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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

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Abstract

To mimic a hydrophobic protein binding domain, which is a region on the surface of a protein that has a preference or a specificity to interact with a complementary surface, we have designed amphipathic α-helical peptides where the non-polar face interacts with the non-polar surface of a reversed-phase stationary phase. Two series of potentially amphipathic α-helical peptides, a native Ala peptide (AA9) and a native Leu peptide (LL9), were designed where the native peptide contains 7 residues of either Ala or Leu, respectively, in its non-polar face. 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 the substitutions Ala, Leu, Lys and Glu, 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. This procedure was further extended to the remaining amino acids commonly found in proteins and it was determined that this general principle applies to all 20 amino acids. These results have major implications to understanding the hydrophilicity/hydrophobicity of amino acid side-chains and the role side-chains play in the folding and stability of proteins.

1. Introduction

One of the most interesting developments of liquid chromatography analysis lies in the employment of reversed-phase liquid chromatography (RPLC) as a physicochemical model of biological systems. Studies in this area have generally centred on attempting to correlate the retention behaviour of peptides [1] or proteins [2–6] during RPLC with their conformational stability; the rationale behind this approach lies

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in the assumption that the hydrophobic interactions between peptides and proteins with the non-polar stationary phase characteristic of RPLC [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 in RPLC may be a reasonable mimic for the hydrophobic environment created internally by proteins, e.g., as a probe of how the pK_a values of potentially ionizable side-chains in the hydrophobic interior of a protein, frequently important in catalytic groups, are influenced by their environment. Indeed, RPLC 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 RPLC 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 RPLC 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 [14–20]; amphipathic helices putatively have a role in the activation of G proteins (trimeric GTP-binding regulatory proteins) by membrane receptors and peptides [21,22], including mastoparan [23]; the high amphipathic helical content of the antibiotic family of peptides known as magainins enables them to interact strongly with bacterial and acidic model membranes [24,25]; finally, other functions of amphipathic helices in ligand–receptor interaction include their involvement in T-cell recognition [26], lipid-associating domains of apolipoproteins and lipoproteins [27,28] and the hydrophobic domains of coiled-coil proteins that bind to DNA (the so-called leucine-zipper proteins) [29,30]. 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 [31,32]. 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 RPLC retention behaviour.

In the present study, we describe a simple model ligand–receptor system based on observing the retention behaviour during RPLC 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.

2. Experimental

2.1. Materials

HPLC-grade water and acetonitrile were obtained from BDH (Poole, UK). Trifluoroacetic acid (TFA) was obtained from Aldrich (Milwaukee, WI, USA). Trifluoroethanol (TFE) was obtained from Sigma (St. Louis, MO, USA).

2.2. Instrumentation

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

The analytical HPLC system consisted of an HP1090 liquid 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 masses of peptides were confirmed by time-of-flight mass spectroscopy on a BIOION-20 Nordic (Uppsala, Sweden).

Circular dichroism (CD) spectra were recorded on a JASCO J-500C spectropolarimeter (Easton, MD, USA) attached to a JASCO DP-500N data processor and a Lauda (Model RMS) water bath (Brinkman Instruments, Rexdale, 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 nitrogen flushing was employed.

2.3. Peptide synthesis

Peptides were synthesized by the solid-phase technique (SPPS) on co-poly(styrene–1% divinylbenzene) benzhydrylamine–hydrochloride resin (0.92 mmol/g resin) as previously described [8]. The cleaved peptide–resin mixtures were washed with diethyl ether (3 × 25 ml) and the peptides extracted with neat acetic acid (3 × 25 ml). The resulting peptide solutions were then lyophilized prior to purification.

2.4. Columns and HPLC conditions

Crude peptides were purified on a semi-preparative Synchropak RP-P C_{18} reversed-phase column (250 × 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 on an Aquapore RP-300 C_8 reversed-phase column (220 × 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.

2.5. Calculation of accessible surface areas

All peptide structures were generated in an idealized conformation using equilibrium bond lengths, and angles and dihedral angles (Insight II, Biosym Technologies, San Diego, CA, USA). Backbone dihedral angles were set to ideal α-helical values of -67 for ϕ and -44 for ψ [33]. 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 [34]. The minimizations were performed with Discover (Biosym) and the consistent valence force field (CVFF) 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 [35]. Individual surface areas

per atom were summed to yield hydrophobic, hydrophilic and charged surface areas according to the definition by Eisenberg and McLachlan [36].

3. Results and discussion

3.1. 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. [37], 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 occurring 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.

