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Reversed-phase liquid chromatography as a tool in the determination of the hydrophilicity/hydrophobicity of amino acid side-chains at a ligand–receptor interface in the presence of different aqueous environments
I. Effect of varying receptor hydrophobicity

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Abstract

We have developed further 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 retention behaviour of de novo designed model 18-residue amphipathic α-helical peptides (representing the hydrophobic binding domain of a ligand) on reversed-phase packings by varying hydrophobicity (representing a receptor protein with a hydrophobic binding pocket). Mutants of the ''native'' peptide ligand (which contains seven Leu residues in its non-polar face) were designed by replacing one residue in the center of the extremely non-polar face of the amphipathic α-helix. Through reversed-phase liquid chromatography of these peptides at pH 2.0 on cyano and C_{18} columns, we have demonstrated how an increase in receptor hydrophobicity (represented by an increase in column stationary phase hydrophobicity; cyano→C_{18}) significantly enhances hydrophilicity of polar amino acid side-chains at the ligand–receptor interface while moderately enhancing the hydrophobicity of non-polar side-chains. The addition of salt (100 m*M* sodium perchlorate) to the aqueous environment surrounding the binding site of receptor and ligand was also shown to have a profound effect on side-chain hydrophilicity/hydrophobicity in the binding interface. This effect was particularly dramatic for the positively charged side-chains Arg, Lys and His, whose significant enhancement of hydrophobicity in the presence of the cyano column contrasted with their increase in hydrophilicity in the presence of the considerably more hydrophobic C_{18} stationary phase. Our results have major implications to understanding the influence of hydrophobic and aqueous environment on hydrophilicity/hydrophobicity of amino acid side-chains and the role side-chains play in the folding and stability of proteins.

Keywords: Hydrophilicity; Hydrophobicity; Ligand–receptor interfaces; Interfaces, ligand–receptor; Amino acids; Peptides

1. Introduction

The tremendous benefit to researchers in the peptide and protein field afforded by the development of high-performance chromatographic techniques over the past two decades is, of course, well known and well documented [1–4]. The advancement in instrumentation and column packings has

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been of particular relevance to peptide separations, where classical column chromatography generally offered unsatisfactory resolution and sensitivity. An interesting offshoot of liquid chromatography analysis in recent years has been the employment of high-performance techniques as probes of protein structure and stability. Thus size-exclusion chromatography (SEC) has been used to analyze protein folding [1,5–10]; in addition, conformational changes of proteins have been monitored by hydrophobic interaction chromatography (HIC) [1,11–14]. Reversed-phase liquid chromatography (RP-HPLC) has been particularly useful as a physicochemical model of biological systems, where studies have generally centered on correlating the retention behaviour of proteins [11,15–21] or peptides [22–27] during RP-HPLC with their conformational stability. The assumption with such RP-HPLC studies is that the hydrophobic interactions between peptides and proteins with the non-polar stationary phase characteristic of RP-HPLC [1–4] reflects the hydrophobicity and interactions between non-polar residues which are the major driving forces for protein folding and stability. It has also been suggested that the hydrophobic environment of RP-HPLC may also be a reasonable mimic of the hydrophobic environment created internally by proteins [19]; specifically, RP-HPLC has been used as a probe of how the pK_a values of potentially ionizable side-chains, frequently important as catalytic groups, are influenced by their microenvironment [28]. RP-HPLC has also proven to be an ideal system for measuring hydrophobicity and amphipathicity of α-helical and β-sheet molecules [4,29–33] promising to provide critical information in the process for rational de novo design of α-helical and β-sheet antimicrobial peptides. The significance of these applications lies in the fact that the original purpose for method development of a particular mode of HPLC was transcended by its employment in a different field, where it became not just a means to an end (i.e. merely a purification tool), but an end in itself.

In addition to its growing role as a useful probe of protein structure, RP-HPLC is also likely to be a good model for ligand–receptor interactions since, in a similar manner to their importance in folding and stabilization of proteins, hydrophobic interactions also play a key role in the binding of ligands to their receptors. We have previously described the design and development of a simple model ligand–receptor system based on observing the retention behaviour of de novo designed single-stranded amphipathic α-helical peptides representing peptide ligands binding to a complementary receptor (RP-HPLC stationary phase) [34]. In this initial appraisal of the model system, it was 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. These results were obtained by varying only one surface of the ligand–receptor model, i.e. the hydrophobic surface of the ligand, represented by the hydrophobic face of the synthetic amphipathic α-helical peptide analogues, was varied, while the non-variable hydrophobic surface of the receptor was represented by the C_8 stationary phase of an RP-HPLC column.

Much of the efficacy of RP-HPLC, both for purification purposes and in the aforementioned novel applications, lies in the wealth of stationary phases and/or mobile phase conditions open to the researcher. Thus, in the present study, we extend our earlier work [34] by examining the effect on side-chain hydrophilicity/hydrophobicity in the center of the non-polar face of a peptide ligand (represented by a model amphipathic α-helical peptide) of varying both the non-polar surface of a model receptor (represented by RP-HPLC packings by varying hydrophobicity) as well as the aqueous environment (represented by the RP-HPLC mobile phase) surrounding the ligand–receptor binding site.

2. Experimental

2.1. Materials

HPLC-grade water and acetonitrile were obtained from BDH (Poole, UK). ACS-grade orthophosphoric acid was obtained from Anachemia (Toronto, Canada). Trifluoroacetic acid (TFA) was obtained from Aldrich (Milwaukee, WI, USA). Sodium perchlorate ($NaClO_4$) was obtained from BDH.

2.2. Instrumentation

Peptide synthesis was carried out on an Applied

Biosystems peptide synthesizer Model 430A (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 HP1040A 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 analyzer (Beckman Instruments, Fullerton, CA, USA).

The correct primary ion molecular masses of peptides were confirmed by electrospray mass spectrometry on a Fisons Quattro (Fisons, Pointe-Claire, Canada).

