu-face) [c]
Leu	1.00	0.59
Ile	1.00	0.64
Phe	0.92	0.47
Trp	0.84	0.18
Val	0.79	0.58
Met	0.74	0.35
Cys	0.52	0.36
Tyr	0.49	— [d]
Ala	0.47	0.35
Thr	0.13	0.06
Glu	0.08	— [d]
Gly	0.00	0.00

[a] Hydrophobic amino acid side-chains are defined as side-chains resulting in an increase in peptide retention time relative to the mutant Gly-substituted peptide (i.e., AG or LG).

[b] The *maximum* relative hydrophobicity is defined as the ratio of Δt_R(AX-AG) values for the hydrophobic amino acid side-chains obtained from the *Ala-face mutants* and the maximum hydrophobicity value obtained for a side-chain in the *Ala-face* (Leu = 8.5 min, Table III-1).

[c] The *minimum* relative hydrophobicity is defined as the ratio of Δt_R(LX-LG) values for the hydrophobic amino acid side-chains obtained from the *Leu-face mutants* and the maximum hydrophobicity value obtained for a side-chain in the *Ala-face* (Leu = 8.5 min, Table III-1).

[d] Glu and Tyr side-chains are not hydrophobic relative to Gly in the Leu-face peptide and therefore, they do not have a minimum relative hydrophobicity by our definition.

environment around the mutation, the apparent *hydrophilicity* of the side-chain *increases* significantly.

Correlation of RPC retention behaviour with non-polar accessible surface area of model peptides. Computer modeling was used to study the α-helices of all analogues of the native Ala- and Leu-face. The side-chains were energy minimized and the non-polar accessible surface area (NPASA) was calculated for the non-polar face of these peptides. From Table III-4, the ratio of the NPASA of LL9 (810 Å^2) to AA9 (442 Å^2) is 1.83. The

Table III-3 Relative hydrophilicity of hydrophilic amino acid side-chains.

Hydrophilic amino acid side-chains [a]	Maximum relative hydrophilicity (Leu-face) [b]	Minimum relative hydrophilicity (Ala-face) [c]
Gly	0.00	0.00
Tyr	-0.10	— [d]
Ser	-0.12	-0.06
Glu	-0.43	— [d]
Asp	-0.48	-0.17
Gln	-0.62	-0.17
Asn	-0.63	-0.37
Arg	-0.90	-0.24
His	-0.93	-0.39
Lys	-1.00	-0.34

[a] Hydrophilic amino acid side-chains are defined as side-chains resulting in a decrease in peptide retention time relative to the mutant Gly peptide (i.e., AG or LG).

[b] The *maximum* relative hydrophilicity is defined as the ratio of Δt_R(LX-LG) values for the hydrophilic amino acid side-chains obtained from the *Leu-face mutants* and the maximum hydrophilicity value obtained for a side-chain in the *Leu-face* in absolute terms (Lys = 9.23 min, Table III-1).

[c] The *minimum* relative hydrophilicity is defined as the ratio of Δt_R(AX-AG) values for the hydrophilic amino acid side-chains obtained from the *Ala-face mutants* and the maximum hydrophilicity value obtained for a side-chain in the *Leu-face* in absolute terms (Lys = 9.23 min, Table III-1).

[d] Glu and Tyr side-chains are not hydrophilic relative to Gly in the Ala-face peptide and, therefore, they do not have a minimum relative hydrophilicity by our definition.

similar ratio of 2.09 (LL9/AA9) for the % acetonitrile required to elute these peptides from the column strongly suggested a correlation between the increase in retentiveness of LL9 by the column relative to AA9 and the concomitant increase in NPASA. This correlation becomes even clearer when allowance is made for the small polar surface areas on the hydrophobic faces of LL9 (31 $Å^2$) and AA9 (47 $Å^2$) which may offset to a small extent the non-polar contribution to retention. From Table III-4, once these polar contributions have been subtracted from their non-polar counterparts, the resulting ratio of modified NPASA

of LL9 to AA9 (779 Å^2/395 Å^2 = 1.97) is now in excellent agreement with the % acetonitrile ratio of 2.09.

Table III-4 Accessible surface area of peptides AA9 and LL9.

Peptide	Non-polar (A) (Å^2)	Polar (B) (Å^2)	Net (A-B)	t_R-t_g [a] (min)
AA9	442	47	395	23.85
LL9	810	31	779	49.90
Ratio LL9/AA9	1.83	—	1.97	2.09

[a] t_g denotes gradient delay time, i.e., the time for the solvent front to travel from the solvent mixer to the top of the column (0.93 min at 1 ml/min). At a gradient-rate of 1% acetonitrile/min, t_R-t_g is then equal to the % acetonitrile required to elute the peptide from the column.

The results of Table III-4 suggested that the non-polar accessible surface area is a major factor in determining the retention behaviour of our model peptides. Taking this further, we now wished to determine whether the change in apparent hydrophilicity /hydrophobicity of a specific side-chain in the centre of one hydrophobic domain compared to another, e.g., between a side-chain in the Ala-face compared to the Leu-face, was related to a corresponding change in non-polar accessible surface area between these domains. The NPASA values for 14 of the analogues in both series are shown in Table III-5. The NPASA values for the glycine mutant in both the Ala-face and Leu-face peptides were now subtracted from each of the values for the remaining residues (Δ NPASA), to produce a designated NPASA value for the substituted side-chain at mutant position 9 only. From the results shown in Table III-5, it can be seen that, in an analogous manner to the calculated apparent side-chain hydrophilicity/hydrophobicity values (or coefficients) reported in Table III-1 (Δt_RAX-AG and Δt_RLX-LG), the ΔNPASA of each side-chain was lower in the more hydrophobic Leu-face compared to the Ala-face.

Figure III-4 plots the difference between the ΔNPASA values of 13 side-chains in the two hydrophobic domains (Ala-face values *minus* Leu-face values, denoted

Table III-5 Comparison of the non-polar accessible surface area in the Ala- and Leu-face peptides.

Amino acid substitution [a]	Non-polar accessible surface area (NPASA) ($Å^2$)				ΔΔNPASA [c]	$\Delta\Delta t_R$ [d]
	Ala-face		Leu-face			
	NPASA	ΔNPASA[b]	NPASA	ΔNPASA[b]		
Leu (L)	499	79	810	45	34	3.49
Ile (I)	494	74	797	32	42	3.10
Phe (F)	493	73	792	27	46	3.88
Trp (W)	501	81	786	21	60	5.55
Val (V)	486	66	802	37	29	1.85
Met (M) [e]	493	73	808	43	30	3.33
Cys (C) [e]	466	46	784	19	27	1.35
Tyr (Y)	464	44	770	5	39	3.24
Ala (A)	442	22	780	15	7	0.94
Thr (T)	455	35	773	8	27	0.55
Gly (G)	420	0	765	0	0	0.00
Ser (S)	430	10	769	4	6	-0.56
Gln (Q)	418	-2	727	-38	-36	-4.23
Asn (N)	410	-10	740	-25	-15	-2.37

[a] Represents the amino acid substituted into position 9 of either the Ala- or Leu-face mutants (Figure III-1).

[b] Non-polar surface area of amino acid side-chain in either the Ala- or Leu-face, obtained by subtracting the non-polar surface area of the Gly-substituted peptide from the corresponding mutant peptide.

[c] Non-polar surface area change in the amino acid side-chain that occurs when the side-chain is substituted from the Ala-face to the Leu-face. Value is obtained by subtracting the non-polar surface area of the side-chain in the Ala-face, i.e. ΔNPASA, from the non-polar surface area of the side-chain in the Leu-face, i.e. ΔNPASA. Since the ΔNPASA values of Gln and Asn are negative in both the Ala- and Leu-face, the absolute value of each ΔNPASA is taken before the subtraction.

[d] The change in retention time that is observed for a substitution in going from the Ala-face to the Leu-face. The value is obtained by subtracting the absolute value of the retention time (Δt_R) of the peptide, relative to the Gly peptide, in the Leu-face, i.e. (LX-LG), from the retention time of the peptide, relative to the Gly peptide, in the Ala-face, i.e. (AX-AG); Table III-1.

[e] The sulphur atom of Met and Cys is calculated as a non-polar atom [33].

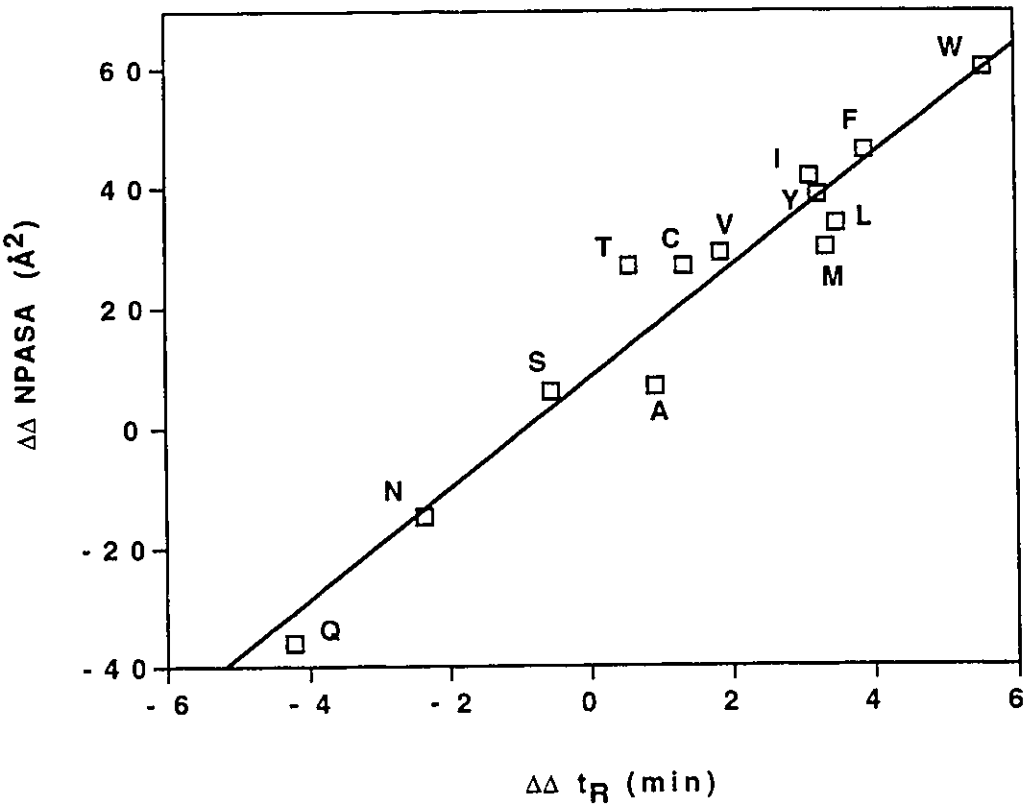

Figure III-4 Plot of ΔΔNPASA *vs* $\Delta\Delta t_R$ (see Table III-4) for amino acid side-chains. The single-letter code represents the amino acid substitution at position 9 of the peptide sequence (see Figure III-1).

ΔΔNPASA in Table III-5 and Figure III-4) *versus* the difference in apparent side-chain hydrophilicity/ hydrophobicity of the side-chains in these domains (Ala-face *minus* Leu-face values, denoted $\Delta\Delta t_R$ in Table III-5 from Δt_R values reported in Table III-1). From Figure III-4, it can be seen that there is an excellent correlation ($r = 0.967$) between these two parameters for most of the amino acid side-chains. These results suggest strongly that the change in apparent hydrophilicity/hydrophobicity of a specific side-chain in environments of varying hydrophobicity is directly related to the concomitant change in non-polar accessible surface area expressed by the side-chain. Interestingly, the values for

the acidic (glutamic acid, aspartic acid) and basic (lysine, arginine, histidine) side-chains did not correlate well. It is possible that the polar constituents in these side-chains are sterically shielding the non-polar accessible surface areas of these residues [59], thus reducing the expected magnitude of interaction of these side-chains with the reversed-phase matrix.

CONCLUSIONS

The present study describes the design and development of a chromatographic model for studying the hydrophobic interactions which characterize the way a ligand binds to its receptor. This model is based on observing the reversed-phase retention behaviour of *de novo* designed model amphipathic α-helical peptides representing the hydrophobic binding domain of a receptor protein and/or ligand. In this initial appraisal of the ligand-receptor model system, we have shown that the hydrophobicity of the environment surrounding a site in the interface of a binding domain affects the apparent hydrophilicity/ hydrophobicity of the amino acid side-chain substituted into the site. In addition, our results suggest that this effect is related to a variation of non-polar accessible surface area expressed by the side-chains in different hydrophobic environments. Such results may have major implications in understanding protein folding and stability, as well as ligand-protein binding and protein-protein interactions, by delineating the role that individual side-chains play in these systems. Thus, the model system described here should prove to be useful not only as a mimic of ligand-receptor interactions, but also as a general chromatographic probe of hydrophobic interactions involved in protein folding and stability.

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CHAPTER IV

Selectivity effects due to conformational differences between amphipathic α-helical and non-helical peptides in reversed-phase chromatography

This work has been submitted to the Journal of Chromatography for publication.

INTRODUCTION

The emergence over the past decade of reversed-phase chromatography (RPC) as the most widely-used mode of high-performance liquid chromatography (HPLC) [1] has also seen a concomitant interest in method development protocols to maximise the excellent resolving power of this technique [2-6]. Indeed, this laboratory has been active for several years in developing empirical approaches to correlate the reversed-phase retention behaviour of peptides during linear gradient elution. This work has focused in two major areas: (1) prediction of peptide retention times during RPC from sequence information alone, based on the assignment of a specific hydrophobicity value to each amino acid side-chain [7-9]; (2) prediction of the effect of varying mobile phase components (e.g., ion-pairing reagent) [10] or run conditions (gradient-rate, flow-rate) [11] on peptide separations. From such studies, we are able to predict, with considerable accuracy, how manipulation of the mobile phase or run conditions will affect the selectivity (and, hence, resolution) of a particular peptide separation. These predictive approaches both rely on relating the predicted retention times of peptides of interest to that of the observed behaviour of a peptide standard [7-11]. This approach to the optimization of peptide resolution assumes that, when not subject to conformational restraints, the chromatographic behaviour of a peptide is mainly or solely dependent on amino acid composition. When one also takes into account the effect of peptide chain length, also independent of any conformational considerations, this assumption holds up well for most practical purposes,

as evidenced by the successful use of a computer-based HPLC method development program, ProDigest-LC, derived from these principles [3,12].

It is well known that the general rule of thumb whereby it is assumed that the resolution between all peaks in a peptide mixture will increase with increased gradient time is not necessarily valid [5]. Indeed, variation of selectivity with gradient slope is commonly observed for peptides and proteins [13-18]. A very important, practical example is the occasional reversal of elution order of peptides when attempting to optimize the separation of peptide fragments from a protein digest [13], i.e., different peptides are affected to a different extent by changes in gradient slope. These selectivity variations arise from the way an individual peptide interacts with the hydrophobic stationary phase, a factor not taken into account in a purely empirical approach to prediction and optimization of peptide retention behaviour. Clearly, to enhance the value of such empirically-derived predictive methods even further, it is necessary to take into account more stringently the way individual peptide solutes interact with a reversed-phase packing, i.e., a form of fine-tuning of predictions derived solely from the knowledge of peptide primary structure information. A prime resource for such fine-tuning is represented by the *linear solvent strength (LSS)* theory of gradient elution [19-23], which enables the researcher to assign parameters to peptidic solutes reflecting differences in both the magnitude and overall affinity of the hydrophobic contact area between the peptide and the hydrophobic stationary phase. The practical value of LSS theory has already been demonstrated by its application in the development of the DryLab HPLC optimization program [2,4,24].

Clearly, many and varied influences will impact on the way a particular peptide will interact with a reversed-phase packing. These will include characteristics of the peptide itself, e.g., amino acid composition [7,9], residue sequence [9,25,26,27], peptide length [8] and the presence of any secondary structure (α-helix or β-sheet) which will affect profoundly the way in which residues are orientated with respect to the stationary phase [9]; mobile phase (e.g., type of organic modifier, ion-pairing reagent, pH) [1,10,28,29]

and run conditions (e.g., temperature) [29,30] make their own contribution to peptide chromatographic behaviour; finally, the effect of the stationary phase (e.g., type of ligand, ligand density, silica- *versus* non-silica-based) must also be considered [31,32]. In order to delineate the relative contribution of different peptide characteristics to its orientation with a reversed-phase packing, it is necessary to reduce as much as possible the number of variables which affect its retention behaviour.

The present study represents our initial investigation into how the presence of a defined structure (α-helix) affects the magnitude of the contact area of a peptide with a hydrophobic stationary phase, as expressed by LSS theory, and how this effect relates to selectivity differences between families of secondary structure (amphipathic *versus* non-amphipathic α-helices) as well as to differences between such structures and peptides with no ordered higher levels of structure (random coil). The importance of gauging the contribution of α-helical structure to the selectivity of peptide separations can be easily appreciated in such aforementioned applications as optimization of the separation of peptide fragment mixtures from chemical or proteolytic digests of proteins; such mixtures typically contain peptides with α-helical potential. Thus, in the present study, RPC was applied, under defined mobile phase and run conditions, to linear gradient elution of a series of synthetic model α-helical and non-helical peptides on an analytical reversed-phase column. We believed that observation of the retention behaviour of such peptide models would offer insight into the way such structures affect separation selectivity and, hence, how such information may be applied to the rational development of separation prediction and optimization protocols.

EXPERIMENTAL

Materials

HPLC-grade water and acetonitrile were obtained from BDH Chemicals Ltd. (Poole, England). ACS-grade orthophosphoric acid was obtained from Anachemia

(Toronto, Canada). Trifluoroethanol (TFE) was obtained from Sigma (St. Louis, MO, USA).

Instrumentation

Peptide synthesis was carried out on an Applied Biosystems peptide synthesizer Model 430 (Foster City, CA, USA). Crude peptides were purified by RPC using an Applied Biosystems 400 solvent delivery system connected to a 783A programmable absorbance detector.

The analytical HPLC system consisted of an HP1090 lquid chromatograph (Hewlett-Packard, Avondale, PA, USA), coupled to an HP 1040A detection system, HP9000 series 300 computer, HP9133 disc drive, HP2225A Thinkjet printer and HP7460A plotter.

Amino acid analyses of purified peptides were carried out on a Beckman Model 6300 amino acid analyser (Beckman Instruments, Fullerton, CA, USA).

The correct primary ion molecular weights of peptides were confirmed by time of flight mass spectroscopy on a BIOION-20 Nordic (Uppsala, Sweden).

Peptide Synthesis

Peptides were synthesized by the solid-phase technique (SPPS) on co-poly (styrene- 1% divinyl-benzene) benzhydrylamine-hydrochloride resin (0.92 mmole of amino groups/g of resin) as described previously [33]. The cleaved peptide/resin mixtures were washed with diethylether (3 x 25 ml) and the peptides extracted with neat acetic acid (3 x 25 ml). The resulting peptide solutions were then lyophilized prior to purification.

Columns and HPLC Conditions

Crude peptides were purified on a semi-preparative Synchropak RP-P C_{18} reversed-phase column (250 x 10 mm I.D., 6.5-μm particle size, 300-Å pore size) from Synchrom, Lafayette, IN, USA. The peptides were purified at pH 2 by linear AB gradient elution (0.5% B/min) at a flow-rate of 5 ml/min, where Eluent A is 0.1% aqueous TFA and Eluent B is 0.1% TFA in acetonitrile.

Analytical runs were carried out at pH 2 on an Aquapore RP-300 C_8 reversed-phase column (220 x 4.6 mm I.D., 7-μm particle size, 300-Å pore size) from Applied Biosystems, by employing linear AB gradient elution (0.5, 1, 2, 4 % acetonitrile/min) at a flow-rate of 1 ml/min, where Eluent A is 20 mM aqueous phosphoric acid and Eluent B is 20 mM phosphoric acid in 50% aqueous acetonitrile.

Circular Dichroism (CD) Measurements

CD measurements were performed on a Jasco J-720 spectropolarimeter (Jasco Inc., Easton, MD, USA). The cell was maintained at 25° C with a Lauda RMS circulating water bath (Lauda, Westbury, NY). The instrument was routinely calibrated with d(+)-10-camphorsulphonic acid at 290.5 nm and with pantoyllactone at 219 nm by following the procedures outlined by the manufacturer. CD spectra were the average of 10 scans obtained by collecting data from 250 to 190 nm. The molar ellipticity is reported as mean residue molar ellipticity ($[\theta]$, with units of deg•cm^2/dmol) and calculated from the following equation: $[\theta] = [\theta]_{obs}$ (mrw)/10lc , where $[\theta]_{obs}$ is the observed ellipticity in degrees, mrw is the mean residue weight (molecular weight of the peptide divided by the number of amino acid residues), c is the peptide concentration in grams/milliliter and l is the optical path length in cm (0.0195 cm). Peptide concentrations were determined by amino acid analysis where the stock solution of the "native" model amphipathic α-helical peptide, designated AA9 (see *Design of synthetic model peptides)* was determined to be 5.62 x 10^{-4} M and that of a non-amphipathic α-helical peptide, designated naA, was determined as 1.99 x 10^{-4} M. CD spectra were measured of peptides dissolved in 40 mM aqueous phosphoric acid - TFE (1:1, v/v), where TFE is a solvent that induces helicity in single-chain potentially α-helical peptides [34,35].