3.2. 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 (Fig. 1, right). In the design of this peptide, leucine, glutamic acid and lysine residues were selected in light of their highly intrinsic helical propensities [38–40]; leucine as a non-polar 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 [41–43] 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 [44,45] at neutral pH values.

Fig. 1 (right) 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 [32,41,46–48], involving 5 hydrophobic residues at positions 2, 5, 9, 12 and 16. It was felt that the wider hydrophobic face of our model peptide

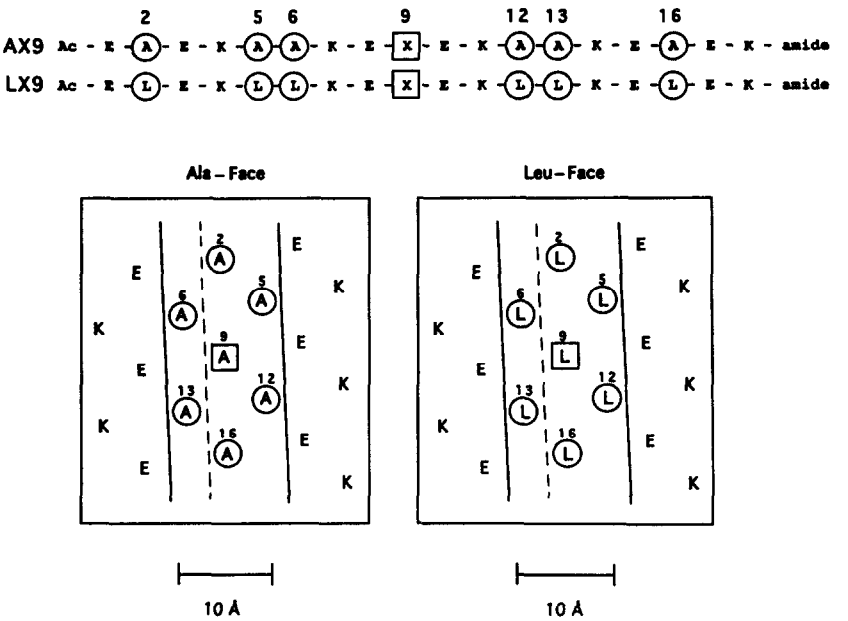

Fig. 1. Design of model synthetic peptides. Top: sequence of mutant peptides, AX9 and LX9, where the first letter represents amino acid residues used in the hydrophobic face of the peptide, the X represents each of the 20 amino acids (boxed) (single letter code given in Table 1) substituted at position 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 2.5 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).

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 below.

3.3. Conformation and helicity of model peptide ligands

The α-helicity of the "native" peptide ligands was determined by CD, with the CD spectrum measured in 0.1 *M* KCl + 50 m*M* potassium phosphate buffer (pH 5.2)–TFE (1:1, v/v), a solvent that induces helicity in single-chain potentially α-helical peptides [49,50]. Studies [51,52] 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 degree 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 degree cm^2/dmol calculated for a 100% α-helical 18-residue peptide [53].

3.4. 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 [54]; 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.

3.5. Retention behaviour of amphipathic α-helices during RPLC

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. [52] 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.

3.6. 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 [55]. 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).

3.7. 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 (Fig. 1). The most hydrophobic series of analogues was based on the "native" model peptide described above (Fig. 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; Fig. 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" (Fig. 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 consid-

erations: (1) alanine, like leucine, has a high intrinsic helical propensity [38–40]; (2) alanine is considerably less non-polar than leucine [56], 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 Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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.

3.8. 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 $-28\,196 \pm 510$ for the Ala-face series, excluding peptide AP9; in addition, analogues of the Leu-face were also shown to exhibit similar high α-helicity. In addition, when Eisenberg and co-workers' [57,58] 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 [58]. 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; apolipoproteins, 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 [31,59]. 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) [60]. 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 affect the helicity of the peptide in a non-polar environment as shown above. In addition, we have previously shown that α-heli-

cal peptides with Gly residues every seventh residue can still be completely α-helical even in benign medium in two-stranded α-helical coiled-coils [61]. The strong interhelical hydrophobic interactions stabilizing the coiled-coil override the destabilizing effect of Gly due to its intrinsic low helical propensity value [61].

3.9. Reversed-phase chromatography of synthetic peptide analogues

Fig. 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 Fig. 2A, 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 Fig. 2A, 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 $t_{R,AL9}$ minus $t_{R,AG9}$, i.e., a retention time difference of 8.5 min; in the Leu-face, this value is $t_{R,LL9}$ minus $t_{R,LG9}$, 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.