2.3. Peptide synthesis

Amphipathic α-helical peptides, their hydrophobic faces representing the hydrophobic face of the ligand in the ligand–receptor model, 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 [28]. The cleaved peptide–resin mixtures were washed with diethyl ether (3×25 ml) and the peptides extracted with neat acetic acid (3×25 ml) [28]. The resulting peptide solutions were then lyophilized prior to purification.

2.4. Columns

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 A–B 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.

Two RP-HPLC analytical columns were employed to represent the hydrophobic surface of the receptor in the ligand–receptor model: (1) Zorbax SB-CN (diisopropyl-3-cyanopropyl bonded phase) (150×4.6 mm I.D., 5-μm, particle size, 100-Å pore size); (2) Zorbax 300SB-C_{18} (diisobutyl-*n*-octadecyl bonded phase) (150×4.6 mm I.D., 3.5 μm, 300 Å). Both columns were obtained from Agilent Technologies (Newport, DE, USA).

2.5. Conditions

Three sets of RP-HPLC mobile phases were employed: mobile phase 1, where eluent A is 10 m*M* aqueous orthophosphoric acid, pH 2.2, and eluent B is 10 m*M* orthophosphoric acid in acetonitrile (to be referred to as our phosphoric acid low ionic strength mobile phase, with a hydrophilic anionic phosphate counterion); mobile phase 2, where eluent A is 10 m*M* aqueous orthophosphoric acid, pH 2.2, containing 100 m*M* sodium perchlorate and eluent B is 10 m*M* aqueous orthophosphoric acid in 60% (v/v) aqueous acetonitrile containing 100 m*M* sodium perchlorate (to be referred to as our phosphoric acid high ionic strength mobile phase, with hydrophilic anionic phosphate and perchlorate counterions); and mobile phase 3, where eluent A is 10 m*M* aqueous TFA, pH 2.0, and eluent B is 10 m*M* TFA in acetonitrile (to be referred to as our TFA mobile phase, with a more hydrophobic anionic trifluoroacetate counterion). Peptides were eluted by a linear A–B gradient (1% acetonitrile/min) at a flow-rate of 1 ml/min and a temperature of 25 °C.

3. Results and discussion

3.1. Design of ligand–receptor model system

Although a detailed description of our approach to the design of a ligand–receptor model system, including the choice of using synthetic amphipathic α-helical peptides has been described previously [34], some aspects of this model need to be reiterated for the present study concerning the choice of model peptide analogues as well as the stationary and mobile phases.

3.1.1. Design of model synthetic amphipathic α-helical peptides (model ligands)

Fig. 1 (top) shows the sequence of our model 18-residue peptide ligands, known to have a high potential to form amphipathic α-helices [34,35]. The periodic distribution of hydrophobic residues along the polypeptide chain (X–N–X–X–N–N–X–X–N–X–X–N–N–X–X–N–X–X) (where N denotes a non-polar residue, the very hydrophobic leucine [36] in this case) ensures a wide hydrophobic face on the amphipathic helix, with hydrophobic residues at positions 2, 5, 6, 12, 13 and 16 surrounding position 9, which is substituted by all 20 amino acids. Thus, the residue substituted at position 9, in the center of the non-polar face, is surrounded by a very hydrophobic environment of leucine residues. Helical net representations of the ''native'' Leu-face peptide, denoted LL, is shown in Fig. 1 (bottom).

The general denotion of this Leu-face series is LX (Fig. 1, top), with X referring to the central residue at position 9, hence the denotion of the ''native'' peptide with leucine at this position as LL (Fig. 1); with glycine at this position, it is denoted LG, etc.

The high and similar α-helicities of this series of peptide analogues, determined by circular dichroism spectroscopy (CD) (in 50% trifluoroethanol), and the considerable amphipathic character of these peptides have been reported previously [34]. Only the proline-substituted analogue, LP, showed relatively low helical character, which was not surprising considering the known helix-disrupting tendency of this residue. Thus, the LP peptide was not included in this study.