THEORETICAL CONSIDERATIONS

The LSS model describing mathematically the retention behaviour of solutes under gradient elution conditions has been reported in detail elsewhere [19-23] and only the appropriate equations used will be discussed here.

An important quantity in LSS theory is the gradient steepness parameter, *b*, which is a function of the separation conditions, e.g., the gradient time, flow rate and mobile phase composition. The retention times of a peptide, t_{g1} and t_{g2}, obtained under two different gradient times, t_{G1} and t_{G2}, may be used to determine the gradient steepness parameter through the following expression:

$$b = t_0 \log\beta \,/\, [t_{g1} - (t_{g2}/\beta) + t_0'(t_{G1} - t_{G2})/t_{G2}] \qquad (1)$$

where β is the ratio of gradient times, t_{G2}/t_{G1}, t_0 is the column dead time and t_0' is the column dead time plus the gradient elapse time (gradient elapse time = t_d, time it takes a change in mobile phase composition to move from the pump through the mixer and injector to the column inlet). The t_0 value, in min, was obtained by injecting a sample containing 1% TFA with the column in place [36]. The gradient elapse time was obtained by removing the column and measuring the time for the gradient to reach the detector when a switch from 0% to 50% B is made [36]. The t_0' value was calculated as the column dead time (t_0) plus the gradient elapse time (t_d). For our HPLC system, $t_0' = 3.73$ min and $t_0 = 2.83$ min.

Once a *b* value has been obtained, the median capacity factor, $\bar{k}$, (i.e., the capacity factor when the solute is at the midpoint of the column during a gradient run) and the median volume fraction of organic solvent, $\bar{\phi}$ (i.e., the volume fraction of organic solvent when the solute is at the midpoint of the column during a gradient run) associated with the elution of each peptide may be determined from the following relationships:

$$\bar{k} = 1/1.15b \qquad (2)$$

$$\bar{\phi} = \phi_0 + [t_{g1} - t_0' - 0.3(t_0/b)](\Delta\phi/t_{G1}) \qquad (3)$$

where $\Delta\phi = (\phi_f - \phi_0)$ and ϕ_0 is the initial value at time zero and ϕ_f is the final value, i.e., at the end of the gradient. It has been stated that in order to optimize a particular separation, the log $\bar{k}$ value should be within a narrow range of values, i.e., $0 \leq \log \bar{k} \leq 1$ [19,20]. It should be noted that the 4 different gradient rates used in this study for the separation of the synthetic peptides results in a log $\bar{k}$ value that approximates this range of values. The $\bar{k}$ and

$\bar{\phi}$ values obtained from equations (2) and (3) may be related through the following expression:

$$\log \bar{k} = \log k_0 - S\bar{\phi} \quad (4)$$

Linear plots of $\log \bar{k}$ *versus* $\bar{\phi}$ were obtained in each case over the experimental range of conditions used and the reported S and $\log k_0$ values were obtained by analysing these data by linear regression. The parameters S and $\log k_0$ are related respectively to the hydrophobic contact area of the peptide and the affinity of this contact region for the hydrophobic stationary phase.

Although, strictly speaking, system-to-system consistency requires that solute resolution under gradient conditions requires a calculation that is different from isocratic conditions [19], for the sake of simplicity we have used the isocratic form of the equation to report resolution: $R_S = 1.176\Delta t/(W1 + W2)$, where Δt is the difference in retention time between the two peaks and W1 and W2 are the widths of the peaks at half height for the corresponding peaks [36].

RESULTS AND DISCUSSION

Design of model synthetic peptides

The suggestion that the solute parameter, S, is dependent on conformation, e.g., random coil *versus* ordered or native *versus* denatured has been considered previously [20]. For instance, in an attempt to determine the relationship between structure and the solute parameter, the S values of synthetic peptides of human growth hormone (hGH) were determined [37]; in another study [38], variations in S values of the peptides bombesin, β-endorphin and glucagon with increasing temperature were followed and related to temperature-induced conformational changes. Other studies produced observations that S values may vary considerably for series of peptides which differ markedly in length and sequence [22,39]; whilst only small S-value variations were observed for minor changes in the sequence of peptide analogues of myosin light chain [40]. In these latter studies, the

conformation of the peptides was not specifically defined or related to the resulting *S* values.

We believed the best initial approach to delineating the effect of α-helical structure on the solute parameter was to compare the retention behaviour of peptides with extremes of structure, i.e., either with as close to 100% α-helical conformation as possible or with the complete absence of α-helix. To this end, two series of peptides designed to exhibit markedly different conformational characteristics during RPC were synthesized, the sequences of which are shown in Figure IV-1. The peptide series designed to exhibit negligible α-helical structure (denoted X1 in Figure IV-1), has the sequence Ac-Xxx-Leu-Gly-Ala-Lys-Gly-Ala-Gly-Val-Gly-amide, where Xxx represents the amino acid substituted at position 1; thus, G1 represents a 10 residue peptide with a glycine residue substituted at position 1. Based on the same sequence as peptide G1, the peptide designated $(G1)_2$, a 20 residue peptide (Figure IV-1), was also synthesized in order to determine any molecular weight (or chain length) effect on the *S* value.

The second series of peptides, denoted AX9 (Figure IV-1), has the sequence Ac-Glu-Ala-Glu-Lys-Ala-Ala-Lys-Glu-Xxx-Glu-Lys-Ala-Ala-Lys-Glu-Ala-Glu-Lys-amide, where Xxx represents the substituted position, a sequence known to have a high potential to form an α-helix, specifically an amphipathic α-helix [41,42]. In a similar manner to the designation of the X1 series of peptides, the analogues of this second series are identified by the substituted residue; thus, the designation AA9 refers to an alanine residue substitution at position 9 of the sequence, etc. An additional peptide, designated naA (Figure IV-1), with the same composition as peptide AA9 but a different sequence was also synthesized. This peptide, also with high α-helical potential, represents a non-amphipathic α-helical control peptide to assess any effect of amphipathicity, as opposed to strictly α-helical conformational influences, on the *S* value of an α-helical peptide.

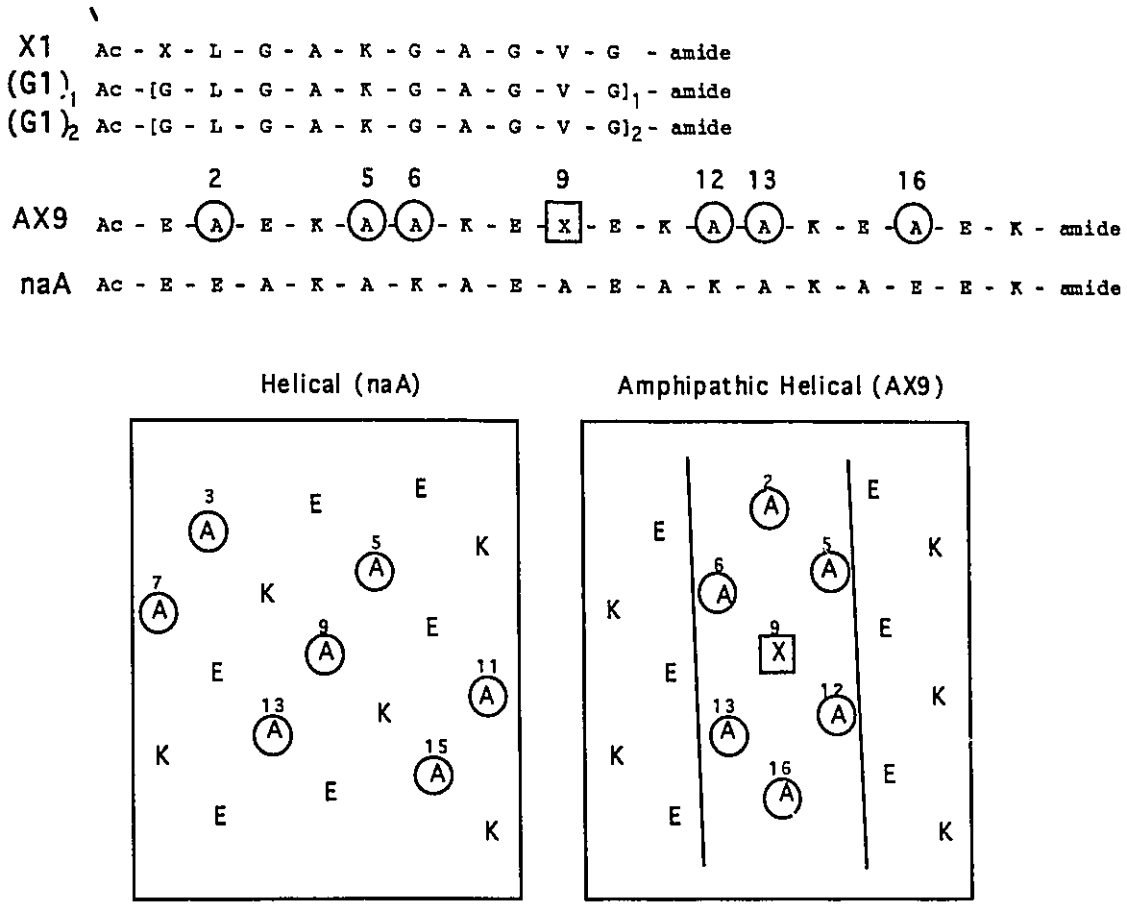

Figure IV-1 Design of synthetic peptides. Top: Sequence of the non-helical peptide analogues, denoted X1, the amphipathic α-helical peptides, denoted AX9, and the non-amphipathic α-helical peptide naA. In the X1 series, X refers to the amino acid substituted at position 1 of the 10 residue peptide. In the AX9 series, A represents the amino acid Ala which makes up the hydrophobic face (circled residues labelled 2, 5, 6, 12, 13, and 16) of the α-helical peptide and X refers to the amino acid substituted at position 9 (boxed residue labeled 9) in the α-helical peptide. The Lys (K) and the Glu (E) acid residues make up the hydrophilic face of the helix. The non-amphipathic α-helical peptide, naA, is of the same sequence as the AA9 peptide, except that the amino acid sequence is different such that the Ala residues are not all in the hydrophobic face as in peptide AA9. Bottom: Non-amphipathic α-helical and amphipathic α-helical peptides are represented as α-helical nets.

Conformation and helicity of model peptides

Conformation of the peptides was determined by CD in aqueous solution in the presence of 50% (v/v) TFE. As noted above, this solvent promotes helix formation only in regions of a polypeptide with some helical propensity [34, 43-45]. This concentration of

TFE is also a good mimic of the hydrophobic environment of RPC, known to induce and stabilize α-helical structure in potentially helical molecules [9].

It has been shown previously by Zhou et al. [9] that the sequence of the peptide series denoted X1 (Figure IV-1) exhibits the desired negligible α-helical structure required for the present study.

From Figure IV-2, it can be seen that both peptides AA9 and naA exhibit significant α-helical content; in addition, the $[\theta]_{220} / [\theta]_{207}$ ratio value is less than 1, suggesting that, in the presence of 50% TFE, peptides AA9 and naA are single stranded α-helices [46,47]. It has been shown previously [48] that even 29- and 36-residue synthetic amphipathic α-helical peptides which form very stable coiled-coil structures are chromatographed as monomers during size exclusion HPLC in 0.1% aqueous TFA containing 50% TFE. Taken together, these observations suggest that peptide AA9, as well as the non-amphipathic naA, will be chromatographed as a single-stranded α-helix during RPC.

RPC retention behaviour of amphipathic versus non-amphipathic α-helical peptides

Figure IV-1 also shows the structures of the amphipathic α-helical series of peptides, AX9, and the non-amphipathic naA presented as α-helical nets. The amphipathic nature of the AX9 series is quite clear, with the circled alanine residues at positions 2,5,6,12,13 and 16 and the substituted residue at position 9 making up the hydrophobic face of the peptide. The orientation of hydrophobic residues along a helix in such a manner also gives rise to a preferred binding domain in RPC, whereby the observed peptide retention time is greater than would be expected from predictions based on sequence and chain length information alone [9,49]. In contrast, the helical net presentation of peptide naA, which has the same amino acid composition as peptide AA9, demonstrates a distribution of alanine residues throughout the helix, such that no preferred binding domain is formed; hence, naA would be expected to be eluted prior to AA9 from an RPC column [9,49].

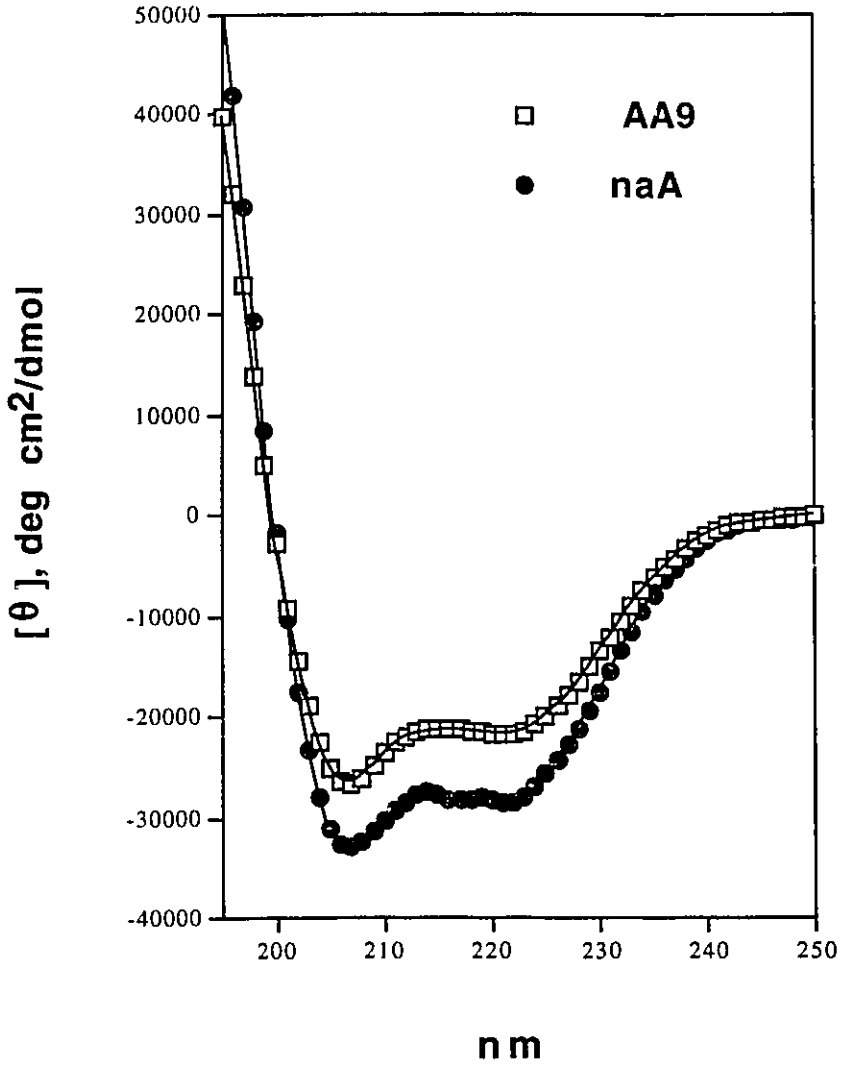

Figure IV-2 CD spectra of amphipathic (AA9) and non-amphipathic (naA) α-helical peptides. The sequences of AA9 and naA are shown in Figure IV-1. The CD spectra of the peptides were performed in 50% TFE in 20 mM aqueous H_3PO_4 (open squares, AA9 and filled circles, naA).

Selectivity differences between non-helical and amphipathic α-helical peptides

Four analogues of the non-helical peptide series (peptides A1, L1, Y1and F1) and four analogues of the amphipathic α-helical series (peptides AA9, AL9, AY9 and AF9) were run on a reversed-phase column at different linear gradient rates of 0.5%, 1%, 2%

and 4% acetonitrile/min. From these data, the median capacity factor, $\bar{k}$, was calculated using equations (1) and (2) and the median volume fraction, $\bar{\phi}$, was calculated using equation (3), as described in the Experimental section.

Figures IV-3, IV-4 and IV-5 show plots of log $\bar{k}$ *versus* $\bar{\phi}$ for selected pairs of non-helical and amphipathic α-helical peptides; *S* and log k_0 values subsequently obtained from these plots are reported in Table IV-I. Such peptide pairs were chosen as being representative of the type of peptide conformational extremes typically encountered when attempting to optimize separations of peptide mixtures obtained from protein digests.

From Table IV-1, it can be seen that the *S* values obtained for the non-helical and amphipathic α-helical peptides represent a small range of values within each series of peptides (i.e., 10.5 to 13.0 and 23.4 to 27.2, respectively); in contrast, there is a significant difference in *S* values between the two series of peptides. According to Snyder [19], the separation of solutes of different *S* values may result in three different types of log $\bar{k}$ *vs* $\bar{\phi}$ plots; in addition, it is also suggested by Snyder and Stadalius [20] that any condition that affects the value of $\bar{k}$ (e.g., the gradient time, t_G) will result in a change in $\bar{\phi}$, this change in $\bar{\phi}$ subsequently resulting in a change in selectivity. Aguilar et al. [39] and Hearn et al. [15] also suggest that selectivity differences between poylpeptides will be related to their respective *S* values.

The elution profiles shown in Figures IV-3, IV-4 and IV-5, where selected pairs of peptides (each pair including one peptide from each peptide series) are separated under different gradient conditions, represent excellent practical examples of the three types of log $\bar{k}$ *vs* $\bar{\phi}$ plot discussed by Snyder [19]. Thus, very different effects of varying gradient rate on the selectivity of the separation of the peptide pairs are observed in Figures IV-3 to IV-5.

From Figure IV-3 (left), it can be seen that the non-helical peptide, L1, and the amphipathic α-helical peptide, AY9, have the same median capacity factor at a gradient rate

Table IV-1 Retention time, *S* and log k_0 values of non-helical and amphipathic α-helical peptides.

	t_g(min) [b]					
t_G (min) [a]	100	50	25	12.5	*S* [c]	log k_0 [c]
gradient rate [a]	0.5%	1%	2%	4%		
Helical Peptides [d]						
naA	30.10	17.79	11.19	7.70	33.2	4.34
AA9	39.65	22.72	13.77	9.04	27.2	4.93
AL9	48.15	27.06	16.06	10.20	24.1	5.44
AY9	40.25	23.04	14.02	9.18	24.9	4.62
AF9	47.25	26.60	15.90	10.12	23.4	5.20
Non-helical peptides [d]						
$(G1)_1$	21.85	14.73	10.34	7.66	13.2	1.53
$(G1)_2$	34.69	20.70	13.09	8.87	17.4	2.91
A1	23.47	15.61	10.80	7.90	13.0	1.63
L1	35.25	21.89	14.16	9.67	11.1	2.17
Y1	31.37	19.66	12.91	8.97	12.3	2.07
F1	38.13	23.35	14.93	10.12	10.5	2.24

[a] t_G represents the length of the linear AB gradient, in min, for a change from 0% to 100% B and the rate represents the equivalent % acetonitrile/min for the corresponding gradient time. For conditions, see Experimental.

[b] t_g represents the retention time of each peptide under linear AB gradient conditions.

[c] *S* and log k_0 are determined by linear regression of the data from the log $\bar{k}$ *vs* $\bar{\phi}$ as described by Synder and Stadalius [20], (see Experimental).

[d] For details of peptide designations, see Design of model synthetic peptides and Figure IV-1.