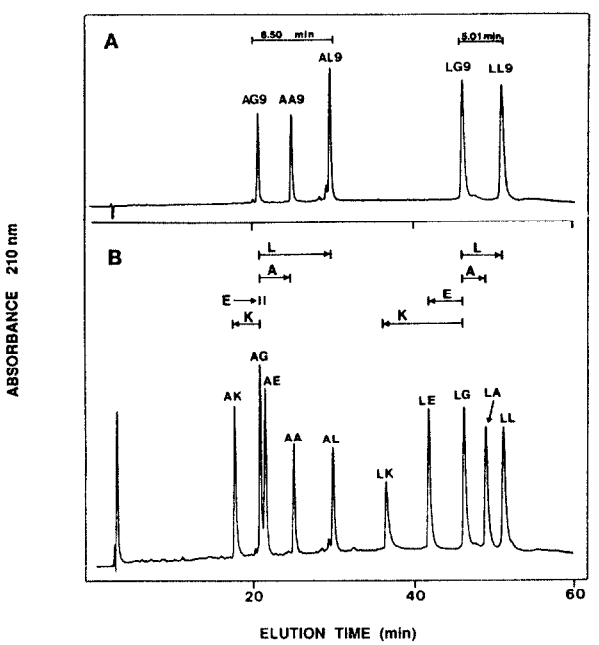

Fig. 2. RPLC of model synthetic peptides. (A) Separation of the "native" Ala-face peptide (AA9) and the "native" Leu-face peptide (LL9) from mutant peptides AG9, AL9 and LG9. (B) separation of the "native" Ala-face (AA) and the "native" Leu-face peptide (LL) from selected mutant analogues [since all residue substituitions were made at the same position in the peptide sequence (see Fig. 1) the number "9" has been omitted from the peptide designations for the sake of clarity]. The bars above the peptides in (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.

Fig. 2B, 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 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 pep-

tide (AE), glutamic acid is more hydrophobic compared to glycine (peptide AG).

Table 1 summarizes the reversed-phase retention behaviour of all 40 peptide analogues. The retention times of the Ala-face peptides (column denoted $t_{R,AX}$ in Table 1) were now plotted against those of the Leu-face peptides (column denoted $t_{R,LX}$ in Table 1). From Fig. 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.

The order of amino acid substitutions shown in Table 1 was based on decreasing retention time of the Ala-face mutants ($t_{R,AX}$), 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 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

Table 1
RPLC retention times of Ala- and Leu-face mutant peptides

Amino acid[a] substitution	Ala-face mutants		Leu-face mutants	
	$t_{R,AX}$ (min)[b]	$\Delta t_{R,AX-AG}$ (min)[c]	$t_{R,LX}$ (min)[b]	$\Delta t_{R,LX-LG}$ (min)[c]
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 (Fig. 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 1 ml/min.

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

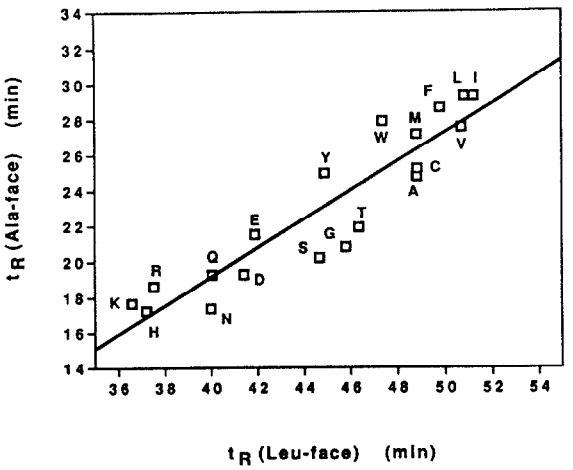

Fig. 3. Plot of $t_{R,AX}$ vs. $t_{R,LX}$, where AX and LX represent mutants of either the Ala- or Leu-face peptides. Retention time (t_R) data taken from Table 1. The single-letter code represents the amino acid substitution at position 9 of the peptide sequence (see Fig. 1).

observed reversed-phase retention behaviour of a series of octapeptide analogues reported by Guo et al. [56]. 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 [56].