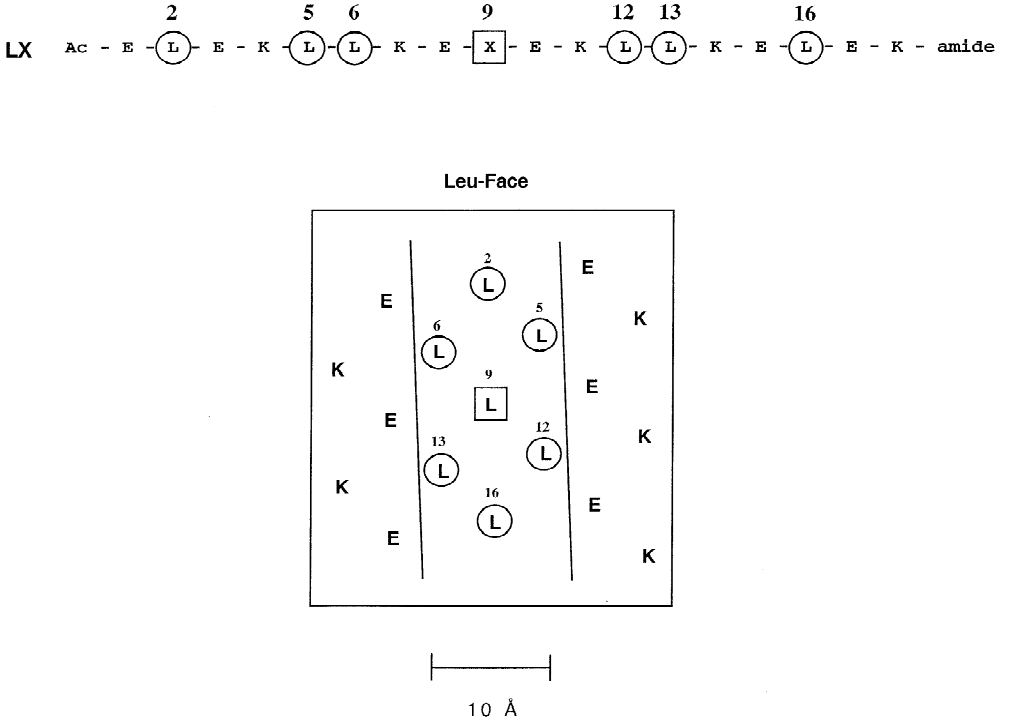

Fig. 1. Design of model synthetic peptides. Top: sequence of mutant peptides, denoted LX series, where the first letter represents amino acid residues used in the hydrophobic face of the peptide and the X represents each of the 20 amino acids (boxed) (single letter code given in the tables) substituted at position 9. The residues that are circled or boxed and labeled 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'' Leu-face (LL) model peptide represented as α-helical net. 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 lines on the α-helical net represents the hydrophobic face of the peptide.

3.1.2. Hydrophobic stationary phases (model receptors)

Silica-based C_{18} and cyano stationary phases were chosen to represent receptors of differing hydrophobicity for the following reasons: (1) the stability (particularly at low pH) and efficiency of such columns, particularly the Stablebond columns used in the present study, makes them particularly advantageous for peptide separations [37–41]; and (2) the C_{18} and cyano functionalities offered a wide range of stationary phase hydrophobicity (in order of increasing hydrophobicity, cyano$<C_{18}$).

3.1.3. RP-HPLC of amphipathic α-helices

On binding to a reversed-phase column, the hydrophobicity of the stationary phase stabilizes secondary (α-helical) structure. Indeed, Zhou et al. [42] demonstrated that amphipathic peptides remain α-helical when bound to a reversed-phase column and, due to the preferred binding domain created by the nonpolar face of the α-helix, are considerably more retentive than peptides of the same composition but lacking the preferred binding domain.

Our previous study [34] demonstrated that the peptides interact with the column matrix as single-stranded amphipathic α-helices during RP-HPLC, interacting with the stationary phase through preferential binding with their hydrophobic faces. The position of the substituted side-chain at position 9 of the peptides, i.e. in the center of the hydrophobic face binding preferentially to the reversed-phase packing, ensures that it is buried between the hydrophobic face of the ligand (peptide) and that of the receptor (stationary phase).

For the present study, a control set of model peptide analogues, with negligible secondary structure [28,42], was also subjected to the same RP-HPLC runs as were applied to the amphipathic α-helical model peptides. The sequence of these ''random coil'' peptides is: Ac–X–Leu–Gly–Ala–Lys–Gly–Ala–Gly–Val–Gly–amide, where X is substituted by the 20 amino acids found in proteins.

3.1.4. RP-HPLC mobile phases

The three mobile phases (mobile phases 1, 2 and 3, described in the Experimental section) were chosen to examine how the aqueous environment surrounding the binding site of the ligand and receptor affects the hydrophilicity/hydrophobicity of amino acids in the binding interface.

3.2. Effect of increasing hydrophobicity of receptor on hydrophilicity/hydrophobicity of side-chains at the ligand–receptor interface: constant ligand hydrophobicity

Table 1 summarizes the reversed-phase retention behaviour of the LX series of peptide analogues (with the exception of the proline analogue, LP), run with mobile phase 1 (phosphoric acid low ionic strength mobile phase; see Experimental) on cyano and C_{18} columns. Table 2 summarizes the retention behaviour of the analogues when run with mobile phase 2 (phosphoric acid high ionic strength mobile phase; see Experimental). In general, results obtained with mobile phase 3 (TFA mobile phase; see Experimental) were similar to those obtained with mobile phase 1. Thus, for the sake of clarity only the results obtained from mobile phases 1 and 2, which differ significantly in ionic strength, will be discussed in detail.

The order of amino acid substitutions shown in Tables 1 and 2 (and subsequent tables) was generally based on decreasing retention times of the Leu-face mutants (t_R, LX) under mobile phase 1 conditions on the cyano column, starting with the highest retention time for the isoleucine-substituted analogue (LI; 44.61 min) and ending with the least retained lysine-substituted analogue (LK; 32.01 min). The only exception (save that of the Gly analogue which represented an internal standard in each mixture of peptides applied to RP-HPLC) is that of the phenylalanine-substituted analogue (LF; 43.27 min), which is placed as shown due to a secondary grouping of side-chains into various categories. Thus, Ile and Val contain hydrophobic, β-branched aliphatic side-chains; Leu, Cys, Met and Ala were classed as having hydrophobic, aliphatic side chains; Phe, Trp and Tyr contain aromatic side-chains; Thr and Ser contain polar hydroxyl groups; Glu and Asp contain (at pH 2) uncharged carboxyl groups; Gln and Asn contain polar side-chain amide groups; and Arg, His and Lys all exhibit, at pH 2, a full positive charge on their side-chains. In this way, it was hoped to identify any effects which may be common to a group of side-chains with similar characteristics.