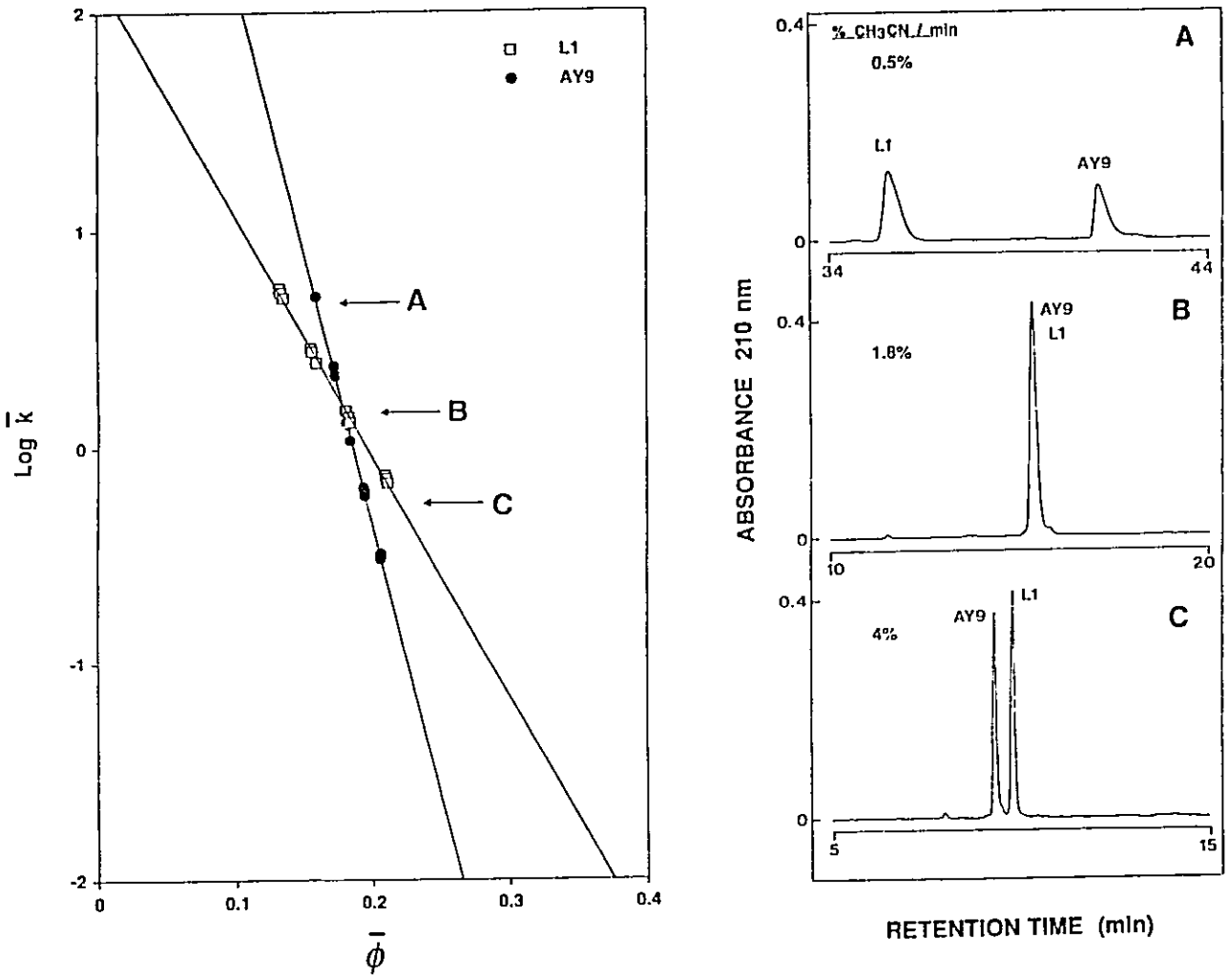

Figure IV-3 Plot of log $\bar{k}$ *vs* $\bar{\phi}$ and reversed-phase elution profiles of peptides L1 and AY9. Plot: Values of log $\bar{k}$ and $\bar{\phi}$ were obtained by separating L1 and AY9 at 4 different gradient rates (see Table IV-1) and applying equations (1), (2) and (3) to the retention time data as described in Experimental. Straight line plots were obtained by linear regression of the data. The labels A, B and C refer to the elution profiles shown in the right hand panels. RPC: Peptides L1 and AY9 were separated at 0.5%, 1.8% and 4% acetonitrile/min at a flow rate of 1ml/min where Eluent A was 20 mM phosphoric acid and eluent B was 20 mM phosphoric acid in 50% aqueous acetonitrile.

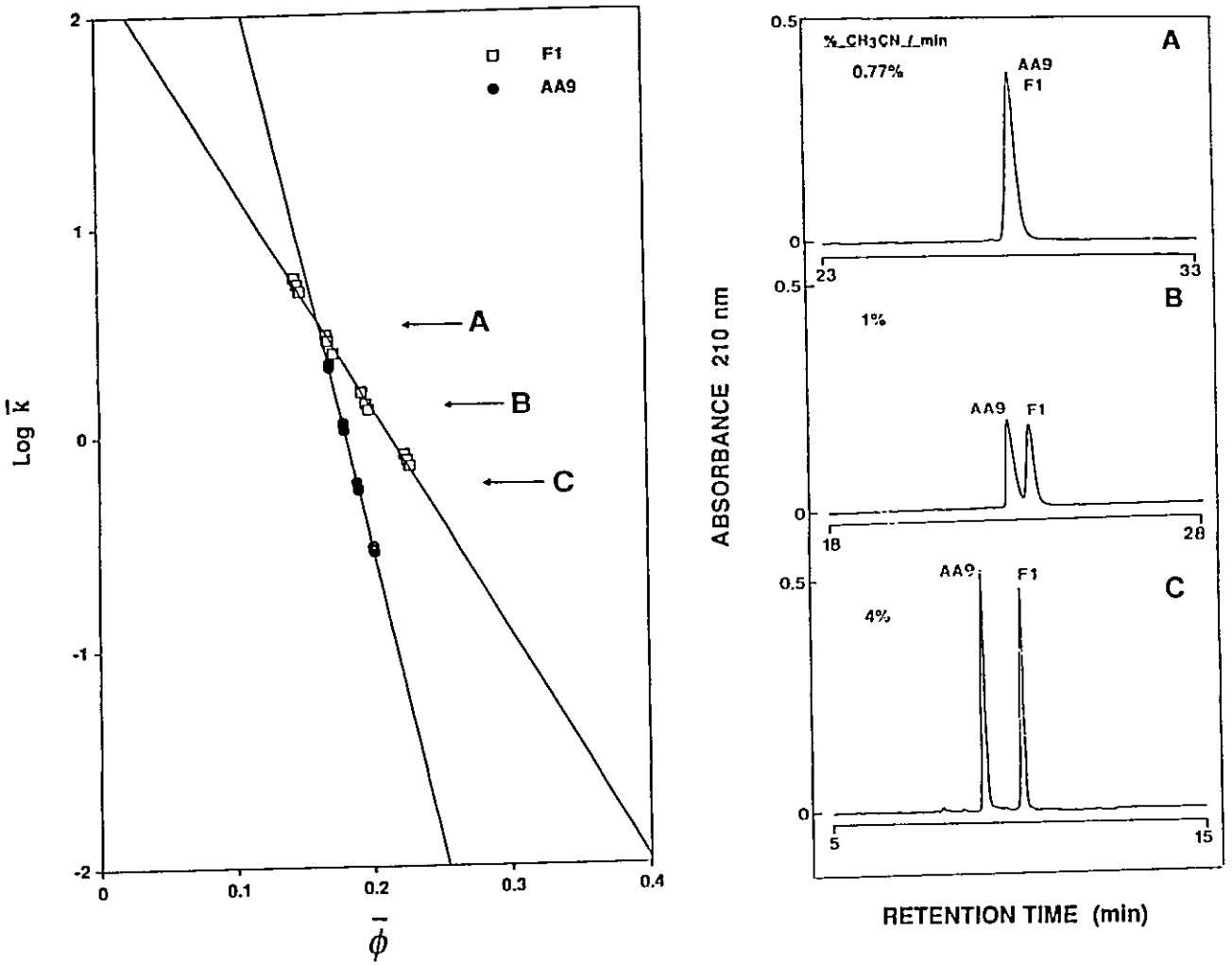

Figure IV-4 Plot of log $\bar{k}$ *vs* $\bar{\phi}$ and reversed-phase elution profiles of peptides F1 and AA9. Plot: obtained as in Figure IV-3. RPC: conditions as in Figure IV-3, except that gradient rates of 0.77%, 1.0% and 4% acetonitrile/min were used .

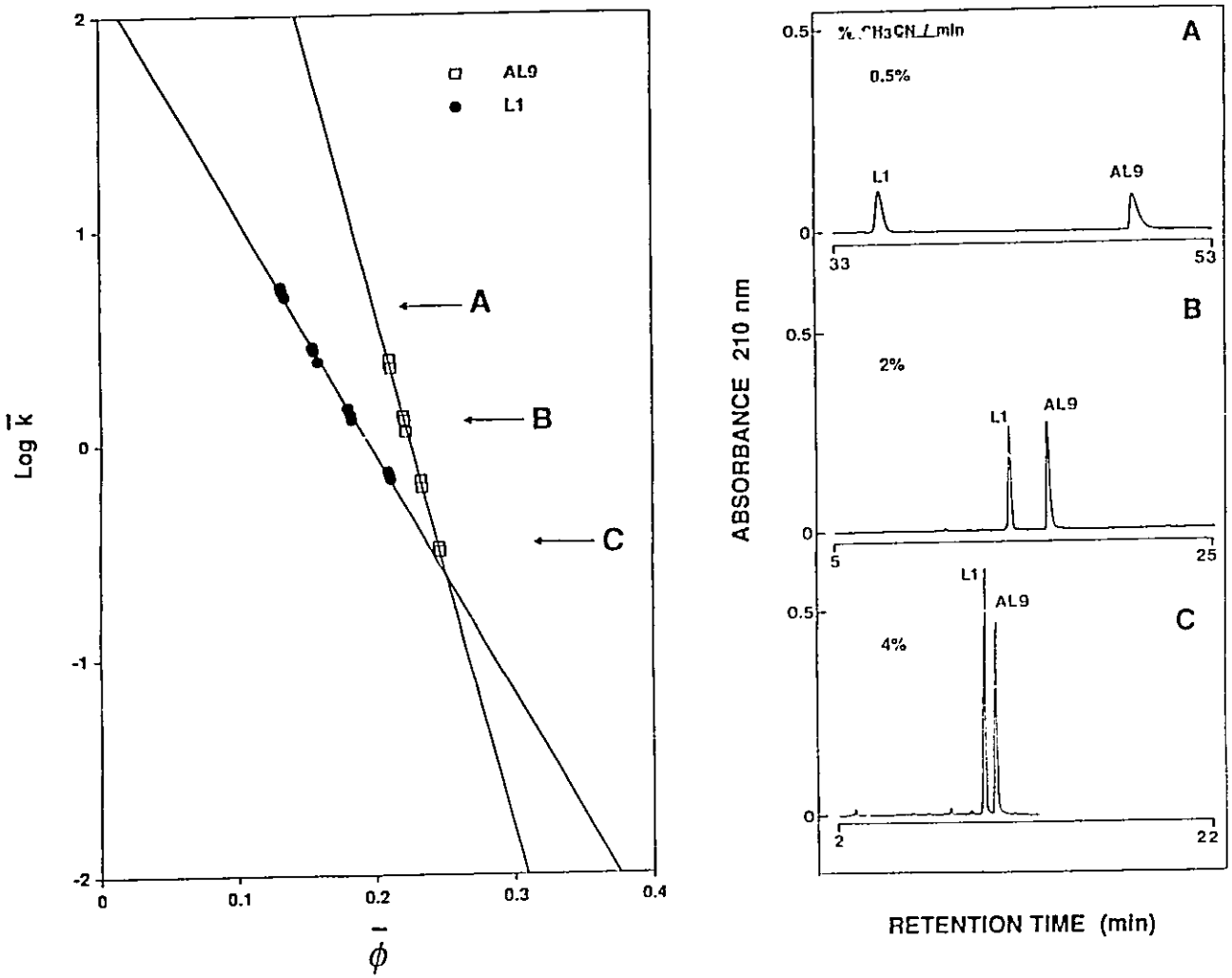

Figure IV-5 Plot of log $\bar{k}$ *vs* $\bar{\phi}$ and reversed-phase elution profiles of peptides L1 and AL9. Plot: obtained as in Figure IV-3. RPC: conditions as in Figure IV-3, except that gradient rates of 0.5%, 2.0% and 4% acetonitrile/min were used.

of 1.8% acetonitrile/min, i.e., the point where the two plots intersect; thus, the two peptides are coeluted, as seen in Figure IV-3 (right), panel B. Since the plots of the two peptides intersect at a point in the centre of the workable range of median capacity factor, i.e., where peptide retention times are neither too short nor too long for practical purposes, separation of the two peptides may be achieved either by decreasing the gradient rate (the more traditional approach) (Figure IV-3 [right], panel A), or increasing the gradient rate (panel C) [19]. Thus, by decreasing the gradient rate from 1.8% acetonitrile/min (panel B) to 0.5% acetonitrile/min (panel A), peptides L1 and AY9 were separated by five minutes (from Table IV-1: AY9, t_g = 40.25 min; L1, t_g = 35.25 min). Alternatively, by increasing the gradient rate from 1.8% acetonitrile/min (panel B) to 4% acetonitrile/min (panel C), peptides L1 and AY9 are separated by 0.49 min (Table IV-1: L1, t_g = 9.67 min; AY9, t_g = 9.18 min). Note the reversal of peptide elution order between the two extremes of gradient rate, a consequence of the intersecting plots shown in Figure IV-3 (left) and an excellent example of changes in separation selectivity due to significantly different peptide *S* values [13,17].

Figure IV-4 represents a second type of log $\bar{k}$ *vs* $\bar{\phi}$ plot [19], where the lines for the two peptides intersect at the higher end of practically favourable median capacity factors. Thus, at a gradient rate of 0.77% acetonitrile/min, the median capacity factor for the non-helical/amphipathic α-helical peptide pair F1/AA9 is the same (Figure IV-4, left); therefore, there is no separation selectivity and the peptides are coeluted (Figure IV-4 [right], panel A). Again in this case, the peptide *S* values are substantially different (Table IV-1: AA9, *S* = 27.2 and F1, *S* = 10.5) and a change in selectivity may be achieved by varying the gradient rate [19]. For this peptide pair, the best option clearly is to increase the gradient to separate the peptides within a reasonable run time. Thus, the separation improved progressively as the gradient rate was increased from 0.77% acetonitrile/min (panel A) to 1% acetonitrile/min (panel B) and, finally, to 4% acetonitrile/min (panel C). In

addition, this separation was achieved within a short run time (< 10 min) and resulted in sharp solute peaks.

Figure IV-5 shows the separation of the peptide pair L1/AL9, which also exhibit significantly different S values (Table IV-1: L1, S = 11.1 and AL9, S = 24.1). These peptides produce the third type of log $\bar{k}$ *vs* $\bar{\phi}$ plot [19], where the individual peptide plots intersect at the lower end of the practical working range of log $\bar{k}$ values. Clearly, for this peptide pair, a decreasing gradient rate would be the appropriate method to improve the separation. Thus, decreasing the gradient rate from 4% acetonitrile/min (Figure IV-5 [right], panel C) to 2% acetonitrile/min (panel B) and, finally to 0.5% acetonitrile/min (panel A) results in a progressive improvement in peptide separation; at a gradient rate of 0.5% acetonitrile/min, the peptides are separated by 12.90 min (Table IV-1: AL9, t_g = 48.15; L1, t_g = 35.25).

Contribution of conformational differences to selectivity of peptide separations

We now set out to determine the role that conformation played in the selectivity differences apparent between the non-helical and amphipathic α-helical peptides demonstrated in Figures IV-3 to IV-5 and Table IV-1. The peptide pairs of Figures IV-3 to IV-5 not only differed in conformation (non-helical X1 series *versus* α-helical AX9 series), but also in molecular weight (or polypeptide chain length) (10-residue X1 series *versus* 18-residue AX9 series). Thus, it was now necessary to determine the contribution, if any, of hydrophobicity and molecular weight to the magnitude of the S values obtained for the two series of peptides in order to delineate the effect of α-helical conformation on separation selectivity.

Despite the significant range of hydrophobicities of the non-helical X1 series of peptides, as expressed by their retention times (23.47 to 38.13 min at 0.5% acetonitrile/min; Table IV-1), the corresponding range of S values was relatively small (10.5 to 13.0; Table IV-1); similar observations were made for the amphipathic α-helical

AX9 series of peptides where the retention times varied from 39.65 to 48.15 min with a small S value range of 23.4 to 27.2 (Table IV-1).

It has been observed that S is dependent on MW through the following relationship: $S = a(MW)^b$ [40]. However, it has been found that this relationship is very much dependent on the peptides used in the determination; thus, Stadalius et al. [50] reported a relationship of $S = 0.48M^{0.44}$, Aguilar et al. [39] reported that $S = 2.99M^{0.21}$ and Hearn and Aguilar [40] determined that $S = 0.11(MW)^{0.68}$. It has therefore been suggested [22] that it is not the molecular weight *per se* that is important in determining S; rather, the S value directly relates to the hydrophobic contact area established by the solute at the stationary phase rather than there being a strict relationship with solute molecular weight. This is also consistent with the previous suggestion [20] that S is dependent on conformation, i.e., random coil *versus* an ordered structure.

From Figure IV-1, non-helical peptides $(G1)_1$ and $(G1)_2$ were now chromatographed at different gradient rates and their S values calculated from plots of $\log \bar{k}$ *vs* $\bar{\phi}$. Peptide $(G1)_1$ is the glycine-substituted 10-residue analogue of the X1 peptide series; whereas, $(G1)_2$ is a 20-residue polymer of $(G1)_1$ and represents a non-helical control peptide similar in length to the 18-residue α-helical series of peptides (AX9). From Table IV-I, the $(G1)_1$ peptide has an S value (13.2) that is similar to the range of values determined for other non-helical 10-residue peptides, A1, L1, Y1 and F1 (10.5 to 13.0). In addition, it can also be seen that an increase in molecular weight (or chain length) from the 10-residue $(G1)_1$ to the 20-residue $(G1)_2$ ($S = 17.4$), resulted in an increase in S which is consistent with a previous suggestion that the S value may increase with MW through simple peptide elongation [22], as in this example. Thus, although peptide chain length may contribute to some extent, the magnitude of the S value increase observed between the 10-residue and 20-residue peptides (13.2 to 17.4; Table IV-1) is not large enough to account for the significant differences in the range of S values (and, hence, selectivity differences) between the non-helical 10-residue X1 peptide series (S = 10.5 to 13.0; Table

IV-1) and the 18-residue amphipathic α-helical AX9 peptide series (S = 23.4 to 27.2; Table IV-1). Despite the amino acid composition differences between the peptide series, we feel this is strong evidence that the significantly different S values for the AX9 series of peptides in comparison to the non-helical X1 series is due mainly to the influence of the α-helical conformation of the former.

We now wished to determine whether the amphipathicity had any significant contribution to peptide S values separate from the conformational influence of the helix. The non-amphipathic α-helical peptide, naA (Figure IV-1), and the amphipathic α-helical peptide AA9 have hydrophobic moments of 0.04 and 0.59, respectively, determined using the method and normalized consensus hydrophobicity scale of Eisenberg et al. [51]). The hydrophobic moment is a measure of amphipathicity, where the greater the value, the greater the amphipathicity [52]. Peptide naA exhibits a significantly different S value (33.2; Table IV-1) compared to the non-helical X1 peptides (a range of 10.5 to 13.0; Table I). Instead, the value for peptide naA is of a magnitude similar to that of its amphipathic analogue, peptide AA9 (27.2; Table IV-1). Clearly, the amphipathicity of these α-helical peptides is not a major factor influencing peptide S values and, hence, separation selectivity, as compared to the conformational differences between non-helical and helical peptides.

Resolution between mixtures of non-helical and amphipathic helical peptides

Resolution under gradient conditions is a function of selectivity, α_g, and a capacity factor term, Q, where $Q = \bar{k}/(1+\bar{k})$ [19]; Q represents the fraction of molecules in the stationary phase [53]). Snyder [19] indicates that if the gradient steepness parameter (b) values for 2 solutes are the same, then selectivity is constant and resolution is then proportional to Q, where maximum resolution can be obtained using large gradient times, i.e., a shallow gradient rate results in a large capacity factor which increases Q and, thus, increases resolution. In addition, Hearn and Aguilar [40] suggest that a change in

selectivity with a change in gradient rate can still be observed for solutes with a small difference in S value (e.g., S = 16.00 and 16.82).

From Figures IV-6 and IV-7, left panels, it can be seen that peptides of similar structure exhibit similar log $\bar{k}$ *vs* $\bar{\phi}$ plots and, therefore, a similar S value; this can also be seen from Table IV-1, where, as noted above, the non-helical and amphipathic α-helical peptides have a small intra-series range of S values (10.5 to 13.0 and 23.4 to 27.2, respectively). Since S for each series of peptides is represented by a small range of values, this suggests that improvement in resolution between peptides of the same series (and, thus the same conformation) may be obtained by a decreasing gradient rate, where any such improvement may be mainly due to changes in Q plus a lesser contribution of the small selectivity differences between the peptides. Figure IV-6 illustrates this situation, where non-helical peptides L1 and F1, having similar values of S (L1 = 11.1 and F1 = 10.5, respectively; Table IV-1), exhibited a 1.2-fold change in resolution (3.5 to 4.2; Table IV-2) for a 2-fold (1% acetonitrile/min to 0.5%/min) decrease in gradient rate. Similar results can be seen in Figure IV-7, where the two amphipathic α-helical peptides, AF9 and AL9, exhibited a 1.25-fold change in resolution (1.2 to 1.5; Table IV-2) for a 2-fold (0.5% acetonitrile/min to 1% acetonitrile/min) decrease in gradient rate.