From Table 1, for the Leu-face mutants, there is a decrease in $\Delta t_{R,LX-LG}$ 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 Fig. 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 1 were normalized, the value for maximum side-chain hydrophobicity (leucine in the Ala-face, where $\Delta t_{R,AX-AG} = 8.50$ min) being denoted 1.00 and the glycine mutant being assigned a value of 0.0. Table 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 minimum 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 1 were now normalized, the maximum value for side-chain hydrophilicity (lysine in the Leu-face, where $\Delta t_{R,LX-LG} = -9.23$ min) being denoted −1.00 and the glycine mutant again being assigned a value of 0.0. Table 3 compares the resulting relative hydrophilicities of these hydrophilic side-chains (i.e., defined as those

Table 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 $\Delta t_{R.AX-AG}$ 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 1).
[c] The minimum relative hydrophobicity is defined as the ratio of $\Delta t_{R,LX-LG}$ 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 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. From Table 1 these two residues behave in a similar fashion to the other hydrophobic residues by showing a decrease in hydrophobicity in the Leu-face compared to the Ala-face. However, these residues become less hydrophobic than Gly in the Leu-face.

Table 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 $\Delta t_{R.LX-LG}$ 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 1).
[c] The minimum relative hydrophilicity is defined as the ratio of $\Delta t_{R.AX-AG}$ 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 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. From Table 1 these two residues behave in a similar fashion to the other hydrophilic residues by showing an increase in hydrophilicity in the Leu-face compared to the Ala-face. However, these residues become more hydrophobic than Gly in the Ala-face.

which are more hydrophilic than glycine). In a similar manner to the observed behaviour of the hydrophobic residues (Table 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 environment around the mutation, the apparent hydrophilicity of the side-chain increases significantly.

3.10. Correlation of RPLC 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

Table 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.

(NPASA) was calculated for the non-polar face of these peptides. From Table 4, the ratio of the NPASA of LL9 (810 Å^2) to AA9 (442 Å^2) is 1.83. The 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 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.

The results of Table 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 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 (ΔNPASA), to produce a designated NPASA value for the substituted side-chain at mutant position 9 only. From the results shown in Table 5, it can be seen that, in an analogous manner to the calculated apparent side-chain hydrophilicity/hydrophobicity values (or coefficients) reported in Table 1 ($\Delta t_{R,AX-AG}$ and $\Delta t_{R,LX-LG}$), the ΔNPASA of each side-chain was lower in the more hydrophobic Leu-face compared to the Ala-face.

Fig. 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 ΔΔNPASA in Table 5 and Fig. 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 5 from Δt_R values reported in Table 1). From Fig. 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

Table 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)					
	Ala-face		Leu-face		ΔΔNPASA[c]	$\Delta\Delta t_R$ [d]
	NPASA	ΔNPASA[b]	NPASA	ΔNPASA[b]		
Leu	499	79	810	45	34	3.49
Ile	494	74	797	32	42	3.10
Phe	493	73	792	27	46	3.88
Trp	501	81	786	21	60	5.55
Val	486	66	802	37	29	1.85
Met[e]	493	73	808	43	30	3.33
Cys[e]	466	46	784	19	27	1.35
Tyr	464	44	770	5	39	3.24
Ala	442	22	780	15	7	0.94
Thr	455	35	773	8	27	0.55
Gly	420	0	765	0	0	0.00
Ser	430	10	769	4	6	−0.56
Gln	418	−2	727	−38	−36	−4.23
Asn	410	−10	740	−25	−15	−2.37

[a] Represents the amino acid substituted into position 9 of either the Ala- or Leu-face mutants (Fig. 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 Leu-face, i.e. ΔNPASA, from the non-polar surface area of the side-chain in the Ala-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 1.
[e] The sulphur atom of Met and Cys is calculated as a non-polar atom [36].

of these residues [62], thus reducing the expected magnitude of interaction of these side-chains with the reversed-phase matrix.

4. 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

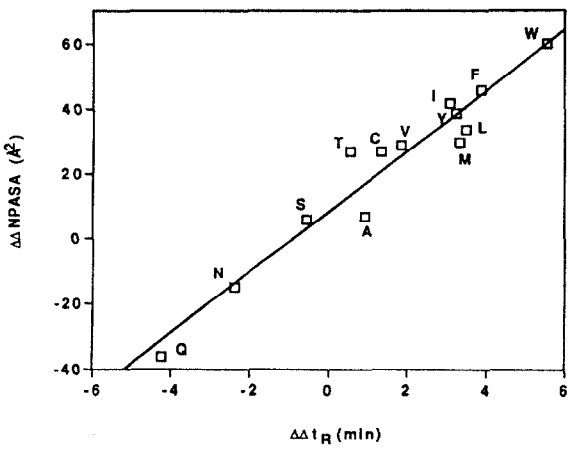

Fig. 4. Plot of ΔΔNPASA vs. $\Delta\Delta t_R$ (see Table 5) for amino acid side-chains. The single-letter code represents the amino acid substitution at position 9 of the peptide sequence (see Fig. 1).

hydrophobic interactions involved in protein folding and stability.

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