Table 1
Effect of increasing hydrophobicity of receptor on hydrophilicity/hydrophobicity of side-chains in the substitution site of the ligand: mobile phase 1 (phosphoric acid low ionic strength mobile phase)

Amino acid substitution	Cyano column[a]		C_{18} column[a]		$\Delta\Delta t_R$[d] (min) (C_{18} minus cyano)
	t_R, LX (min)[b]	Δt_R, LX–LG (min)[c]	t_R, LX (min)[b]	Δt_R, LX–LG (min)[c]	
Ile(I)	44.61	+4.16	49.76	+4.59	+0.43
Val (V)	44.22	+3.77	49.05	+3.88	+0.11
Leu (L)	43.94	+3.50	49.62	+4.42	+0.92
Cys (C)	42.95	+2.50	47.19	+2.14	−0.36
Met (M)	42.57	+2.13	47.25	+2.05	−0.08
Ala (A)	42.39	+1.94	47.05	+1.88	−0.06
Phe (F)	43.27	+2.83	48.69	+3.49	+0.66
Trp (W)	42.12	+1.68	46.12	+0.92	−0.76
Tyr (Y)	39.83	−0.62	42.84	−2.36	−1.77
Thr (T)	41.11	+0.66	44.47	−0.70	−1.36
Ser (S)	39.93	−0.52	43.20	−1.97	−1.45
Glu (E)	37.83	−2.61	39.88	−5.32	−2.71
Asp (D)	37.48	−2.91	39.42	−5.75	−2.84
Gln (Q)	37.23	−3.21	38.23	−6.97	−3.76
Asn (N)	36.74	−3.71	38.28	−6.89	−3.18
Arg (R)	33.20	−7.08	34.08	−10.97	−3.98
His (H)	33.18	−7.27	34.38	−10.79	−3.52
Lys (K)	32.01	−8.44	33.62	−11.55	−3.11
Gly (G)	40.39±0.07	–	45.14±0.07	–	–

[a] Descriptions of columns can be found in Experimental.
[b] Mobile phase 1 (see Experimental) was used; LX refers to the observed retention times (t_R) of the Leu-face analogues; the sequences of the peptides are shown in Fig. 1.
[c] Δt_R refers to the retention time differences between the mutant peptide and the Gly-substituted peptide (LG); the t_R values for the Gly analogue represent the average of three runs, since the peptide was used as an internal standard for mixtures of the other peptides.
[d] $\Delta\Delta t_R$=(Δt_R, LX–LG on C_{18} column)−(Δt_R, LX–LG on cyano column).

This order of amino acids was subsequently retained for all tables.

The retention times of the glycine analogue were now subtracted from the retention times of the other 18 analogues (Δt_R, LX-LG in Tables 1 and 2). The resulting values represent hydrophilicity/hydrophobicity values of the side-chains in the hydrophobic face of the amphipathic peptide ligand determined relative to Gly; the Gly analogue, LG, represents the situation where there is no side-chain present at position 9 (see Fig. 1). Similarly, hydrophilicity/hydrophobicity values of side-chains in the control random coil peptides were also determined relative to the Gly analogue of this peptide series. Plotting the Δt_R, LX–LG values obtained from the C_{18} column against those obtained from the CN column produced the plot shown in Fig. 2A. From Fig. 2A, there is a good correlation between the two sets of data (r=0.997), suggesting that though the magnitude of the hydrophilicity/hydrophobicity values for the side-chains (as expressed by their Δt_R, LX–LG values) are different between the two columns, the directional effect on all side-chains is similar when changing the hydrophobicity of the model receptor. Although the results from mobile phase 2 conditions are shown in Fig. 2, those obtained from mobile phase 1 were essentially identical.

When the Δt_R values obtained from the cyano column are subtracted from the Δt_R values obtained from the C_{18} column, another expression (denoted $\Delta\Delta t_R$ in Tables 1 and 2) is obtained. Thus, $\Delta\Delta t_R$= (Δt_R, LX–LG on C_{18} column)−(Δt_R, LX–LG on cyano column) and represents the effect of increasing surface hydrophobicity of the receptor on hydrophilicity/hydrophobicity of side-chains in the substitution site of the ligand. Similar calculations were

Table 2
Effect of increasing hydrophobicity of receptor on hydrophilicity/hydrophobicity of side-chains in the substitution site of the ligand: mobile phase 2 (phosphoric acid high ionic strength mobile phase)

Amino acid substitution	Cyano column[a]		C_{18} column[a]		$\Delta\Delta t_R$[d] (min) (C_{18} minus cyano)
	t_R, LX (min)[b]	Δt_R, LX–LG (min)[c]	t_R, LX (min)[b]	Δt_R, LX–LG (min)[c]	
Ile(I)	54.02	+4.57	60.12	+5.72	+1.15
Val (V)	53.53	+4.08	59.31	+4.90	+0.82
Leu (L)	53.28	+3.90	60.02	+5.68	+1.78
Cys (C)	52.12	+2.74	56.74	+2.45	−0.29
Met (M)	51.71	+2.28	56.72	+2.37	+0.09
Ala (A)	51.32	+1.88	56.68	+2.28	+0.40
Phe (F)	52.38	+2.96	58.54	+4.19	+1.23
Trp (W)	51.08	+1.65	54.86	+0.51	−1.14
Tyr (Y)	48.53	−0.90	50.82	−3.53	−2.63
Thr (T)	50.18	+0.73	53.50	−0.90	−1.63
Ser (S)	48.92	−0.53	51.83	−2.58	−2.63
Glu (E)	46.52	−2.91	47.11	−7.23	−4.32
Asp (D)	46.49	−2.96	46.98	−7.42	−4.46
Gln (Q)	45.91	−3.52	45.28	−9.07	−5.55
Asn (N)	45.79	−3.66	45.74	−8.66	−5.00
Arg (R)	44.17	−5.20	42.47	−11.82	−6.62
His (H)	44.43	−5.01	43.13	−11.27	−6.26
Lys (K)	43.15	−6.30	41.79	−12.62	−6.32
Gly (G)	49.42±0.03	–	54.35±0.05	–	–

[a] Descriptions of columns can be found in Experimental.
[b] Mobile phase 2 (see Experimental) was used; t_R, LX refers to the observed retention times of the Leu-face analogues; the sequences of the peptides are shown in Fig. 1.
[c] Δt_R refers to the retention time differences between the mutant peptide and the Gly-substituted peptide (LG); the t_R values for the Gly analogue represent the average of three runs, since the peptide was used as an internal standard for mixtures of the other peptides.
[d] $\Delta\Delta t_R$=(Δt_R, LX–LG on C_{18} column)−(Δt_R, LX–LG on cyano column).

also carried out for the control random coil peptides. The $\Delta\Delta t_R$ values obtained from all three mobile phases (mobile phases 1, 2 and 3; see Experimental) for both the LX peptide analogues and the control random coil peptides are compared in Fig. 3.