This is in clear contrast to the situation where changes in selectivity, due to conformational differences between non-helical and amphipathic helical peptides, result in large changes in resolution. For example, in Figure IV-3 (right), a decreasing gradient rate results in a large improvement in resolution for the peptide pair L1/AY9; thus, from Table IV-2, the resolution of this pair increases from 0 to 7.6 for a 3.6-fold decrease in gradient rate (1.8% acetonitrile/min to 0.5%/min). Similarly, in Figure IV-5 (right), the resolution of the peptide pair L1/AL9 increases 2.4-fold (7.2 to 17.5; Table IV-2) for a 4-fold (2% acetonitrile/min to 0.5%/min) decrease in gradient rate. These effects are probably due to changes in both Q and selectivity, the latter now making a vital contribution to the

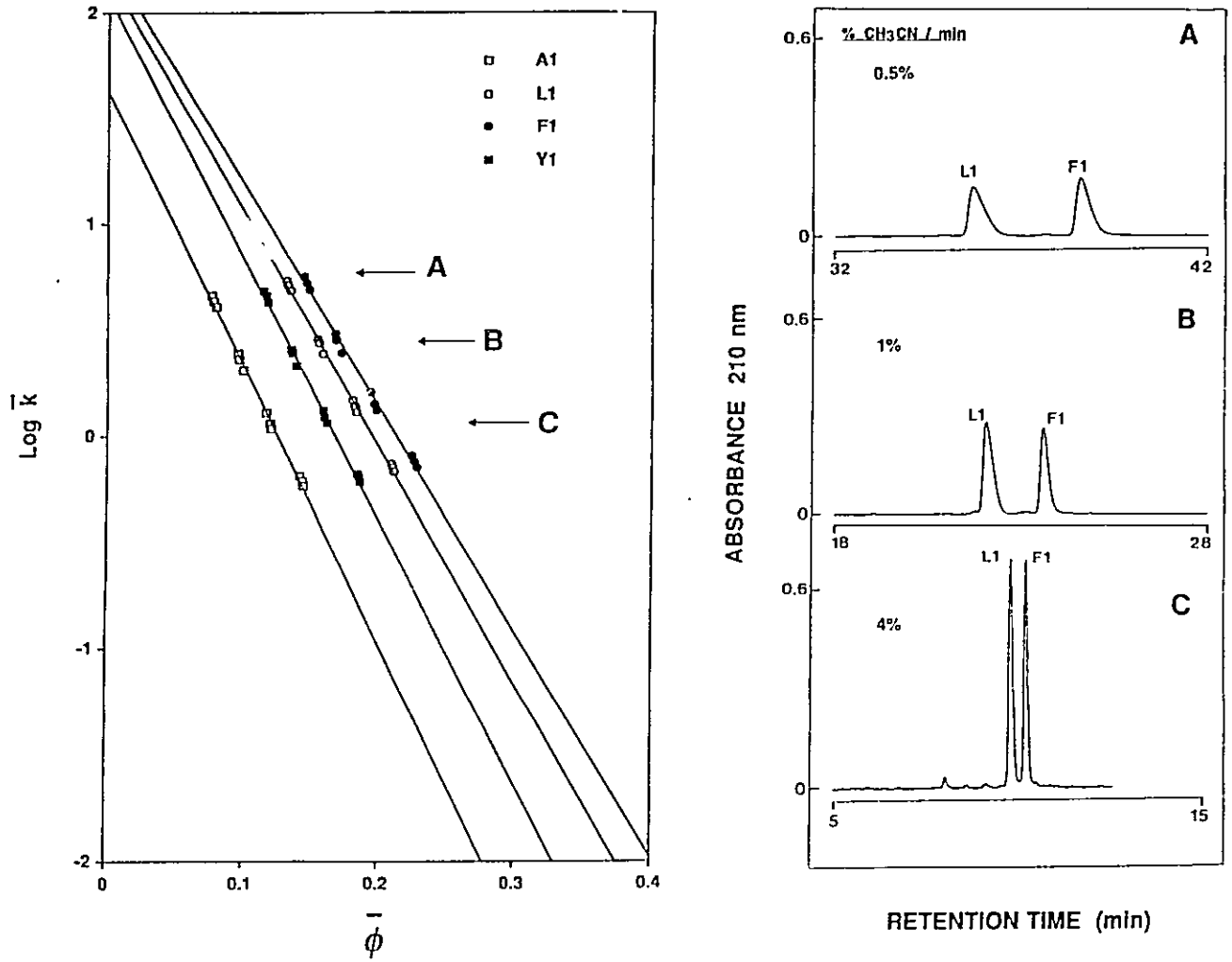

Figure IV-6 Plot of log $\bar{k}$ *vs* $\bar{\phi}$ and reversed-phase elution profiles of the non-helical peptides A1, L1, Y1 and F1. Plot: obtained as in Figure IV-3. RPC: conditions as in Figure IV-3, except that gradient rates 0.5%, 1.0% and 4% acetonitrile/min were used.

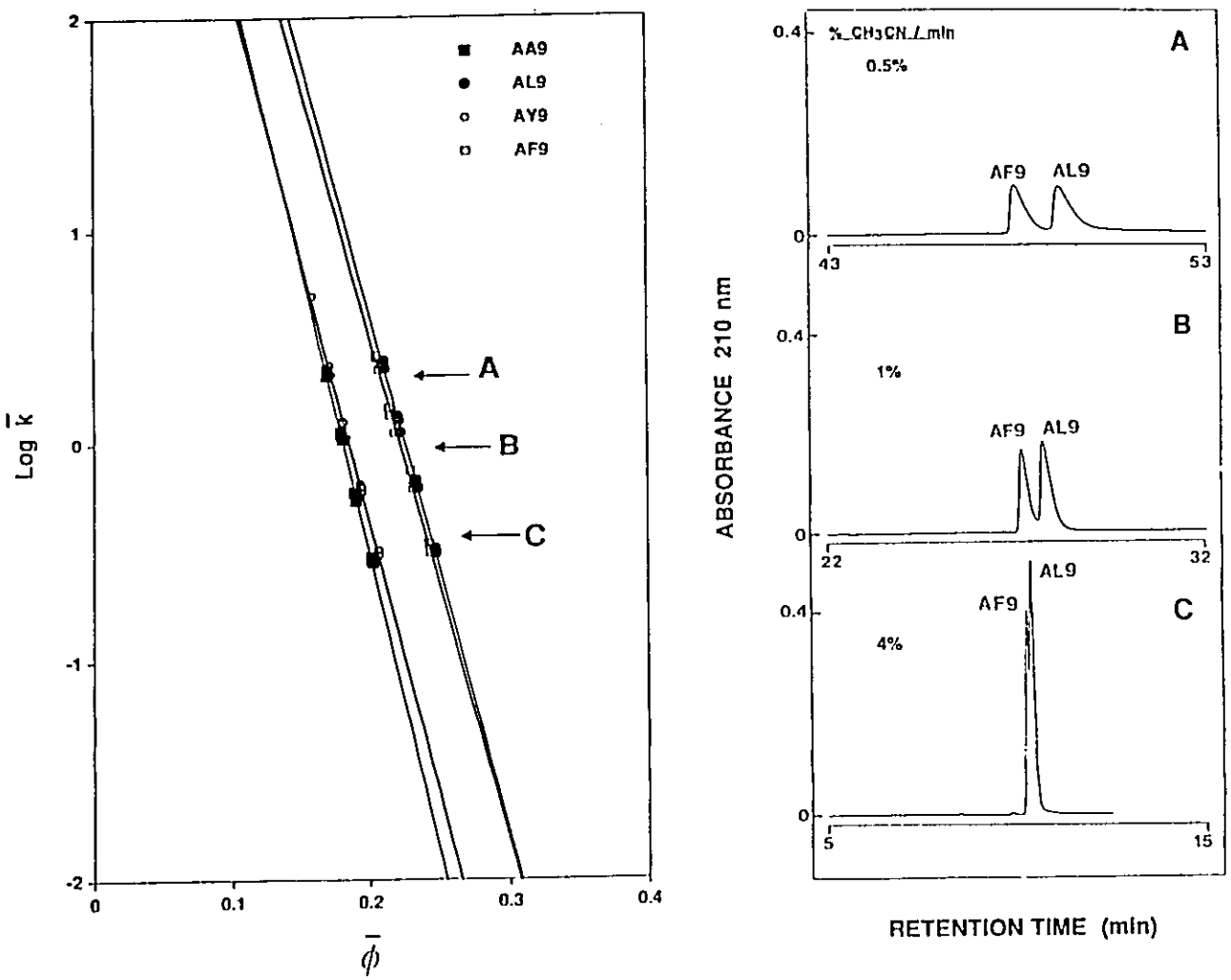

Figure IV-7 Plot of log $\bar{k}$ *vs* $\bar{\phi}$ and reversed-phase elution profiles of the amphipathic α-helical peptides AA9, AL9, AY9 and AF9. Plot: obtained as in Figure IV-3. RPC: conditions as in Figure IV-3, except that gradient rates 0.5%, 1.0% and 4% acetonitrile/min were used.

Table IV-2 Resolution for peptide pairs in Figures IV-3 to IV-7.

Figure [a]	peptide pair [a]	% acetonitrile/min [b]	Resolution (R_S)[c]
IV-3	L1/AY9	0.5	7.6
		1.8	0
		4	2.7
IV-4	F1/AA9	0.77	0
		1	1.5
		4	6.1
IV-5	L1/AL9	0.5	17.5
		2	7.2
		4	2.1
IV-6	L1/F1	0.5	4.2
		1	3.5
		4	2.2
IV-7	AF9/AL9	0.5	1.5
		1	1.2
		4	----

[a] Refers to Figures IV-3 through IV-7 and the corresponding peptide pair separated under the given linear AB gradient conditions (see experimental for conditions).

[b] % acetonitrile used for the separation of the reported peptide pair in panels A, B and C, respectively (lowest to highest gradient rate) of Figures IV-3 to IV-7.

[c] $R_S = 1.176\Delta t / (W1 + W2)$, where Δt is the difference in retention times for the two peaks and W1 and W1 is the width at half height for each corresponding peak.

separation. Increasing gradient rates are also shown to improve resolution in Figures IV-3 and IV-4, where an increase in resolution of the peptide pair L1/AY9 (Figure IV-3) from 0 to 2.7 (Table IV-2) is observed for a 2.2-fold (1.8% acetonitrile/min to 4% acetonitrile/min) increase in gradient rate and a 4.1-fold increase in resolution (1.5 to 6.1) of the peptide pair F1/AA9 (Figure IV-4) is observed for a 4-fold (1% acetonitrile/min to 4% acetonitrile/min) increase in gradient rate. In these cases, the observed distinct improvements in peptide resolution are likely due solely to changes in selectivity.

Table IV-3 reports the gradient steepness parameter (*b*) and median capacity factor ($\bar{k}$) values obtained for 17 analogues of both the non-helical (X1) and α-helical (AX9) series of peptides. It can be seen that the intra-series peptide *b* or $\bar{k}$ values are of similar

magnitude for both series of peptides. This suggests that all of the peptides within each peptide series would exhibit similar plots of log $\bar{k}$ *vs* $\bar{\phi}$ as those shown in Figure IV-6

Table IV-3 Retention time, gradient steepness parameter and median capacity factor of non-helical and amphipathic α-helical peptides.

	Helical peptides				Non-helical peptides			
Amino acid substitution[a]	t_g (min) [b]				t_g (min) [b]			
gradient rate	2% t_{g1}	1% t_{g2}	b [c]	$\bar{k}$ [c]	2% t_{g1}	1% t_{g2}	b [c]	$\bar{k}$ [c]
Ile (I)	16.14	27.13	1.20	0.72	13.72	21.44	0.75	1.16
Leu (L)	16.09	27.20	1.36	0.64	14.08	22.19	0.76	1.14
Phe (F)	15.92	26.78	1.28	0.68	14.83	23.66	0.75	1.16
Trp (W)	15.56	26.23	1.47	0.59	15.24	24.43	0.73	1.18
Val (V)	15.07	25.40	1.69	0.52	12.43	18.91	0.77	1.13
Met (M)	14.88	24.96	1.59	0.55	12.87	19.83	0.78	1.11
Cys (C)	13.97	23.03	1.44	0.60	11.46	16.90	0.74	1.17
Tyr (Y)	13.97	23.20	1.69	0.52	12.70	19.41	0.75	1.15
Ala (A)	13.76	22.81	1.74	0.50	10.66	15.69	0.90	0.97
Thr (T)	12.32	20.00	1.87	0.46	10.52	15.53	0.96	0.91
Glu (E)	12.13	19.77	2.24	0.39	10.73	15.62	0.81	1.08
Gly (G)	11.66	18.77	2.08	0.42	10.27	14.73	0.82	1.06
Ser (S)	11.51	18.36	1.83	0.47	10.12	14.66	0.92	0.94
Asp (D)	11.17	17.67	1.81	0.48	10.43	14.92	0.77	1.13
Gln (Q)	11.17	17.71	1.89	0.46	10.23	14.81	0.89	0.98
Asn (N)	10.14	15.66	1.91	0.45	10.06	14.49	0.90	0.97
Pro (P)	9.94	15.10	1.62	0.54	12.06	18.18	0.77	1.13

[a] Three letter and single letter code represents the amino acid substituted in position 9 of the helical peptide (AX9) or position 1 of the non-helical peptide (X1).

[b] For run conditions, see Experimental.

[c] The gradient steepness parameter, b, and the median capacity factor, $\bar{k}$, is calculated as described by Synder and Stadalius [20], (see Experimental). For a gradient rate of 2% acetonitrile/min, $t_G = 25$ min; for 1% acetonitrile/min, $t_G = 50$ min.

(non-helical peptides) and Figure IV-7 (α-helical peptides). This further suggests that within each series of peptides there would only be small changes in selectivity with changes in gradient rate for any mixtures of these peptides; thus, in a similar manner to that

discussed above for Figures IV-6 and IV-7, improvements in peptide resolution may be made due to its dependence on Q. In contrast, and again in a similar manner to that discussed above, there would be a much larger change in resolution of peptides between the two peptide groups with varying gradient rate due to the large inter-series selectivity differences.

CONCLUSIONS

In this report we have illustrated the use of two series of synthetic model peptides (non-helical or amphipathic α-helical) in order to demonstrate the selectivity that may be obtained in a reversed-phase separation based on conformational differences between the peptides. Peptides within a series, i.e., non-helical or amphipathic α-helical, exhibit very similar plots of log $\bar{k}$ *versus* $\bar{\phi}$ (and, therefore, similar S values), with only small consequent changes in selectivity with changing gradient rate; whereas, peptide mixtures containing peptides from both series of peptides and, hence, containing peptides with large differences in S values, show a correspondingly greater change in separation selectivity with a gradient rate variation. These results are directly applicable to optimizing the separation of mixtures of peptides obtained from such common sources as chemical or proteolytic digests of proteins. Since α-helical structure generally, and amphipathic α-helices (54,55) represent very common structural motifs in proteins, such digests would be expected to contain mixtures of both helical and non-helical peptides. We will be continuing our investigations of the effect of α-helical structure, as well as extending work to assessing the potential effect of β-structure and nearest neighbor effects on peptide retention behaviour.

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CHAPTER V

Future projects

INTRODUCTION

This chapter contains preliminary results on projects that originate directly from work in one of the preceeding chapters or is a new project. The first topic of this chapter will focus on how changes in the amphipathicity of an amphipathic α-helix affects chromatographic behaviour as measured by the chromatographic parameters S and log k_0. The second topic deals with the 2 parameters that affect a charged residue, i.e., the effect of sodium perchlorate on the apparent hydrophilicity of a charged residue and the effect of peptide conformation on a charged residue's ability to express its hydrophilicity. The third topic that will be discussed is the effect of an amino acid substitution between the hydrophilic and hydrophobic face of an amphipathic α-helical peptide on peptide retention behaviour. The fourth topic deals with the effect of position of substitution of an amino acid in the hydrophobic face of the α-helix, which is an extension of the research in chapter III.

RESULTS

Effect of amphipathicity on S and log k_0 values of α-helical peptides: Ala-face *vs* Leu-face

In chapter IV, using non-helical and amphipathic α-helical peptides, it was shown that conformation can have an effect on the S value that is obtained from plots of log $\bar{k}$ *vs* $\bar{\phi}$. As well, it was shown that peptide analogs, that do not vary in conformation, show only small variations in terms of their S value. The following experiments were done to determine the parameters that may affect S values and log k_0 within a set of peptides of the same conformation. The conformation chosen was the amphipathic α-helix and the

peptides used were the AX9 and LX9 series of peptides described in chapter III, see Figure III-1. The basic difference between these 2 series of peptides is that the LX9 series has a preferred binding domain that is considerably more hydrophobic than that of the AX9 series. The end result being that the amphipathicity, which may be estimated by the hydrophobic moment [1], of the Leu series will be greater than the Ala series. As previously indicated in chapter III, the hydrophobic moment of LL9 is 0.73 as opposed to 0.59 for AA9. Four substitutions were made in each face to test the effect of amphipathicity on S values and Table V-1 reports the retention time data as well as the S values obtained from the $\log \bar{k}$ *vs* $\bar{\phi}$ plots. From Table V-1, it can be seen that the S value for each series of peptides are represented by a small range, e.g., for AX9 the range is 23.4 to 27.2 and for the LX9 series the range is 13.4 to 17.7; whereas, there is a significant difference in S values between the 2 sets of peptides (i.e., for a large increase in amphipathicity, the Ala-face to the Leu-face, there is a large decrease in S value). Interestingly, with each series, the relative order of decreasing S values is correlated with increasing hydrophobicity of the side-chain (compare Tyr to Phe and Ala to Leu). An additional peptide naA, a non-amphipathic peptide of the same composition as AA9 with hydrophobic moment of 0.04, was analysed in order to determine the effect of a non-amphipathic peptide, of the same amino acid composition as the amphipathic peptide AA9, on the S value. The S value of naA is even greater than that of AA9 (Table V-1). Since the S value of the non-amphipathic peptide (naA) is larger than the S value of the AX9 series of peptides and the S value of the AX9 series of peptides is larger than the S value of the LX9 series of peptides (i.e., naA > AX9 > LX9), the data suggests that increasing amphipathicity can result in a decreasing S value for these amphipathic α-helical peptides.

An additional term is reported in Table V-1, $\log k_0$, which is the y intercept obtained from the linear regression analysis of the plot as indicated by equation (4) in chapter IV and this value represents the capacity factor of the peptide that would be

Table V-1 Effect of amphipathicity on the S and log k_0 values of Ala-face and Leu-face α-helical peptides.

	t_g(min) [b]					
t_G (min) [a]	100	50	25	12.5	S [c]	log k_0 [c]
rate	0.5%	1%	2%	4%		
Helical Peptides [d]						
naA	30.10	17.79	11.19	7.70	33.2	4.34
AA9	39.65	22.72	13.77	9.04	27.2	4.93
AY9	40.25	23.04	14.02	9.18	24.1	5.44
AL9	48.15	27.06	16.06	10.20	24.9	4.62
AF9	47.25	26.60	15.90	10.12	23.4	5.20
	t_g(min) [b]					
t_G (min) [e]	160	80	40	20	S [c]	log k_0 [c]
rate	0.5%	1%	2%	4%		
LY9	80.37	43.91	24.76	14.60	17.7	7.06
LA9	87.47	47.74	26.73	15.77	14.4	6.35
LL9	91.70	50.15	27.97	16.39	13.8	6.44
LF9	90.07	49.24	27.63	16.21	14.8	6.17

[a] t_G represents the time of the linear AB gradient, in min, for a change from 0% to 100% B and the rate represents the equivalent % acetonitrile/min for the corresponding gradient time. Eluent A is 20 mM phosphoric acid and eluent B is 20 mM phosphoric acid in 50% aqueous acetonitrile.

[b] t_g represents the retention time of each peptide under linear AB gradient conditions.

[c] S and log k_0 is determined by linear regression of the data from the log $\bar{k}$ *vs* $\bar{\phi}$ as described by Synder et al. (see Experimental section in Chapter IV).

[d] Designation for the synthetic amphipathic α-helical peptides AX9 and LX9 where A or L represents the amino acid Ala or Leu that makes up the hydrophobic face of the peptide and X represents the amino acid substitution in postion 9 of the peptide, i.e., Ala (A), Leu (L), Tyr (Y) or Phe (F). The designation naA represents a peptide that is of the same composition as AA9 but a different sequence, i.e., a non-amphipathic (na) helical structure as opposed to an amphipathic structure as in the AX9 series (see Figure IV-1).

[e] same as in footnote (a) except where eluent B is 20 mM phosphoric acid in 80% aqueous acetonitrile.

obtained where the solvent contains no organic modifier [2]. It has been suggested [3] that the log k_0 value obtained from the log $\bar{k}$ *vs* $\bar{\phi}$ plot is indicative of the solute's affinity for the stationary phase in the absence of organic modifier. From Table V-1, it can be seen that the average log k_0 for the LX9 series is 6.51; whereas, for the AX9 series it is 5.05. This suggests that the affinity of the LX9 series of peptides is greater than that of the AX9 series and this is consistent with the retention times obtained for the elution of these peptides, e.g., at a gradient of 1% acetonitrile/min LL9 elutes at 50.15 min and AA9 elutes at 22.72 min. As reported in Table V-1, the log k_0 value for the non-amphipathic peptide is 4.34 which is lower than that of AA9 and consistent with previous studies [4], this peptide is less retentive (at a gradient rate of 1% acetonitrile/min the t_R for naA is 17.79 min as opposed to 22.72 min for AA9) than the AA9 peptide. From the data, it can be seen that if the peptides are aligned in order of increasing amphipathicity, the log k_0 values are also of increasing order, i.e., naA < AX9 < LX9.