From Fig. 3, it is clear that an increase in receptor hydrophobicity (column surface hydrophobicity) enhances hydrophilicity of uncharged polar (Thr, Ser, Gln, Asn, Glu, Asp) and positively charged (Arg, Lys, His) amino acid side-chains on the hydrophobic surface of a preferred binding domain of an amphipathic α-helical peptide ligand. Thus, as the receptor surface becomes more hydrophobic, a hydrophilic amino acid side-chain buried in the ligand–receptor interface becomes dramatically more hydrophilic. This increase in polar side-chain hydrophilicity with increasing receptor hydrophobicity can also be seen in Table 3 where these increases ranged from a 1.4-fold (mobile phase 1) or 2.0-fold (mobile phase 2) in hydrophilicity for Lys up to a 3.8-fold (mobile phase 1) or 4.9-fold (mobile phase 2) for Ser.

An important observation from Fig. 3 is that the $\Delta\Delta t_R$ values for the polar side-chains appear to be dependent on the hydrophilicity of the side-chains, i.e. the more hydrophilic the side-chain (as characterized by the Δt_R, LX–LG values on the C_{18} column; Tables 1 and 2), the greater the $\Delta\Delta t_R$ value, i.e. the greater the effect of increasing receptor hydrophobicity on the hydrophilicity of the side-chain on the face of the ligand. This is most easily visualized by looking at the effects of receptor hydrophobicity on groups of similar side-chains with mobile phase 2 as the aqueous environment. Thus, the Thr and Ser side-chains, with Δt_R, LX–LG values of −0.9 and −2.58 min, respectively (Table 2) exhibit $\Delta\Delta t_R$

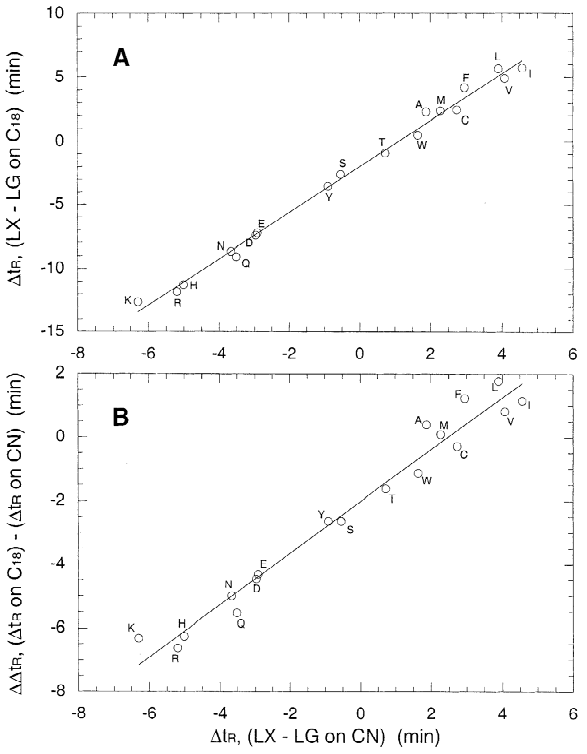

Fig. 2. Plot Δt_R, (LX–LG) on C_{18} column vs. Δt_R, (LX–LG) on cyano column (A) and $\Delta\Delta t_R$, (Δt_R, [LX–LG] on C_{18} minus Δt_R, [LX–LG] on cyano) vs. Δt_R, (LX–LG) on the cyano column (B). Data are taken from Table 2 (mobile phase 2). The single letter code represents the amino acid substitution at position 9 of the peptide sequence (see Fig. 1).

values ranging from −1.63 to −2.63 min (Table 2, Fig. 3); in comparison, the Glu and Asp side-chains with much higher Δt_R values or higher hydrophilicity (Δt_R, LX–LG values of −7.23 and −7.42 min, respectively) exhibit $\Delta\Delta t_R$ values from −4.32 to −4.46 min; similarly the Gln and Asn side-chains (Δt_R, LX–LG values of −9.07 and −8.66 min, respectively) exhibit $\Delta\Delta t_R$ values from −5.55 to −5.0 min; and finally, the positively charged side-chains (Arg, Lys, His; Δt_R, LX–LG values ranging from −11.27 to −12.62 min) exhibit the highest $\Delta\Delta t_R$ values ranging from −6.26 to −6.62 min. Indeed, Fig. 2B demonstrates a clear correlation (r=0.995) between side-chain hydrophilicity/hydrophobicity values (Δt_R, LX–LG values on the C_{18}) and the magnitude ($\Delta\Delta t_R$) of the effect of increasing receptor hydrophobicity (CN→C_{18}) on these values.

From Fig. 3, the $\Delta\Delta t_R$ values for the control random coil peptides containing polar side-chain substituents were either negligible or relatively small compared to results obtained with the amphiphatic LX series of peptides. In addition, they do not show the correlation between side-chain hydrophilicity and the $\Delta\Delta t_R$ values exhibited by side-chains in the center of the model amphipathic α-helical ligand. These peptides lack a preferred binding domain and, thus, the residues substituted at position 1 of this decapeptide series are not situated at the center of a controlled ligand–receptor interface.

In contrast to the polar residues, the effect of increasing receptor hydrophobicity on the hydrophilicity/hydrophobicity of hydrophobic side-chains (Leu, Met, Cys, Ala, Ile, Val) in the ligand face was generally much smaller (Fig. 3, Table 3); the effect on the Met, Cys and Ala side-chains, in particular, was small and/or of similar magnitude to their corresponding random coil control peptides. Again in contrast to the polar residues, the hydrophobic residues Leu, Ile, Val and Phe showed relatively more *hydrophobic* character as the receptor became more hydrophobic [$\Delta\Delta t_R$ values for Leu, Ile, Val and Phe of +0.92, +0.43, +0.11 and +0.66, respectively for mobile phase 1 (Table 1); $\Delta\Delta t_R$ values of +1.78, +1.15, +0.82 and +1.23, respectively, for mobile phase 2 (Table 2)], as opposed to the tendency of the polar residues to become more *hydrophilic*. Table 3 also shows the relative increase in hydrophobicity of these hydrophobes as receptor hydrophobicity increases. Finally, it is worth noting that the four side-chains in this group affected the most by the increase in receptor hydrophobicity (Leu, Ile, Val, Phe) are also the four most hydrophobic residues (see $\Delta\Delta t_R$, LX–LG values for these residues in Table 1 and Table 2). However, the relative increase in hydrophobicity of these four residues (1.1- to 1.5-fold) is small compared to the relative increase in hydrophilicity for the other amino acids (1.4- to 4.9-fold; Table 3).

At first glance, the aromatic side-chains of Tyr and Trp appear to be exhibiting some anomalous behaviour, in that they are responding to increasing receptor hydrophobicity in a manner similar to the polar side-chains, i.e. they exhibit negative $\Delta\Delta t_R$ values. Generally, the aromatic amino acids have been classed as hydrophobic in terms of their

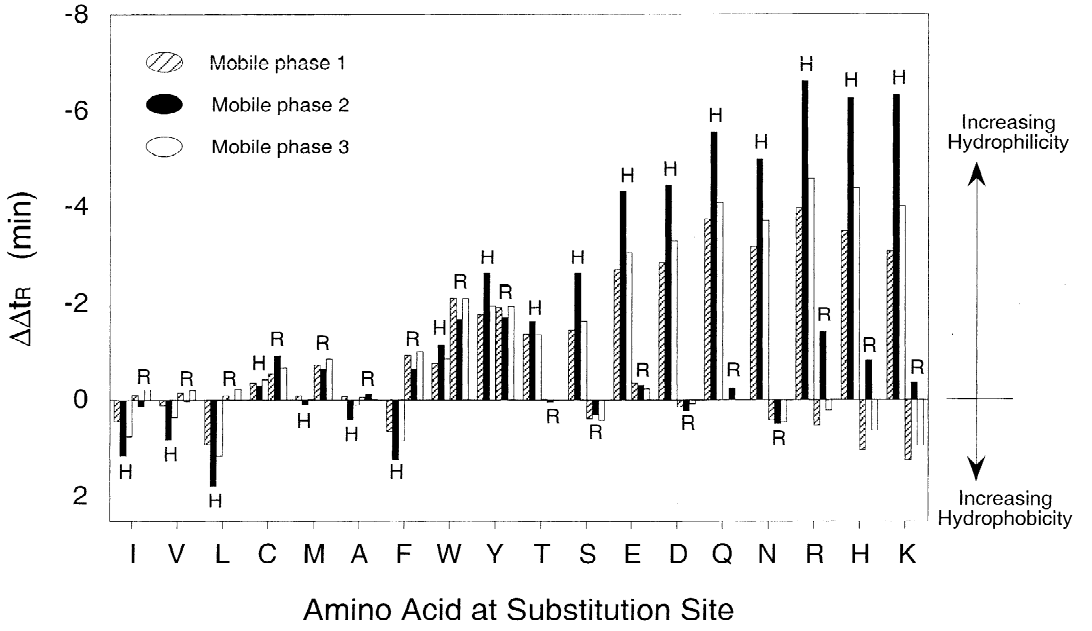

Fig. 3. Effect of varying receptor hydrophobicity on hydrophilicity/hydrophobicity of side-chains substituted in the non-polar face of a model amphipathic α-helical peptide ligand compared to the same side-chain substituted in a random coil peptide. Mobile phases 1, 2 and 3 are described in Experimental. For each side-chain, the three histograms on the left denoted by H (helical ligand) are results obtained from the amphipathic α-helical peptide ligand model system (denoted LX peptides; sequence shown in Fig. 1) and the three on the right denoted by R (random coil ligand) are results obtained from the control random coil peptides (denoted Ac–X series). $\Delta\Delta t_R = \Delta t_R$, (LX–LG) on the C_{18} column minus Δt_R, (LX–LG) on the cyano column (data shown in Tables 1 and 2) for the LX analogues; $\Delta\Delta t_R = \Delta t_R$, (Ac–X–Ac–G) on the C_{18} column minus Δt_R, (Ac–X–Ac–G) on the cyano column for the random coil analogues (sequence shown in text). The single letter code represents the amino acid substitution at position 9 of the peptide sequence (Fig. 1).

retention behaviour during RP-HPLC [36]. Indeed, the Phe side-chain on the hydrophobic face of the amphipathic peptide ligand is becoming more hydrophobic (i.e. it has positive $\Delta\Delta t_R$ values of 0.66 and 1.23 min; Tables 1 and 2, respectively) with increasing receptor hydrophobicity, in a manner similar to hydrophobic side-chains such as Leu, Ile and Val. However, as noted above, Trp and, particularly, Tyr are clearly exhibiting negative $\Delta\Delta t_R$ values (−0.76 and −1.77 min, respectively in Table 1; −1.14 and −2.63 min, respectively in Table 2). Unlike Phe, the side-chains of Tyr and Trp do possess some polar characteristics (side-chain hydroxyl and N–H groups, respectively), in addition to the innate hydrophobicity of the aromatic ring structure. Indeed, from Tables 1 and 2, it can be seen that, in the hydrophobic environment surrounding the mutation site at position 9 of the peptide ligand, the Tyr side-chain is exhibiting hydrophilic characteristics; thus, the Δt_R, LY–LG values of −2.36 and −3.53 min (Tables 1 and 2, respectively) on the C_{18} column are comparable to that of Ser (−1.97 and −2.58 min in Tables 1 and 2, respectively) and the negative $\Delta\Delta t_R$ values of these two residues are also quite similar. In terms of amphipathic peptide ligands, Trp is perhaps the only side-chain showing some anomalous behaviour; thus, although it has Δt_R, LW–LG values on the C_{18} column of +0.92 min (Table 1) and +0.51 min (Table 2) (i.e. it is exhibiting moderately hydrophobic characteristics in the hydrophobic environment of the peptide ligand), it has negative (if small) $\Delta\Delta t_R$ values of −0.76 min (Table 1), and −1.14 min (Table 2) in going from the cyano to the C_{18} column (i.e. it is acting in a manner similar to the more hydrophilic side-chains).