It has been previously suggested [5] that peptides/proteins may be characterized, using *S* and log k_0 values, into 4 different families: (1) large *S*, small k_0, (2) small *S*, small k_0, (3) large *S*, large k_0 and (4) small *S*, high k_0. As indicated in Table V-1, as the amphipathicity of the peptide increased (naA < AX9 < LX9), the *S* value decreased significantly; whereas, with this increase in amphipathicity one observes a large increase in log k_0. It has been suggested that this apparent inverse relationship between *S* and log k_0 values, i.e., low *S* but high k_0 is indicative of self-aggregating membrane proteins [5] or hydrophobic peptide homologs [6]. One possible explanation for this inverse relationship between *S* and log k_0, for the AX9 and LX9 of peptides, is based on the suggestion that the *S* value represents the magnitude of the surface contact area of the solute that is involved in binding to the ligands of the stationary phase [3]. The LX9 series of peptides is highly retentive, due to a very hydrophobic preferred binding domain, i.e., log k_0 is large; therefore, the residues on the hydrophilic side of the helix interact with the stationary phase to a lesser extent, resulting in an apparent reduction in contact area as opposed to the AX9

series in which the hydrophilic residues would be able to interact with the stationary phase to a greater extent due to the less hydrophobic preferred binding domain (thus a larger contact area).

From this, one may state the following hypothesis: for amphipathic α-helices, as the amphipathicity of the peptide increases, its *S* value (contact area) decreases and its log k_0 (affinity for the stationary phase) value increases. This hypothesis could be tested by synthesizing peptides in which the Ala residues in the hydrophobic face of the amphipathic α-helix AA9 are successively replaced by Leu residues, e.g., AL9; AL5,9; AL5,6,9; AL5,6,9,13 and AL5,6,9,12,13.

Effect of sodium perchlorate on the hydrophilicity of residues with charged side-chains.

In this section, data will be presented suggesting that sodium perchlorate, a reagent commonly used in RPC to suppress the interaction of basic solutes with silanols at neutral pH values [7], may be used at low pH (pH 2) resulting in gains in selectivity. It is well known that, at pH 2, the retention time of a peptide containing basic residues may be increased by the addition of hydrophobic ion-pairing reagents such as TFA or HFBA (see Figure I-3) [8]. As well, it is known that if the concentration of these anionic pairing reagents are increased from the normal range of 0.05 to 0.1% to a higher concentration, e.g., 0.8%, the retention time of the peptide will increase, suggesting that the increase in retention time is due to an increase in hydrophobicity of the positively charged groups through a higher concentration of ion-pairs with the hydrophobic ion-pairing reagent (see Figure I-4) [8]. It is also possible that increasing the concentration of even a hydrophilic anionic pairing reagent may increase the retention time not because of the hydrophobicity of the ion-pairing reagent, but by neutralization of the positive charge during ion-pair formation which in turn reduces the hydrophilicity of the charged side-chain.

The peptides used to study this effect were the amphipathic α-helical peptides from the AX9 series, see Figure III-1. Table V-2a reports data showing the effect of 100 mM

perchlorate ion on the retention time of 3 peptides containing charged residues in the hydrophobic face of the peptide. In the absence of the perchlorate ion, all 3 peptides exhibit a retention time that is shorter than the peptides in the presence of perchlorate ion. Since these peptides have 5 positively charged residues in the hydrophilic face, see Figure III-1,

Table V-2a Effect of sodium perchlorate on the hydrophilicity of charged residues in the hydrophobic face of the amphipathic α-helical peptides in the AX9 series.

Peptide [a]	absence of CLO_4 [b]		presence of CLO_4 [b]		
		Δt_R to AQ9 [c]		Δt_R to AQ9 [c]	$\Delta\Delta t_R$ [d]
AQ9	17.71	——	25.16	——	——
AK9	14.69	-3.02	24.09	-1.07	1.95
AR9	15.50	-2.21	25.63	0.47	2.68
AH9	14.26	-3.45	24.52	-0.64	2.81

[a] Peptide designations as in Figure III-1: Ala series AX9, where the first letter represents the residues that comprise the hydrophobic face and X represents the amino acid substituted at position 9.

[b] Represents either the absence (minus CLO_4) or presence (plus CLO_4) of 100 mM sodium perchlorate in eluent A (20 mM H_3PO_4) and eluent B (20 mM H_3PO_4 in 50% acetonitrile). Separations performed on a C_8 RP column at a gradient rate of 1% acetonitrile/min and a flow rate of 1 ml/min.

[c] Δt_R represents the difference in retention time between a peptide with a Gln at position 9, AQ9, and a peptide with a charged substitution, i.e., Lys (K), Arg (R) or His (H).

[d] $\Delta\Delta t_R$ represents the change in hydrophobicity of a charged residue in the presence of perchlorate ion. For example, for the Lys substitution, in the presence of perchlorate this residue appears 1.95 min more hydrophobic than in the absence, i.e., -1.07 - (-3.02) = 1.95 min.

the Lys (AK9), Arg (AR9) and His (AH9) substituted peptides were referenced to a Gln (AQ9) substituted peptide so that the effect of the perchlorate ion on the charged residue in the hydrophobic face could be observed (the difference between the 2 peptides being reported as Δt_R to AQ9). From Table V-2a, it can be seen that in the *absence* of perchlorate ion, the Lys substituted peptide is less retentive than a Gln substituted peptide by 3.02 min (Δt_R is -3.02 min) and in the *presence* of the perchlorate ion, the Lys substituted peptide is less retentive, Δt_R = -1.07 min, than the Gln substituted peptide. This suggests that, upon

ion-pairing of the perchlorate ion with the charged Lys residue, the retention time of the Lys side-chain was increased by 1.95 min, i.e., $\Delta\Delta t_R$ = -1.07 - (-3.02) = 1.95 min; thus the Lys side-chain becomes less hydrophilic. Similarily, the side-chains of the Arg and His in the hydrophobic face of these peptides also become less hydrophilic by 2.68 min and 2.81 min upon ion-pairing with the perchlorate ion.

In addition, we wanted to test the effect of the perchlorate ion on a charged residue in the hydrophilic face of an amphipathic peptide. The peptides that were designed and synthesized are shown in Figure V-1, where changes to the peptide AA9 are made in the hydrophilic face. A series of 5 peptides was made where each Lys (K) residue in the hydrophilic face (at positions 4, 7, 11, 14 and 18) of the AA9 peptide is successively changed to a Gln; therefore, resulting in a series of peptides that vary in charge only by +1

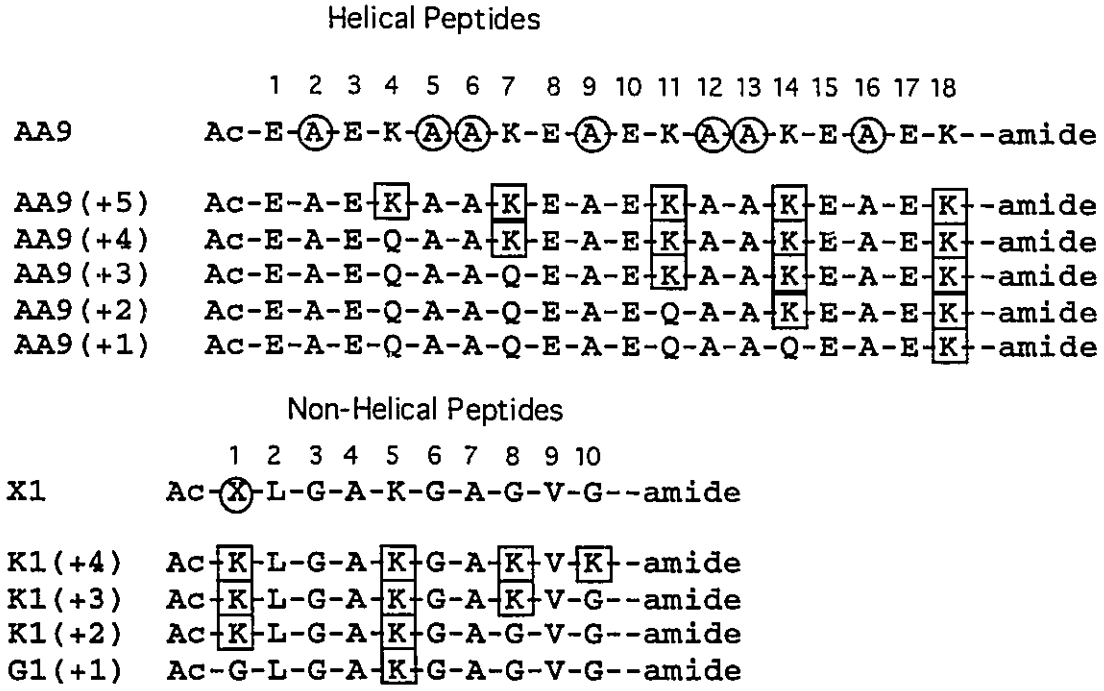

Figure V-1 Design of peptides used to determine the effect of charged side-chains in the hydrophilic face of amphipathic α-helical and non-helical peptides. The net charge on the helical peptides is obtained by successively replacing a Lys residue in the hydrophilic face of the AA9 peptide with a Gln, resulting in a charge difference of +1 between each successive analogue. Similarily, a Gly is substituted in the X1 peptides by a Lys resulting in a charge difference of +1 between each successive analogue.

between successive analogs. The peptides are therefore designated AA9 (+x) where the value in the brackets represents the net charge in the hydrophilic face, e.g., AA9 (+2) where AA9 represents the original starting peptide AA9 and (+2) indicates that 3 of the 5 Lys residues in the hydrophilic face have been replaced by Gln residues, thus a net charge of +2. The retention time data in the absence and presence of perchlorate is presented in Table V-2b. All of the peptides, AA9 (+5) to AA9 (+2), have longer retention times in the presence of the perchlorate ion than in the absence of the perchlorate ion. The retention time difference, reported as Δt_R in Table V-2b, for the peptide in the absence and presence of perchlorate indicates that in each case the peptide will increase in retention time due to the addition the perchlorate ion. The average increase in retention time per charged residue, calculated by dividing Δt_R by the number of Lys residues in the hydrophilic face, due to the presence of the perchlorate ion is 1.29 min. The data therefore suggests that upon ion-pairing of the charged side-chain with the perchlorate ion, the apparent hydrophilicity of the side-chain is reduced.

We next wanted to determine if the effect observed in both the hydrophobic face and hydrophilic face of an amphipathic helix would also occur for positively charged residues in a non-helical peptide. The peptides used for this study are shown in Figure V-1 and are designated X1 (+x) where the (+x) represents the net charge on the peptide at pH 2. To design this series of peptide, the starting peptide was G1 (+1), which has one Lys at position 5 and a Gly substitution at position 1. Peptides of successive increasing charge were designed by replacing, in succession, the Gly residue for a Lys residue at position 1, 8 and 10, see Figure V-1. The retention times of these non-helical peptides are reported in Table V-2b, where it is observed that in the presence of perchlorate, the retention time of the peptide is greater than in the absence of the perchlorate ion (as was observed for a charged residue in the hydrophobic face or hydrophilic face of the amphipathic helix). The difference in retention time for a peptide in the presence and absence of perchlorate is reported as Δt_R and from Table V-2b, where it can be seen that in the presence of

perchlorate, a charged Lys residue appears more hydrophobic (i.e., less hydrophilic), e.g., G1 (+1) the Δt_R = 2.65 min indicating that in the presence of perchlorate, the Lys side-chain is 2.65 min more hydrophobic than in the absence of perchlorate. This same effect can be seen for the other peptides K1 (+4), K1 (+3) and K1 (+2). Also reported in Table V-2b is the effect of the perchlorate ion per charged residue and from the data presented, it can be seen that, on average, the presence of the perchlorate ion will increase the hydrophobicity of the Lys side-chain by 2.64 min in a non-helical peptide.

Table V-2b Effect of sodium perchlorate on the hydrophilicity of charged residues in the hydrophilic face of amphipathic α-helical and non-helical peptides.

Peptide [a]	absence [b]	presence [b]		
Helical	t_R	t_R	Δt_R	Δt_R/charge
AA9 (+5)	22.75	30.09	7.37	1.47
AA9 (+4)	24.29	29.61	5.23	1.33
AA9 (+3)	25.32	28.96	3.64	1.21
AA9 (+2)	26.09	28.37	2.28	1.14
				1.29 = Avg
non-helical	t_R	t_R	Δt_R	Δt_R/charge
K1 (+4)	8.09	18.56	10.47	2.61
K1 (+3)	10.35	18.43	8.08	2.69
K1 (+2)	12.76	17.96	5.20	2.60
G1 (+1)	14.71	17.36	2.65	2.65
				2.64 = Avg

[a] Design of α-helical and non-helical peptides. The net charge on the hydrophilic face of the helical peptides are obtained by successively replacing a Lys residue in the hydrophilic face with a Gln, resulting in a charge difference of +1 between each successive analogue. Similarily, a Gly is substituted by a Lys resulting in a charge difference of +1 between each successive analogue.

[b] Represents either the absence (minus CLO_4) or presence (plus CLO_4) of 100 mM sodium perchlorate in eluent A (20 mM H_3PO_4) and eluent B (20 mM H_3PO_4 in 50% acetonitrile). Conditions: Linear AB gradient at a rate of 1% acetonitrile/min and a flow rate of 1 ml/min. Column: C_8, 300Å pore size and 7 μm particle size; 220 x 4.6 mm.

[c] Δt_R represents the difference in retention time between the peptide in the presence (plus CLO_4) and absence (minus CLO_4) of perchlorate, i.e., for AA9 (+5) Δt_R = 30.09 - 22.75.

[d] Δt_R/charged represents the effect of the perchlorate ion per charged residue in the hydrophilic face of the peptide, i.e., the difference in retention time divided by the number of Lys residues in the hydrophilic face.

From the previous 3 sets of experiments, a charged residue in the hydrophobic face or the hydrophilic face of an amphipathic helix or a non-helical peptide, it is observed that there is an increase in side-chain hydrophobicity (i.e., a reduction in side-chain hydrophilicity) of a charged residue in the presence of perchlorate ion. A general hypothesis that may be stated from these observations is: a charged residue can have an apparent increased hydrophobicity by reduction of the hydrophilicity of the side-chain due to ion-pair formation with a hydrophilic ion-pairing reagent.

Comparison of the effect of the perchlorate ion between the hydrophobic and hydrophilic face

In the previous section, it was shown that the presence of the perchlorate ion resulted in an apparent increase in hydrophobicity of charged side-chains. We now wanted to determine if the magnitude of the perchlorate effect observed would be the same in the hydrophobic and hydrophilic face of the amphipathic α-helical peptides. The data presented in Table V-2c comes from Table V-2a and V-2b and has been reanalysed to determine this effect. From this table, in the *absence* of perchlorate, a Gln to Lys substitution in the hydrophobic face of the peptide, results in a reduction in retention time of 3.02 min; whereas, in the *presence* of perchlorate the same substitution results in a reduction of retention time of only 1.07 min indicating that the perchlorate resulted in a reduction in the hydrophilicty of 1.95 min ($\Delta\Delta t_R$ = 1.95, see discussion of Table V-2a). In a similar manner, a Gln to Lys substitution in the hydrophilic face, AA9 (+4) to AA9 (+5), in the *absence* of perchlorate results in a reduction of retention time of 1.54 min; whereas, in the *presence* of perchlorate there is an increase in retention time of 0.48 min. This indicates that in the hydrophilic face of the peptide, ion-pairing of the perchlorate with a Lys side-chain resulted in a 2.02 min increase in retention time. Both of these numbers taken together suggest that when perchlorate ion-pairs with the positively charged Lys side-chain, the reduction in hydrophilicity that occurs will be of the same magnitude independent of the face of the peptide.

Table V-2c Effect of sodium perchlorate on the hydrophilicity of charged residues in the hydrophobic face and hydrophilic face of amphipathic α-helical peptides.

	absence of CLO_4 [b]		presence of CLO_4 [b]		
hydrophobic face of peptide [a]	t_R	Δt_R for Q→K [c]	t_R	Δt_R for Q→K [c]	$\Delta\Delta t_R$ [d]
AQ9	17.71	——	25.16	——	——
AK9	14.69	-3.02	24.09	-1.07	*1.95*
hydrophilic face of peptide [a]					
AA9 (+4)	24.29	-----	29.61	-----	------
AA9 (+5)	22.75	-1.54	30.09	0.48	*2.02*
		$\Delta\Delta t_R$[e] = *1.48*		$\Delta\Delta t_R$[e] = *1.55*	

[a] Peptide designations as in Figure III-1 for the hydrophobic face: Ala-face series AX9, where the (A) represents the Ala residues that comprise the hydrophobic face and X represents the amino acid substituted at position 9 or Figure V-1 for the hydrophilic face: designation AA9 (+x) represents the Ala-face peptide in which Gln to Lys substitutions are made in the hydrophilic face such that the net charge is indicated by (+x).

[b] Represents either the absence (- CLO_4) or presence (+ CLO_4) of 100 mM sodium perchlorate in eluent A (20 mM H_3PO_4) and eluent B (20 mM H_3PO_4 in 50% acetonitrile). Separations performed on a C_8 RP column at a gradient rate of 1% acetonitrile/min and a flow rate of 1 ml/min.

[c] Δt_R represents the retention time difference for a Gln to Lys substitution in either the hydrophobic or hydrophilic face, e.g., for this substitution in the hydrophobic face, Δt_R = 14.69 - 17.71 = -3.02.

[d] $\Delta\Delta t_R$ represents the effect of the perchlorate ion on the hydrophobicity of a charged residue. For example, for the Lys substitution, in the presence of perchlorate this residue appears 1.95 min more hydrophobic than in the absence, i.e., $\Delta\Delta t_R$ = -1.07 - (-3.02) = 1.95 min.

[e] $\Delta\Delta t_R$ represents the difference of the Gln to Lys substitution in the hydrophobic face *vs* the hydrophilic face. For example, -3.02 - (-1.54) = 1.48 min.

Fom the previous discussion, a general hypothesis may be stated from these observations: when a charged residue is ion-paired, the apparent increased hydrophobicity

by reduction of the hydrophilicity of the side-chain due to ion-pair formation will be independent of whether the ion-pair is in the hydrophobic or hydrophilic face of the peptide.

Effect of sodium perchlorate on all 20 amino acid substitutions

Table V-2d reports the effect of 100 mM perchlorate on the retention behaviour of all 20 analogs of the AX9 series of peptides studied in Chapter III. The data further suggests that the presence of the perchlorate ion in a separation will result in an increase in hydrophobicity of a charged side-chain due to the ion-pairing effect. For example, considering the helical peptides with non-charged substitutions, the average increase in retention time due to the perchlorate (i.e., the average of the Δt_R values obtained from the retention times of the peptides in the absence and presence of perchlorate) is 8.05 min. If this value is divided by 5, the number of Lys residues in the hydrophilic face of the peptide, the increase in retention time is 1.61 min which is in reasonable agreement with the value of 1.29 min as obtained in TableV-2b. Similarily, for the non-helical peptides an average of 2.88 min is obtained for Δt_R which is also in reasonable agreement with the value of 2.64 min obtained in Table V-2b.

Use of sodium perchlorate to effect selectivity in RPC. The previous discussion has shown that the presence of the hydrophilic perchlorate ion may result in the reduction of hydrophilicity due to the ion-pairing effect on charged residues in peptides separated by RPC. It was shown that this effect can occur for charged residues in either the hydrophobic or hydrophilic face of an amphipathic helix or in non-helical peptides. Figures V-2a and V-2b show examples of HPLC profiles of these helical and non-helical peptides where selectivity may be obtained through the use of sodium perchlorate. In panels A, C and E, a mixture of 4 peptides is separated at pH 2 using eluents that do not contain perchlorate; whereas, panels B, D and F show the same mixture of peptides and eluents with the exception that both eluents contain 100 mM perchlorate. Panel A shows that the α-helical

Table V-2d Sodium perchlorate effect on all 20 amino acid substitutions in the amphipathic helix as compared to non-helical peptides.

	helical peptides (AX9)			non-helical peptides (X1)		
Amino acid [a] substitution	t_R [b]		Δt_R [c]	t_R [b]		Δt_R [c]
	absence	presence		absence	presence	
Ile (I)	27.13	35.73	8.60	21.44	24.82	3.38
Leu (L)	27.20	35.55	8.35	22.19	25.42	3.23
Phe (F)	26.78	35.23	8.45	23.66	26.56	2.90
Trp (W)	26.23	34.60	8.37	24.43	27.35	2.92
Val (V)	25.40	34.04	8.64	18.91	22.10	3.19
Met (M)	24.96	33.32	8.36	19.83	22.78	2.95
Cys (C)	23.03	31.20	8.48	16.90	20.17	3.27
Tyr (Y)	23.20	31.20	8.00	19.41	22.44	3.03
Ala (A)	22.81	30.31	7.50	15.69	18.52	2.83
Thr (T)	20.00	28.17	8.17	15.53	17.92	2.39
Glu (E)	19.77	27.27	7.50	15.62	18.18	2.56
Gly (G)	18.77	26.38	7.61	14.73	17.55	2.82
Ser (S)	18.36	26.16	7.80	14.66	17.10	2.44
Arg (R)	15.50	25.63	*10.13*	13.03	19.23	*6.20*
Asp (D)	17.67	25.42	7.75	14.92	17.62	2.70
Gln (Q)	17.71	25.16	7.45	14.81	17.31	2.50
His (H)	14.26	24.52	*10.26*	12.66	18.43	*5.70*
Lys (K)	14.69	24.09	*9.40*	12.82	18.17	*5.35*
Asn (N)	15.66	23.42	7.76	14.49	17.13	2.64
Pro (P)	15.10	22.98	7.88	18.18	21.32	3.14
			Avg = 1.61			Avg = 2.88

[a] Peptide designations for the helical peptides as in Figure III-1: Ala series AX9, where the first letter represents the residues that comprise the hydrophobic face of the peptide and X represents the amino acid substituted at position 9. Peptide designations for the non-helical peptides as in chapter II: where X represents the amino acid substituted at the N-terminal position in a 10 residue peptide.

[b] Represents the retention time of the peptide in either the absence (minus) or presence (plus) of 100 mM sodium perchlorate in eluent A (20 mM H_3PO_4) and eluent B (20 mM H_3PO_4 in 50% acetonitrile). Conditions: Linear AB gradient at a rate of 1% acetonitrile/min and a flowrate of 1 ml/min. Column: C_8, 300Å pore size and 7 µm particle size; 220 x 4.6 mm).

[c] Δt_R represents the effect of the perchlorate ion, i.e., the difference in retention time for the peptide in the absence and presence of perchlorate.

and non-helical peptide pair A1 and AN9 coelute and W1 and AM9 are poorly resolved; whereas, with the addition of 100 mM perchlorate, all 4 peptides are baseline resolved. A similar situation is seen in the 2 panels C and D where the peptides D1 and AP9 coelute;

whereas, when 100 mM perchlorate is added, all 4 peptides are again baseline separated.

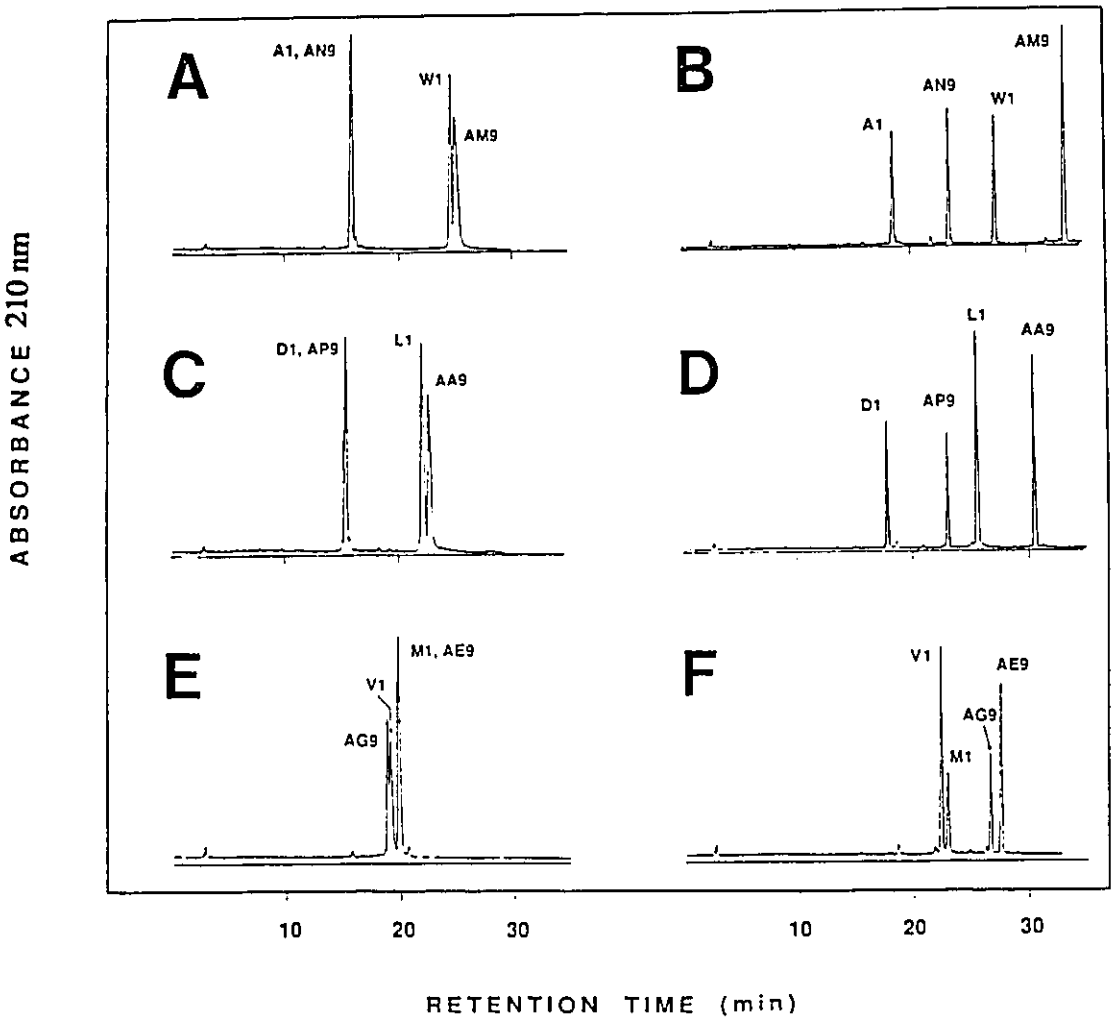

Figure V-2a Selectivity changes between non-helical and amphipathic α-helical peptides upon addition of perchlorate. Amphipathic α-helical peptides are designated as AX9, where A represents Ala residues in the hydrophobic face and X represents the amino acid substituted at position 9. The non-helical peptides are designated as X1 where X represents the amino acid substituted at the N-terminal position. Conditions: Linear AB gradient elution at a rate of 1% acetonitrile/min and a flowrate of 1ml/min. Panels A, C and E: Eluent A is 20 mM H_3PO_4 and eluent B is 20 mM H_3PO_4 in 50% aqueous acetonitrile; Panels B, D and F: same as A, C and E except that eluents contain 100 mM perchlorate. Column: C_8, 300Å pore size and 7 μm particle size, 220 x 4.6 mm.

Panels E and F show a mixture of 4 peptides V1, M1, AG9 and AE9 which is poorly separated at pH 2 in the absence of perchlorate, but is baseline resolved with the addition of perchlorate. An important observation can be made from panel F of Figure V-b, where the amphipathic α-helical peptides AG9 and AE9 and the non-helical peptides V1 and M1 have

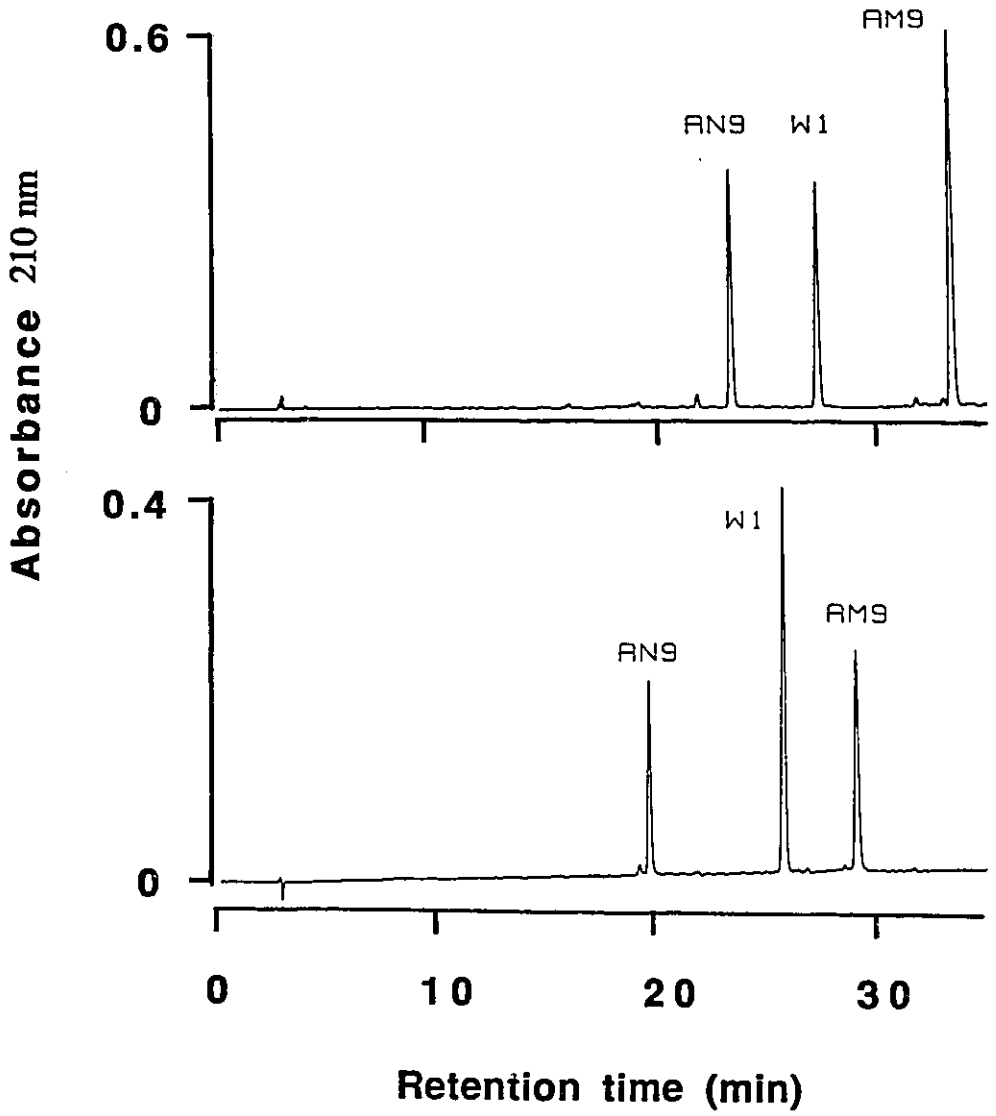

Figure V-2b Selectivity changes for non-helical and amphipathic α-helical peptides between phosphate/100mM perchlorate and TFA. Amphipathic α-helical peptides are designated as AX9 and the non-helical peptides are designated as X1. Conditions: Upper panel, same as in Figure V-2a in which the eluents contain perchlorate. Lower panel, Linear AB gradient elution, 1% acetonitrile/min, where A is 0.1% aqueous TFA and B is 0.1% TFA in acetonitrile, at a flowrate of 1ml/min.

been separated in the presence of perchlorate. It can be seen that the retention time difference between AG9 and AE9 and the retention time difference between V1 and M1 has not improved upon the addition of perchlorate; although a large separation is achieved between the amphipathic α-helical and the non-helical peptides, suggesting that peptides that vary in charge, as in this case, may result in a large gain in selectivity by the addition of

sodium perchlorate to the elution buffers. Since there was a difference in the effect of perchlorate on the reduction of the hydrophilicity of a charged residue in amphipathic helices, hydrophilic face (Table V-2b = 1.29 min; Table V-2d = 1.61 min), and non-helical peptides (2.88 min), the results suggest that selectivity may be achieved due to conformation as well as the number of charged residues in a peptide.

Figure V-2b shows HPLC profiles indicating the selectivity difference that may be obtained by using the perchlorate ion in a phosphate buffer as opposed to using the hydrophobic ion-pairing reagent TFA. Panel A of Figure V-2b shows that in phosphate/perchlorate the difference in retention time (Δt_R) between AN9 and W1 is 3.95 min and between W1 and AM9 it is 6.02 min; whereas, in TFA the Δt_R are 6.13 and 3.29 respectively, indicating that a selectivity change may be obtained for a separation using 100 mM perchlorate in a phosphate buffer as opposed to that obtained using aqueous TFA.

Effect of amino acid substitution between the hydrophobic and hydrophilic face of amphipathic α-helical peptides

The discussion in this section deals with the effect of an amino acid substitution in the hydrophobic face as opposed to the same substitution in the hydrophilic face of an amphipathic α-helix. It has been previously shown that all amino acids contribute to the retention behaviour of an amphipathic helix [4], suggesting that residues on both the hydrophobic and hydrophilic surface are interacting with the stationary phase. It will be shown in this section that the effect of an amino acid substitution, on the retention behaviour of a peptide, in the hydrophobic face can be different from the effect of the same substitution in the hydrophilic face of these peptides.

Data presented in Table V-2c, in the discussion in the previous section, indicates that a Lys residue in the hydrophobic face will have a different effect on the retention behaviour of the peptide than a Lys in the hydrophilic face. From Table V-2c it is observed that a Gln to Lys substitution in the hydrophobic face results in a reduction in retention time of the peptide by 3.02 min (Δt_R = -3.02 min in the absence of perchlorate); whereas, a Gln

to Lys substitution in the hydrophilic face results in a reduction in retention time for the peptide of only 1.54 min (Δt_R = -1.54 min in the absence of perchlorate). This suggests that when the charged residue is in the hydrophilic face of the peptide, it will have a lesser effect on peptide retention behaviour than a charged residue in the hydrophobic face by 1.48 min ($\Delta\Delta t_R$ = -1.48 min in the absence of perchlorate). The effect of the substitution between the hydrophobic and hydrophilic face is independent of the presence or absence of perchlorate (i.e., $\Delta\Delta t_R$ = 1.55 min in the presence of perchlorate). The data further suggests that even though a preferred binding domain exists in this peptide, i.e., the Ala residues that comprise the hydrophobic face, the residues in the hydrophilic face are still able to interact with the stationary phase (e.g., the Lys substitution in the hydrophilic face results in a reduced retention time of 1.54 min); but, the residues in the hydrophilic face may not be interacting with the stationary phase to the same extent as the residues in the hydrophobic face resulting in the observed retention behaviour.

Figure V-3 shows the two series of peptides that were used to determine the effect of an amino acid being placed in the hydrophobic or hydrophilic face of an amphipathic α-helix. From the helical net representations given in Figure V-3, it can be seen that the substitution at position 9 in the AX9 series of peptides is in the center of the residues that comprise the hydrophobic face; whereas, in the AX7 series the substitution at position 7 will be surrounded by the hydrophilic residues, Glu and Lys.

Figure V-4a shows the elution profiles of all 20 amino acid substitutions in the hydrophobic face of the AX9 series of peptides and Figure V-4b shows the elution profiles of 15 amino acid substitutions in the hydrophilic face of the AX7 series of peptides separated at pH 2. From Figure V-4a, it can be seen that the retention times of these substituted peptides are generally in agreement with the hydrophobicity scale of Guo et al. [9]; for example, the hydrophobic substitutions such as Trp, Phe, Leu and Ile result in a retention time that is greater than hydrophilic substitutions such as Arg, His and Lys. As well, the retention time range for the most hydrophobic to the most hydrophilic amino acid

obtained by Guo et al. [9] is approximately 10.9 min and from Figure V-4a, this same range of amino acid substitutions result in a retention time range of approximately 12 min.

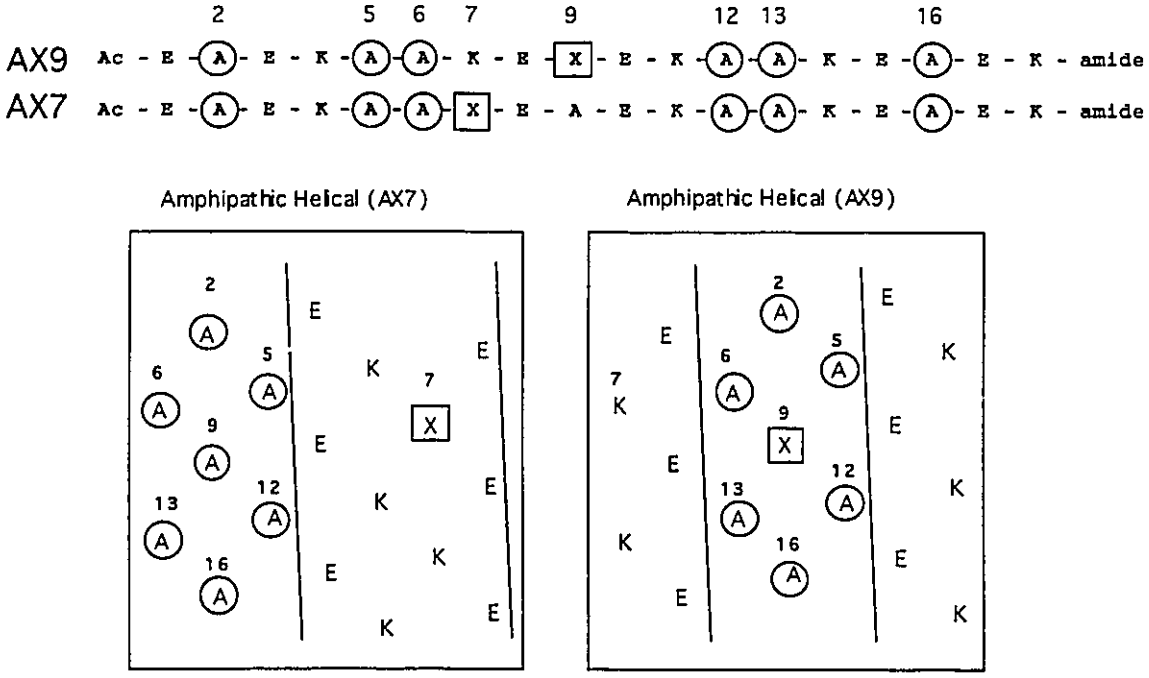

Figure V-3 Design of model synthetic peptides. Top: Sequence of synthetic peptides, AX9 and AX7, where the first letter represents amino acid residues used in the hydrophobic face of the peptide, the X represents each of the 20 amino acids (boxed) (single letter code given in Table V-2c) substituted at position 9 or 7. The residues that are labeled 2, 5, 6, 9, 12, 13 and 16, the circled residues, are in the hydrophobic face of the amphipathic α-helical peptide. Lysine and glutamic acid residues make up the hydrophilic face of the amphipathic α-helix. Bottom: Ala-face (AX7, left) and (AX9, right) model peptides represented as α-helical nets.

This observation is expected since the substitutions in these peptides are situated in the preferred binding domain of the peptide which allows these residues to interact with the stationary phase to a large extent [4]. In contrast to the hydrophobic face, the profiles of the hydrophilic face in Figure V-4b show that the retention time range of these peptides is much smaller (e.g., with the exception of the Pro substitution, the approximate range of times is 2.3 min) resulting in a separation which has considerably poorer resolution than

in the case of the substitutions in the hydrophobic face. This small range of retention times in comparison to the hydrophobic face suggests that the residues that are substituted in the hydrophilic face are interacting with the stationary phase to a lesser extent than the residues in the hydrophobic face, i.e., the substitutions in the hydrophilic face do not affect retention behaviour to the same extent as evidenced by the very similar retention times of these peptides. This is consistent with the findings as indicted in the previous discussion with the charged residue Lys and is also consistent with the suggestion [4] that some residues in an amphipathic helix may not be contributing to the same extent to the overall hydrophobicity of the peptide.