Some insight into the behaviour of the Trp side-chain may be gained by examining the $\Delta\Delta t_R$ values of the control random coil peptides containing

Table 3
Relative increases in hydrophilicity or hydrophobicity of amino acid side-chains in the hydrophobic face of a peptide ligand as the receptor surface becomes more hydrophobic[a]

Amino acid substitution	Relative increase in hydrophilicity[b]		Relative increase in hydrophobicity[b]	
	Mobile phase 1[c]	Mobile phase 2[c]	Mobile phase 1[c]	Mobile phase 2[c]
Ile(I)	–	–	1.1	1.3
Val (V)	–	–	1.0	1.2
Leu (L)	–	–	1.3	1.5
Cys (C)	1.2	–	–	–
Met (M)	1.0	–	–	1.0
Ala (A)	1.0	1.1	–	1.2
Phe (F)	–	–	1.2	1.4
Trp (W)	1.8	3.2	–	–
Tyr (Y)	3.8	3.9	–	–
Thr (T)	2.1	2.2	–	–
Ser (S)	3.8	4.9	–	–
Glu (E)	2.0	2.5	–	–
Asp (D)	2.0	2.5	–	–
Gln (Q)	2.2	2.6	–	–
Asn (N)	1.9	2.4	–	–
Arg (R)	1.5	2.3	–	–
His (H)	1.5	2.3	–	–
Lys (K)	1.4	2.0	–	–

These results are adapted from those shown in Table 1 (mobile phase 1) and Table 2 (mobile phase 2).

[a] Refers to amino acid side-chains substituted at position 9 of the model peptide ligand (LX series; see Fig. 1 for sequence).

[b] Refers to the increase in hydrophilicity or hydrophobicity of an amino acid side-chain on the hydrophobic face of the peptide ligand as the receptor surface hydrophobicity is increased, i.e. cyano to C_{18} column. For example, from Table 1, the increase in *hydrophobicity* of the Ile side-chain is calculated by (Δt_R, LI–LG on C_{18})/(Δt_R, LI–LG on cyano column)=+4.59/+4.16=1.10, i.e. a 1.1-fold increase in hydrophobicity; similarly, the increase in *hydrophilicity* of the Ser side-chain is calculated by (Δt_R, LS–LG on C_{18})/(Δt_R, LS–LG on cyano column)=−1.97/−0.52=3.78, i.e. a 3.8-fold increase in hydrophilicity. A decrease in hydrophobicity for a side-chain is also an increase in hydrophilicity, e.g. from Table 1, the decrease in hydrophobicity (i.e. increase in *hydrophilicity*) of the Trp side-chain is calculated by (Δt_R, LW–LG on cyano column)/(Δt_R, LW–LG on C_{18} column)=+1.68/+0.92=1.82, i.e. a 1.8-fold increase in hydrophilicity; similarly, the decrease in hydrophobicity (i.e. increase in *hydrophilicity*) of the Thr side-chain is calculated by (Δt_R, LT–LG on cyano column+$|\Delta t_R$, LT–LG on $C_{18}|$)/(Δt_R, LT–LG on cyano column)=+0.66+|−0.70|/+0.66=+1.36/0.66=2.06, i.e. a 2.1-fold increase in hydrophilicity. A value of 1.0 represents no change in relative hydrophilicity/hydrophobicity between the two columns.

[c] Mobile phase 1 and mobile phase 2 are described in Experimental.

aromatic amino acid substitutions at position 1. The two random coil peptide analogues showed $\Delta\Delta t_R$ values of similar (Tyr) or greater (Trp) magnitude than the model ligand analogues. In their comparison of cyano vs. a C_8 column, Zhou et al. [38] demonstrated significant selectivity differences between these two functional groups for specific peptide separations, including between Trp- and Phe-substituted octapeptide analogues with negligible secondary structure. Thus, any apparent anomalous behaviour exhibited by the aromatic side-chains in Fig. 3, particularly when substituted in the random coil controls, may simply be a reflection of subtle selectivity differences between the cyano and C_{18} groups for these amino acids.

3.3. Effect of aqueous environment on hydrophilicity/hydrophobicity of side-chains at the ligand–receptor interface: constant ligand hydrophobicity

A point of interest when comparing the data shown in Tables 1 and 2 concerns the relative retention times of peptides on the cyano and C_{18} columns when run in different mobile phase environments. Thus, from Table 1, all 19 peptides shown are

eluted later on the C_{18} column compared to the cyano column, as may have been expected considering the lesser hydrophobic character of the latter packing. In contrast, from Table 2, the Gln, Asn, Arg, Lys and His analogues are now eluted earlier on the C_{18} column compared to the cyano column in the presence of 100 m*M* sodium perchlorate. This reversal in elution was not observed with the random coil peptides. This result appeared to stem from the considerable enhancement in relative hydrophilicity of these residues (see Discussion below), situated at the hydrophobic ligand–receptor interface, in the salt-containing environment (Tables 2 and 3), which presumably was able to more than compensate for the overall greater hydrophobicity of the C_{18} stationary phase. Note that all peptides are eluted later on the two columns in mobile phase 2 (Table 2) compared to mobile phase 1 (Table 1). On the cyano column, the retention times range from 32 to 44 min in the absence of salt and 43 to 54 min in the presence of salt, showing an increase in hydrophobicity due to salt of ~10 min. Similarly, the same effect is observed on the C_{18} column where retention times range from 33 to 49 min in the absence of salt and 42 to 60 min in the presence of salt, showing an increase in hydrophobicity due to salt of ~11 min. This increase in peptide retention times observed on addition of salt to the mobile phase is analogous to an increase in affinity of a ligand and its receptor. Thus, the buried hydrophilic side-chains become substantially more hydrophilic when the ligand–receptor is in an aqueous environment of higher ionic strength.