Table V-3 reports the retention times of the peptides separated in Figures V-4a and V-4b. From Figure V-3, it can be seen that in the AX9 series of peptides, a substitution essentially replaces an Ala residue at position 9 in the hydrophobic face; whereas, in the AX7 series of peptides a substitution essentially replaces an Lys residue at position 7 in the hydrophilic face of the α-helix. In order to determine the effect of a substitution between the 2 faces, the retention times of the substitutions in the hydrophobic face, at position 9, and the hydrophilic face, at position 7, are referenced to the retention time of the Gly substitution, in either the hydrophobic or hydrophilic face. For example, a Met substitution in the hydrophobic face is more retentive than an Gly substitution in the hydrophobic face by 6.39 min (Δt_R = 6.39 min); whereas, a Asn substitution is less retentive than an Gly substitution by 3.53 min (Δt_R = -3.53 min). Similarily, for a substitution in the hydrophilic face, Met is more retentive than a Gly substitution in the hydrophilic face by 2.16 min (Δt_R = 2.16 min) and as well Asn is more retentive than a Gly substitution by 0.55 min (Δt_R = 0.55 min). The effect of a substitution between the 2 faces is then reported as $\Delta\Delta t_R$ in Table V-3. From the values reported in this table, it can be seen that a residue in the hydrophobic face will have a significantly different effect on the retention behaviour than a residue in the hydrophilic face. For example, a Leu in the hydrophilic face will cause the

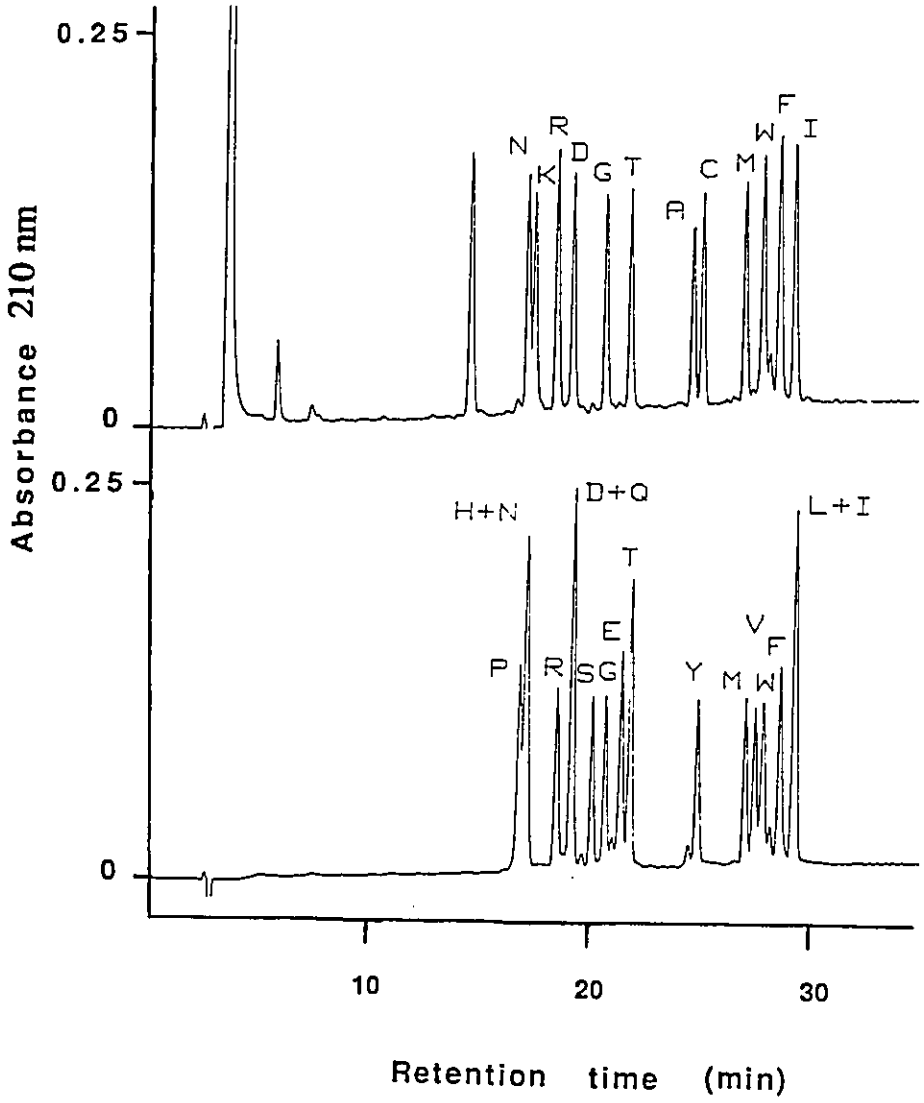

Figure V-4a HPLC profiles of peptides with amino acid substitutions at position 9 in the hydrophobic face of the amphipathic α-helix at pH 2. Conditions: Eluent A is 0.1% aqueous TFA and eluent B is 0.1% TFA in acetonitrile with linear AB gradient elution (1% acetonitrile/min) at a flow-rate of 1 ml/min. Column: same as in Figure V-2a.

peptide to be 6.17 min less retentive than a Leu in the hydrophobic face ($\Delta\Delta t_R$ = -6.17 min); whereas, a Lys in the hydrophilic face will cause the peptide to be 4.07 min more retentive than a Lys in the hydrophobic face ($\Delta\Delta t_R$ = +4.07 min). This futher supports the suggestion that a substitution in the hydrophobic face will have a different effect on retention behaviour of the peptide than a residue in the hydrophilic face.

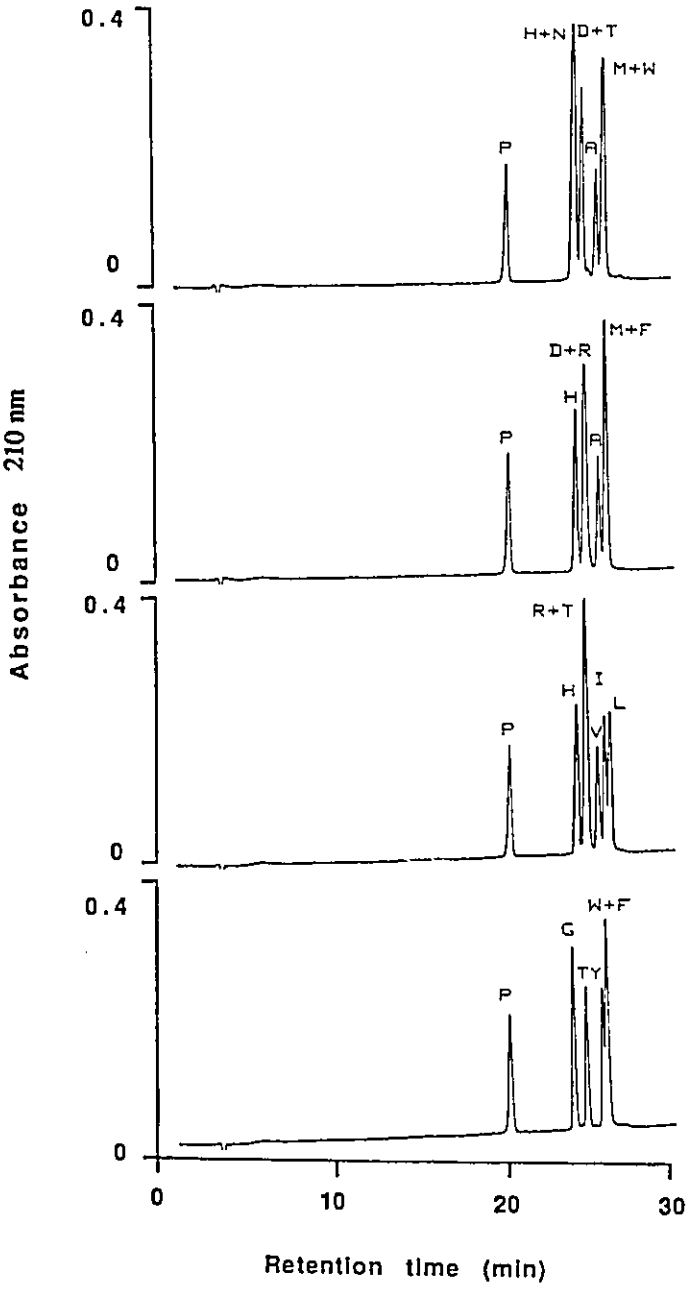

Figure V-4b HPLC profiles of peptides with amino acid substitutions at position 7 in the hydrophilic face of the amphipathic α-helix at pH 2. Conditions and column as in Figure V-4a.

Figure V-5 plots the data presented in Table V-3. From this figure it can be seen that for the amino acid substitutions having the largest effect (Ile) to the smallest effect (Asn), the substitution in the hydrophilic face (filled circles) represents a small change in

Table V-3 Effect of amino acid substitution between the hydrophobic and hydrophilic face of amphipathic α-helical peptides.

	hydrophobic face [a]		hydrophilic face [a]		
Amino acid [b] substitution	t_R [c]	Δt_R to Gly [d]	t_R [c]	Δt_R to Gly [d]	$\Delta\Delta t_R$ [e]
Trp (W)	27.93	7.10	26.00	2.04	-5.06
Phe (F)	28.63	7.80	26.05	2.09	-5.71
Leu (L)	29.32	8.49	26.28	2.32	-6.17
Ile (I)	29.38	8.55	25.65	1.69	-6.86
Met (M)	27.22	6.39	26.12	2.16	-4.23
Val (V)	27.43	6.60	25.53	1.57	-5.03
Tyr (Y)	25.07	4.24	25.79	1.83	-2.41
Cys (C)	25.21	4.38	25.37	1.41	-2.97
Pro (P)	17.03	-3.80	20.33	-3.63	0.17
Ala (A)	24.78	3.95	25.63	1.67	-2.28
Glu (E)	21.55	0.72	25.84	1.88	1.16
Thr (T)	21.95	1.12	24.80	0.84	-0.28
Asp (D)	19.34	-1.49	24.84	0.88	2.37
Gln (Q)	19.33	-1.48	25.61	1.65	3.13
Ser (S)	20.27	-0.56	24.96	1.00	1.56
Gly (G)	20.83	0.00	23.96	0.00	0.00
Arg (R)	18.46	-2.37	24.99	1.03	3.40
Asn (N)	17.30	-3.53	24.51	0.55	4.08
His (H)	17.32	-3.51	24.45	0.49	4.00
Lys (K)	17.49	-3.34	24.69	0.73	4.07

[a] Represents the face, either the hydrophobic or hydrophilic, of the peptide in which the substitution is made.

[b] Amino acid code for the substitution in either the hydrophobic or hydrophilic face of the peptide.

[c] Represents the retention time of the peptide. Conditions: Linear AB gradient at a rate of 1% acetonitrile/min and a flowrate of 1 ml/min where eluent A is 0.1% aqueous TFA and eluent B 0.1% TFA in acetonitrile. Column: C_8, 300Å pore size and 7 μm particle size; 220 x 4.6 mm).

[d] Δt_R is obtained by subtracting the retention time of the Gly substituted peptide from the retention time of the peptide in which a substitution is made.

[e] $\Delta\Delta t_R$ represents the effect on retention behaviour of an amino acid substitution between the hydrophobic and hydrophilic face of an amphipathic helix and is obtained by subtracting the Δt_R value in the hydrophilic face from the Δt_R value in the hydrophobic face.

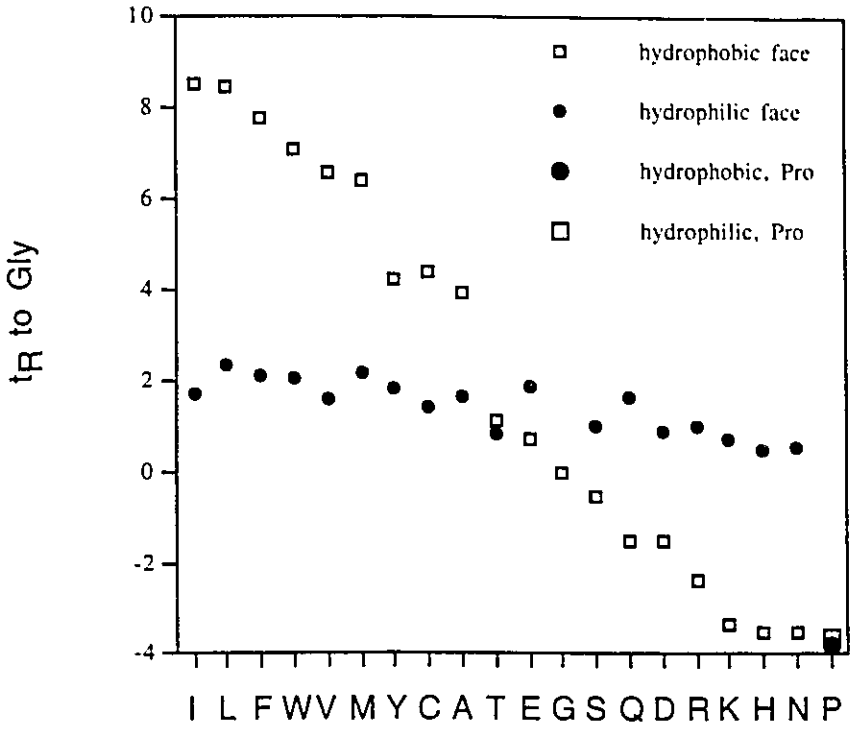

Figure V-5 Plot of Δt_R to Gly *vs* amino acid substitution in either the hydrophobic or hydrophilic face of an amphipathic α-helix. Data from Table V-3.

retention time. Whereas, compared to the same substitutions in the hydrophobic face (open boxes), a large range of retention times are observed (approximately 8 min to -4 min). It is also noticed that the Pro substitution results in a retention time, relative to Gly, that is approximately the same for either a substitution in the hydrophobic or hydrophilic face. Since it is known that a Pro residue disrupts the α-helix, from the Pro substitution to the terminal end of the peptide (AA9, $[\theta]_{220}$ = -29 150; AP9, $[\theta]_{220}$ = -14 600, where the ellipticity of peptides were recorded in the presence of pH 7 buffer containing 50% TFE) [11], one might expect that the retention behaviour of these 2 peptides would be similar because the Pro is no longer in an α-helix and the amphipathicity is thus lost.

From this discussion a general hypothesis may be made: the effect on the retention behaviour of the peptide of an amino acid substitution in the hydrophobic face will be

different than the effect of the same amino acid substitution in the hydrophilic face of an amphipathic helix.

Due to the fact that the results presented in Table V-3 and the hydrophobicity scale of Guo et al. [9] do not coincide exactly, this suggests that the numbers in Table V-3 may represent a hydrophobicity scale for amino acid side-chains in amphipathic α-helices as opposed to that of a random coil as expressed by Guo et al. for an octapeptide, or that reported in Table II-1, column labeled H, for a decapeptide (chapter II).

Effect of position of substitution in the hydrophobic face of the amphipathic α-helix

In chapter III, we examined the effect of environment on the side-chain hydrophobicity of each of the 20 amino acids by placing the substitution in the hydrophobic face of 2 amphipathic α-helices differing in the hydrophobicity of the preferred binding domain. The substitutions were placed in position 9 because this position is the center of the hydrophobic face and is surrounded by the maximum number of hydrophobic residues.

It is known that there are positional effects within the helix [11]; therefore prior to the study in chapter III, a study was done to examine the effect of position within the hydrophobic face. Since the Ala-face and Leu-face peptides were to be used in the chapter III study, a series of peptides were designed and synthesized in which a Gly and a Leu residue were "walked" through either the Ala- or Leu-face; these peptides designated AGx and ALx represent either a Gly or Leu substitution at position x in the hydrophobic face of the peptide. For example, AG2 represents a Gly replacing an Ala at position 2 with all other positions in the hydrophobic face (5, 6, 9, 12, 13, and 16) containing an Ala residue. The Gly walk through the Ala-face is presented in Figure V-6. In the same way, a Leu is walked through the Ala-face and a Gly is walked through the Leu-face and is also presented in Figure V-6.

(1) Gly walk in the Ala face

	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
AA9	Ac-E-A-E-K-A-A-K-E-A-E-K-A-A-K-E-A-E-K--amide
AG2	Ac-E-G-E-K-A-A-K-E-A-E-K-A-A-K-E-A-E-K--amide
AG5	Ac-E-A-E-K-G-A-K-E-A-E-K-A-A-K-E-A-E-K--amide
AG6	Ac-E-A-E-K-A-G-K-E-A-E-K-A-A-K-E-A-E-K--amide
AG9	Ac-E-A-E-K-A-A-K-E-G-E-K-A-A-K-E-A-E-K--amide
AG12	Ac-E-A-E-K-A-A-K-E-A-E-K-G-A-K-E-A-E-K--amide
AG13	Ac-E-A-E-K-A-A-K-E-A-E-K-A-G-K-E-A-E-K--amide
AG16	Ac-E-A-E-K-A-A-K-E-A-E-K-A-A-K-E-G-E-K--amide

(2) Leu walk in the Ala face

	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
AA9	Ac-E-A-E-K-A-A-K-E-A-E-K-A-A-K-E-A-E-K--amide
AL2	Ac-E-L-E-K-A-A-K-E-A-E-K-A-A-K-E-A-E-K--amide
AL5	Ac-E-A-E-K-L-A-K-E-A-E-K-A-A-K-E-A-E-K--amide
AL6	Ac-E-A-E-K-A-L-K-E-A-E-K-A-A-K-E-A-E-K--amide
AL9	Ac-E-A-E-K-A-A-K-E-L-E-K-A-A-K-E-A-E-K--amide
AL12	Ac-E-A-E-K-A-A-K-E-A-E-K-L-A-K-E-A-E-K--amide
AL13	Ac-E-A-E-K-A-A-K-E-A-E-K-A-L-K-E-A-E-K--amide
AL16	Ac-E-A-E-K-A-A-K-E-A-E-K-A-A-K-E-L-E-K--amide

(3) Gly walk in the Leu face

	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
LL9	Ac-E-L-E-K-L-L-K-E-L-E-K-L-L-K-E-L-E-K--amide
LG2	Ac-E-G-E-K-L-L-K-E-L-E-K-L-L-K-E-L-E-K--amide
LG5	Ac-E-L-E-K-G-L-K-E-L-E-K-L-L-K-E-L-E-K--amide
LG6	Ac-E-L-E-K-L-G-K-E-L-E-K-L-L-K-E-L-E-K--amide
LG9	Ac-E-L-E-K-L-L-K-E-G-E-K-L-L-K-E-L-E-K--amide
LG12	Ac-E-L-E-K-L-L-K-E-L-E-K-G-L-K-E-L-E-K--amide
LG13	Ac-E-L-E-K-L-L-K-E-L-E-K-L-G-K-E-L-E-K--amide
LG16	Ac-E-L-E-K-L-L-K-E-L-E-K-L-L-K-E-G-E-K--amide

Figure V-6 Gly and Leu walk in the Ala-face and Leu-face peptides. The native Ala-face peptide is designated AA9 and the peptides used to determine the effect of position are synthesized by successively replacing an Ala residue with a Gly residue in each position (i.e., 2, 5, 6, 9, 12, 13 and 16) in the hydrophobic face of the AA9 peptide resulting in 7 different peptides. In the same manner a Leu residue is walked through the hydrophobic face of the AA9 peptide. The native Leu-face peptide is designated LL9 and as for the AA9 peptide, a Gly residue is walked through the hydrophobic face of the LL9 peptide.

Due to the small range of retention times obtained for the peptides within a walk, these peptides were separated at a gradient rate of 0.5% acetonitrile/min in order to facilitate the identification of each peptide in the chromatogram. From the data presented in Table V-4, it can be seen that some positional substitutions result in identical retention times, e.g., in the AGx series AG5, AG9, AG12 and AG13 coelute at 40.54 min and AG6 and AG16 coelute at 41.48 min. Similarily, this is observed with the ALx and LGx series of peptides.

In order to determine the effect of the hydrophobic environment on side-chain hydrophobicity, a Gly to Leu substitution in the Ala-face is compared to a Gly to Leu substitution in the Leu-face, taking the positional effect into account. For example, AG2 to AL2 results in an 11.39 min retention time difference (Δt_R = 11.39 min); whereas, AG5 to AL5 results in a 13.5 min retention time difference. Thus, from Table V-4, it can be seen that the retention time difference of a Gly to Leu substitution in the Ala-face will be dependent on the position in the hydrophobic face. A similar effect is seen for a Gly to Leu substitution in the Leu face, where the positional effect of the Gly is referenced to the native LL9 peptide. For the Ala-face, a maximum difference in retention time is seen at positions 9 and 13; whereas, for the Leu-face the minimum retention time difference is observed for positions 9 and 6. Therefore, one can see that the maximum difference that may be obtained for a Gly to Leu substitution between an Ala- and Leu-face will be at position 9; the value of 5.37 min being reported as $\Delta\Delta t_R$ representing the effect of the different hydrophobic faces on a Gly to Leu substitution. The reported $\Delta\Delta t_R$ in column of Table V-4 indicate that a smaller difference is observed for positions 5, 6, 12 and 13, i.e., 3.26, 3.86 and 3.55, 2.83 as opposed to 5.37 min. In attempting to determine the effect of the hydrophobic environment on side-chain hydrophobicity, it was decided that the position that should be used would be the position that resulted in the maximum retention time difference ($\Delta\Delta t_R$) in order to accommodate the full range of amino acids that would be tested. The data presented in Table V-4 is consistent with that reported in chapter III, i.e., a Leu substitution

in the Ala-face (Δt_R =14.08 min) will have a greater apparent hydrophobicity than a Leu in the Leu-face (Δt_R = 8.71 min). The Δt_R data from Table V-4 is plotted against the position

Table V-4 Effect of position of substitution in the hydrophobic face of the amphipathic α-helix (Ala- and Leu-face).

Position of [a] substitution	Ala-face [b]			Leu- face [b]		
	t_R [c]			t_R [c]		
	AGx	ALx	Δt_R [d]	LGx	Δt_R [d]	$\Delta\Delta t_R$ [e]
2	43.23	54.62	11.39	85.16	10.80	0.59
5	40.54	54.04	13.50	85.72	10.24	3.26
6	41.48	54.04	12.56	87.25	8.71	3.86
9	40.54	54.62	14.08	87.25	8.71	5.37
12	40.54	53.19	12.65	86.86	9.10	3.55
13	40.54	54.62	14.08	84.71	11.25	2.83
16	41.48	53.19	11.71	83.65	12.31	-0.60
range	2.69	1.43		3.60		

[a] Represents the position of substitution in the hydrophobic face of the peptide, see Figure V-3.

[b] Nomenclature for peptides: The first letter in code, A (Ala) or L (Leu), represents the amino acid that comprises the hydrophobic face and the second letter, G (Gly) or L (Leu) represents the substitution at the designated position x (2, 5, 6, 9, 12, 13, 16) in the helix. Therefore, AG2 represents a peptide with a Gly substituted for an Ala at position 2 in the Ala-face peptide.

[c] Retention time of the corresponding peptide. Conditions: Linear AB gradient elution at a rate of 0.5% acetonitrile /min where A is 0.1% aqueous TFA and B is 0.1% TFA in acetonitrile at a flowrate of 1ml/min.Column: Zorbax C_8, 300Å pore size and 6 μm particle size; 150 x 4.6 mm).

[d] Δt_R represents the difference in retention time between an AGx mutant and an ALx mutant for the Ala face peptides and the difference between an LGx mutant and the peptide LL9 (t_R = 95.96 min) for the Leu face. Essentially this retention time difference represents a Gly to Leu substitution.

[e] $\Delta\Delta t_R$ represents the difference in Δt_R between the Ala face and the Leu face where Δt_R for the Ala face is determined as the difference t_RALx - t_RAGx and Δt_R for the Leu face is determined by the difference t_RLL9 - t_RLGx.

in the helix in Figure V-7 and this plot shows that a Gly to Leu substitution in the Leu face (filled circles) results in a decrease in apparent hydrophobicity; whereas, a Gly to Leu

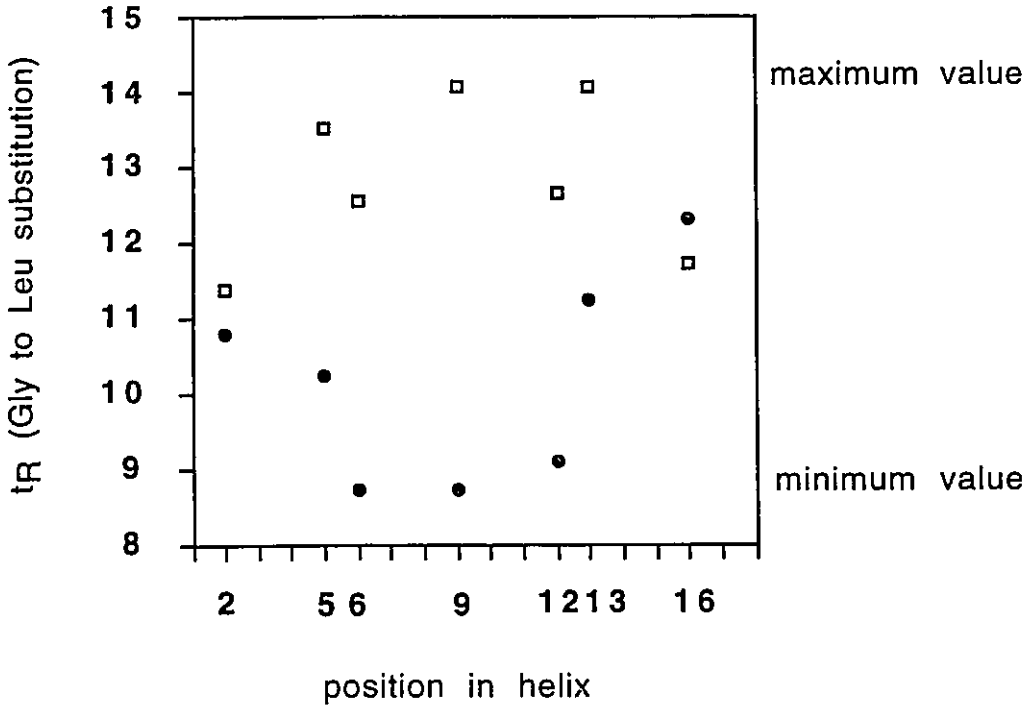

Figure V-7 Plot of Δt_R to Gly substitution *vs* position in the helix. Data from Table V-4, labeled Δt_R: Ala-face (open boxes) and Leu-face (filled circles).

substitution in the Ala-face results in an apparent increase in side-chain hydrophobicity. It should also be noted that position 2 and 16 result in a very small $\Delta\Delta t_R$ values, 0.59 and -0.60.

From the previous discussion, the following hypothesis may be made: The effect of an amino acid substitution in the hydrophobic face of an amphipathic α-helix on the retention behaviour will be dependent on the position in the hydrophobic face, with the greatest effect occuring in the center of the α-helix.

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CHAPTER VI

Summary

INTRODUCTION

This chapter gives a brief summary of the factors affecting the retention behaviour of peptides in reversed-phase chromatography (RPC) as determined in this thesis. Also included in this chapter is a brief discussion on how this information may be applied to RPC, the understanding of protein-ligand interactions or the understanding of how the environment surrounding the substitution site on the protein surface may affect the pK_a of a potentially ionizable group.

DISCUSSION

Summary of the factors affecting the retention behaviour of peptides in RPC

Figure VI-1 is a diagramatic representation of the factors that are known, or have been shown in this thesis, to affect the retention behaviour of peptides in RPC. The items in hatched boxes are factors that have been studied previously which include amino acid composition, peptide chain length and sequence dependent effects [1]. For example, since the hydrophobicity/hydrophilicity of each amino acid side-chain affects the overall hydrophobicity of the peptide, many amino acid side-chain hydrophobicity scales have been developed based on the retention behaviour of peptides during RPC [2-5]. In the absence of conformational effects, the assumption upon which such scales are based is that the chromatographic behaviour of a peptide is mainly dependent on the amino acid composition. In addition, it has been shown that polypeptide chain length (up to 50 residues) affects retention behaviour [6] in that the retention time varies as a function of the logarithm of the number of residues in a peptide and its hydrophobicity, as determined using retention coefficients derived from small peptides. Mant et al. [7] have also been able

to extend this correlation to that of protein retention times for proteins ranging in polypeptide chain length from 30 to 300 residues based on the sum of the hydrophobicity coefficients and the number of residues in the polypeptide chain. Sequence dependent effects can lead to preferential interaction sites for binding to the stationary phase, i.e., preferred binding domains [1]; for instance, it has been shown that an amphipathic α-helical peptide will have a longer retention time than a non-amphipathic α-helical peptide [1]. It has also been shown [8] that ion-paring reagents have predictable effects on retention behaviour (see discussion in chapter I, Figure I-3 and I-4).

The factors numbered 1, 2, 3, 3a, 3b and 4 in Figure VI-1 have been shown in studies presented in this thesis to affect the retention behaviour of peptides. Factor 1 in Figure VI-1 indicates that the presence of a free α-amino group affects retention behaviour in 2 different ways; a free α-amino group may decrease the retention time of a peptide due to its very hydrophilic character as previously suggested [8] and as well, it may decrease the hydrophobicity of the N-terminal side-chain of some residues, predominantly hydrophobic residues, while it may increase the hydrophobicity of the N-terminal side-chain of other residues, predominantly hydrophilic (see Chapter I), which is consistent with that suggested by other researchers [9,10]. It has also been shown (factor 2 in Figure VI-1, Chapter III) that the environment surrounding a residue may exert an effect on the side-chain hydrophobicity/hydrophilicity of that residue. The data presented suggest that an environment of increasing hydrophobicity results in a decrease in side-chain hydrophobicity for all of the common amino acids. As a consequence of environmental effects the pK_a of an ionizable group may be affected (factor 2a in Figure VI-1, Chapter II), where it has been shown that the pK_a of an α-amino group may be decreased if it is in a non-polar environment, such as a reversed-phase stationary phase, or by the increasing hydrophobicity of the side-chain in the N-terminal position. In addition, it has been shown that the

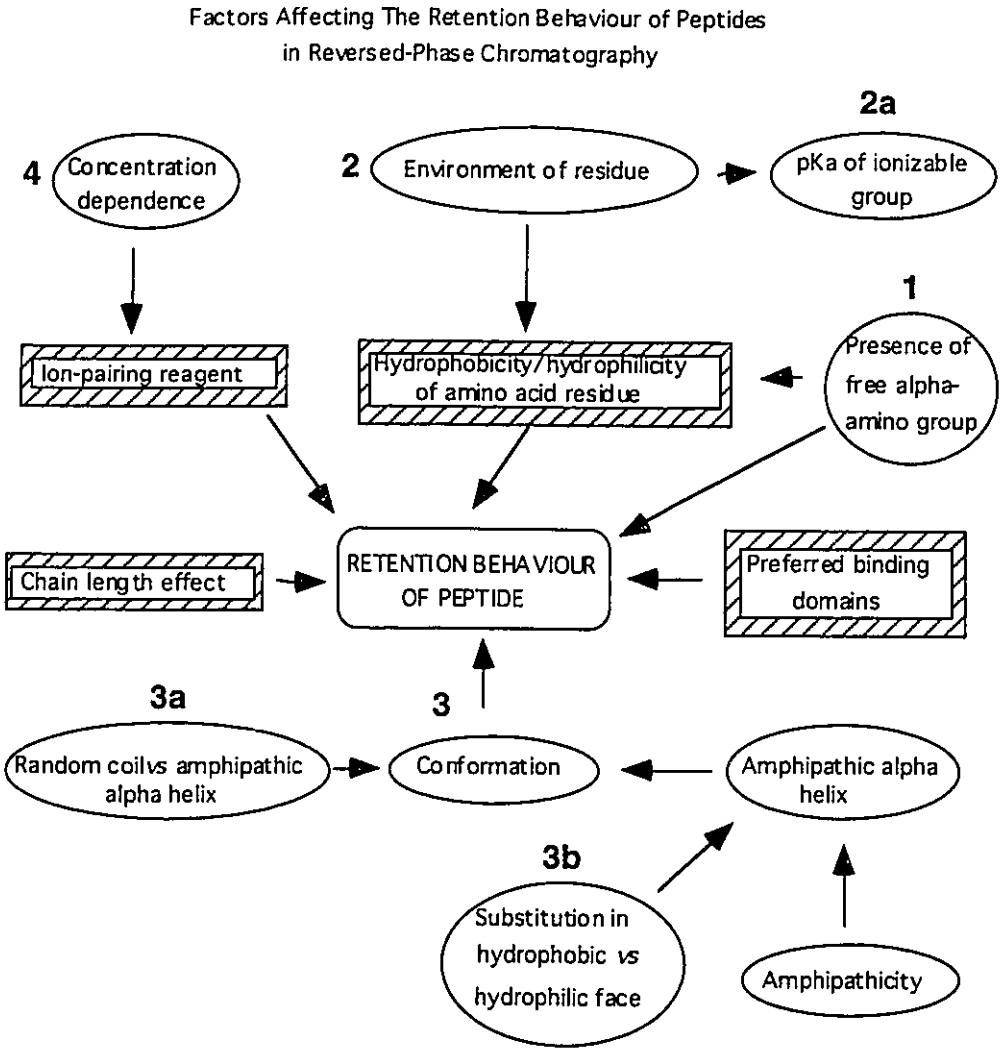

Figure VI-1 Factors that affect the retention behaviour of peptides in RPC. Factors shown to affect retention behaviour in this thesis are discussed in the following chapters: factors 1and 2a in Chapter II; factors 2 in Chapter III; factors 3 and 3a in Chapter IV and factors 3b and 4 in Chapter V. The hatched boxes represent factors that have been studied previously and a brief description of their affect on retention behaviour is given in the text.

presence of a potentially positively or negatively charged side-chain in the N-terminal position may also affect the pK_a of the α-amino group.

The third factor shown in this thesis to affect retention behaviour is conformation (factors 3, 3a in Figure VI-1). The discussion in Chapter IV indicates that the selectivity of a separation of peptides of very different conformation may be effected by altering the

gradient rate, suggesting that the conformation is an important factor for the effect observed. In addition, it has been shown (factor 3b in Figure VI-1, Chapter V) that a substitution in the hydrophobic face of an amphipathic α-helical peptide will have a different effect on retention behaviour as compared to the same substitution in the hydrophilic face.

The fourth factor reported in this thesis is the concentration dependence of a hydrophilic ion-pairing reagent. It has been shown that the presence of 100 mM perchlorate at pH 2 may increase the retention time of peptides containing a positively charged group through reduction in hydrophilicity of the charged group, as previously suggested [8]; in addition, this is consistent with the previous observation that peptide retention time increases with increasing amount of phosphate ion at pH 2 [11]. This effect of perchlorate can be used advantageously to increase the selectivity between peptides of different conformations.

Application to reversed-phase separation protocols

As shown in Figure VI-1, three factors that affect the retention behaviour are hydrophobicity/hydrophilicity, conformational effects on selectivity and the ion-pairing effects (concentration dependence of a hydrophilic ion-pairing reagent) and these topics will now be discussed in terms of how this information may be used to develop separation protocols in RPC.

Hydrophobicity/hydrophilicity: Many amino acid side-chain hydrophobicity/hydrophilicity scales have been developed over the years [12,13], using a variety of methods and some of the reasons for the variability between scales has been previously discussed [14]. Generally, researchers have applied computer-calculated regression analysis of the retention times of a wide range of peptides of varied composition. The drawbacks of such an approach include a variation in the frequency of occurrence of amino acids and polypeptide chain length (and perhaps conformation) amongst a random sampling of peptides. In addition, this thesis has clearly shown that the presence of a free α-amino group will affect the hydrophobicity of the side-chain in the N-terminal position, another factor not taken

into account in other researchers' hydrophobicity/hydrophilicity scales. Thus, although HPLC methods for the determination of retention coefficients which use an approach which averages all factors that affect retention behaviour [5] result in retention coefficients that may give reasonable predictions on retention times, the averaging effect of these methods results in the loss of important information which would allow one to understand fully the retention behaviour of peptides in RPC. When one considers that a protein digest may contain peptide fragments of widely varying length, hydrophobicity and conformation (an RPC stationary phase will induce and stabilize α-helical structure) most or all of these fragments also containing a free α-amino group, the need for a thorough understanding of how all these factors may influence peptide RPC retention behaviour becomes clear. The approach of this laboratory in attempting to understand such behaviour has been to examine the retention behaviour of defined model peptide systems, thus delineating the effects of various peptide properties (e.g., chain length, side-chain hydrophobicity, free α-amino acid) one at a time.

For instance, from this thesis, in attempting to predict the retention time of peptides with a free α-amino group, 2 sets of retention coefficients, e.g., values labelled H in Table II-1 for internal residues and values labelled h in Table II-1 for N-terminal residues only, could be used to improve retention time prediction. It has also been shown that the environment of a residue may affect the hydrophobicity/hydrophilicity of a side-chain; therefore, the 2 sets of values presented in Table III-1, Δt_R (AX-AG) and Δt_R (LX-LG), represent 2 scales which are dependent on environment. Thus, this suggests that the effect of environment surrounding a residue may add variability to other researchers hydrophobicity/hydrophilicity scales that do not take this factor into account. Peptide conformation, specifically the difference between the hydrophobic and hydrophilic face of an amphipathic peptide, has also been shown to affect retention behaviour (Table V-3). Thus the 3 sets of values obtained for each of the 20 amino acids given in Table II-1 for a random coil (column labelled H), Table V-3 for an amphipathic α-helix (column labelled

hydrophilic face) and Table V-3 for an amphipathic α-helix (column labelled hydrophobic face) represent 3 different scales, which are therefore a function of conformation.

A computer program, ProDigest LC [15], developed to simulate RPC (as well as size exclusion and ion-exchange) elution profiles to aid in the development of separation protocols, only requires the knowledge of the amino acid composition of the peptides. The development of this program was based on the step-by-step approach noted above of gauging the effect of individual parameters such as peptide chain length and composition on peptide retention behaviour and taking these factors into account in the final simulation. The program is currently being modified to take into account the effects of a free α-amino acid group on peptide retention behaviour during RPC as described in this thesis.

In conclusion, the systematic identification of factors affecting the hydrophobicity/hydrophilicity of amino acids, by using model synthetic peptides, will result in a better understanding of the retention behaviour of peptides in RPC.

Conformational effects on selectivity: In Figure VI-1, label 3 and 3b indicate that the conformation may also affect the retention behaviour of peptides in RPC. The data presented in Chapter IV suggests that the significantly different conformations, random coil *vs* amphipathic α-helix, are an important factor in the selectivity effects observed in the separation of mixtures of peptides containing these two very different conformations.

RPC optimization protocols such as Dry Lab [16] may be described as a hybrid technique [17], since some experimental data, two gradient runs in which the information may be used to determine S values for each solute, are required in addition to the theoretical aspects, as described in Linear Solvent Strength (LSS) theory of gradient elution (Chapter IV). Use of LSS theory allows Dry Lab to optimize a separation; but the physical basis of the chromatographic parameter S is not yet fully understood. As suggested in Chapter IV, the significantly different conformations may result in significantly different S values, which ultimately suggests changes in separation selectivity. Knowledge of the factors that affect S values will be useful. In addition, knowledge of how conformation affects S

values may provide information on the secondary structure of a peptide. Since *S* values have been shown to be dependent on conformation, studies in this area could also be extended to peptides containing β sheet conformation.

The ultimate goal of a program like ProDigest LC is to simulate separation profiles based solely on sequence information, e.g., sequence information could potentially be searched for secondary structure and knowledge of this information in addition to the knowledge of the dependence of conformation on *S* values could be used to make improvements in separations due to selectivity effects.

Ion-pairing effects: Discussion in Chapter V showed that a hydrophilic ion-pairing reagent, at a concentration of 100 mM, resulted in a selectivity difference as compared to the hydrophobic ion-pairing reagent TFA, at 13 mM; thus suggesting that other hydrophilic ion-pairing reagents used at this concentration may supply additional possibilities for varying the selectivity of a separation.

Ligand-receptor interactions

As indicated in Figure VI-1, Factor 2, the environment surrounding a residue may affect its hydrophobicity/hydrophilicity; therefore, one might expect that each residue in a preferred binding domain that is involved in a ligand-receptor interaction is part of a complex set of interactions that require not only the knowledge of the hydrophobicity/ hydrophilicity of that residue, but as well, how the environment affects its hydrophobicity/hydrophilicity will also be an important factor. Discussion in this section will focus on some examples in biological systems where this effect could be important.

The amphipathic α-helix is a common motif and Segrest et al. [18] reviews the types of amphipathic helices that are commonly found in biologically active peptides and proteins and groups them into 2 classes; lipid associating and protein-protein associating. By definition, an amphipathic helix will have a polar and non-polar face [18], e.g., transmembrane helices have a very wide non-polar face, whereas coiled-coils have the smallest non-polar face of the 7 different types as described by Segrest et al. [18].

Therefore, studies described in this thesis on how environment affects the hydrophobicity/hydrophilicity of a residue were carried out with synthetic model amphipathic α-helical peptides.

The following is a set of biological examples involving amphipathic α-helices. It has been previously shown that the stability of synthetic coiled-coils may vary based on the hydrophobic residue substituted in the hydrophobic face of the coiled-coil [19]. In addition, a synthetic coiled-coil with Leu residues substituted in the hydrophobic face resulted in a structure that was more stable than the natural tropomyosin homodimer; therefore, it was suggested that the subtle control of stability, by altering the substitution in the hydrophobic face, results in the appropriate stability for homo- or heterodimer formation which also affects biological activity [19]. Another example of this variability in ligand-receptor interactions was observed by Anantharamaiah [20], where synthetic amphipathic helical peptides are shown to have different lipid associating affinities upon altering the width or length of the hydrophobic face. As well, from the crystal structure of troponin C (TnC) [21], it has been shown that the A helix from the N-terminal domain interacts with the hydrophobic cleft or pocket in the C-terminal domain. This example of binding is used as a model for the binding of other amphipathic helices to TnC.

In conclusion, in assessing the stability of a ligand-receptor interaction in biologically important peptides/proteins, one must be aware not only of the hydrophobicity/ hydrophilicity of the residue being substituted into a particular peptide/protein; but also, it must be known how the environment surrounding this residue will affect the hydrophobicity/hydrophilicity of that residue in order to fully understand how this substitution will affect stability and therefore impact on biological activity.

Effect of environment on the pKa of an ionizable group

In Figure VI-1, Factor 2a, it is shown that the environment of an ionizable group may affect its pK_a and in Chapter II it was shown that the hydrophobic nature of the reversed-phase stationary phase could affect the pK_a of amino acids with ionizable side-

chains. This observation lends support to the observation that some ionizable groups in proteins have anomalous pK_a values which may be affected by the non-polar environment of the protein. A good example is Glu35 in lysozyme, where it has been shown that this group has a pK_a value of 6.1 [22]. It was also shown in this report that if Trp108, which is in van der Waals contact with Glu35, is replaced with groups of lower hydrophobicity, the pK_a value decreases, suggesting that the hydrophobic environment is an important factor in maintaining the appropriate pK_a value of Glu35. An additional example of how a non-polar environment may affect the pK_a of an ionizable group is the substitution of Val66 for a Lys residue [23], resulting in Lys being buried in a hydrophobic core of a protein. In the folded state of the protein, the pK_a of Lys66 is 6.4; whereas, in the unfolded state it is 10.2.

In conclusion, the effect of the non-polar stationary phase on an ionizable group in a synthetic peptide is a good mimic for the effect of a non-polar environment on a ionizable group in a protein.

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