It is clear from Fig. 3 that the aqueous environment surrounding the binding site of ligand and receptor affects considerably the hydrophilicity/hydrophobicity of amino acids in the binding interface. Thus, the most dramatic effects on the changes in side-chain hydrophilicity/hydrophobicity effected by increasing receptor hydrophobicity were seen following the addition of salt (100 m*M* sodium perchlorate; mobile phase 2) to the mobile phase which further enhanced interactions between ligand and receptor. This enhancement is reflected in Table 3 by comparing the relative increases in side-chain *hydrophilicity* between mobile phase 1 and mobile phase 2 as the receptor surface becomes more hydrophobic. Thus, there was a 2-fold increase in hydrophilicity for Lys up to a 4.9-fold increase for Ser in mobile phase 2 compared to lesser values of 1.4- and 3.8-fold for Lys and Ser, respectively, in mobile phase 1. In contrast to the polar and charged residues, the relative increases in side-chain *hydrophobicity* of hydrophobic residues at the substitution site in the centre of the non-polar face of the ligand were generally of lesser magnitude and changed little with the addition of salt. Note that Met and Ala are showing a relative increase in *hydrophobicity* (1.0- and 1.2-fold, respectively) in the presence of salt (mobile phase 2) compared to a relative increase in *hydrophilicity* (both 1.0-fold) in mobile phase 1.

Table 4 now summarizes the hydrophobicity of each side-chain at the substitution site (Δt_R, LX–LG) on the cyano and C_{18} columns in mobile phases 1 and 2. The effect of 100 m*M* sodium perchlorate on the hydrophilicity/hydrophobicity of the side-chains ($\Delta\Delta t_R$) on both columns can then be expressed as $\Delta\Delta t_R = \Delta t_R$, (LX–LG) in mobile phase 2 minus Δt_R, (LX–LG) in mobile phase 1. Such values allow another way of examining the effect of changing or increasing the hydrophobicity of the receptor, since they quantify the change in hydrophilicity/hydrophobicity of amino acid side-chains substituted in the centre of the non-polar face of the peptide ligand when varying the environment around the ligand–receptor interface, the magnitude of such an effect depending on the hydrophobicity of the receptor. When the $\Delta\Delta t_R$ values obtained in mobile phase 2 are plotted against those obtained in mobile phase 1, there is an excellent correlation of the respective series of data ($r = 0.991$) (Fig. 4), showing that, although the hydrophobicity change of the receptor has different effects depending on the mobile phase environment, these effects on hydrophilicity/hydrophobicity of amino acid side-chains are proportional regardless of mobile phase composition.

From Table 4, on the cyano column, where the receptor hydrophobicity is low, the addition of salt to the mobile phase had only small effects on the hydrophilicity/hydrophobicity of the side-chains with the notable exception of the effect on the basic side-chains Arg, His and Lys (Table 4) (an average $\Delta\Delta t_R$ magnitude of only 0.19 when excluding these basic side-chains). Fig. 5A shows a reasonable correlation of the hydrophilicity/hydrophobicity of the side-chains, with the exception of the basic side-

Table 4
Effect on the relative hydrophilicity/hydrophobicity of amino acid side-chains of changing the aqueous environment surrounding the ligand–receptor interface

Amino acid substitution	Cyano column[a]		$\Delta\Delta t_R$ (min)[d] (mobile phase 2 minus mobile phase 1)	C_{18} column[a]		$\Delta\Delta t_R$ (min)[d] (mobile phase 2 minus mobile phase 1)
	Mobile phase 1[b] Δt_R, LX–LG[c] (min)	Mobile phase 2[b] Δt_R, LX–LG[c] (min)		Mobile phase 1[b] Δt_R, LX–LG[c] (min)	Mobile phase 2[b] Δt_R, LX–LG[c] (min)	
Ile(I)	+4.16	+4.57	+0.41	+4.59	+5.72	+1.13
Val (V)	+3.77	+4.08	+0.31	+3.88	+4.90	+1.02
Leu (L)	+3.50	+3.90	+0.40	+4.42	+5.68	+1.26
Cys (C)	+2.50	+2.74	+0.24	+2.14	+2.45	+0.31
Met (M)	+2.13	+2.28	+0.15	+2.05	+2.37	+0.32
Ala (A)	+1.94	+1.88	−0.06	+1.88	+2.28	+0.40
Phe (F)	+2.83	+2.96	+0.13	+3.49	+4.19	+0.70
Trp (W)	+1.68	+1.65	−0.03	+0.92	+0.51	−0.41
Tyr (Y)	−0.62	−0.90	−0.28	−2.36	−3.53	−1.17
Thr (T)	+0.66	+0.73	+0.07	−0.70	−0.90	−0.20
Ser (S)	−0.52	−0.53	−0.01	−1.97	−2.58	−0.61
Glu (E)	−2.61	−2.91	−0.30	−5.32	−7.23	−1.91
Asp (D)	−2.91	−2.96	−0.05	−5.75	−7.42	−1.67
Gln (Q)	−3.21	−3.52	−0.31	−6.97	−9.07	−2.10
Asn (N)	−3.71	−3.66	+0.05	−6.89	−8.66	−1.77
Arg (R)	−7.08	−5.20	+1.88	−10.97	−11.82	−0.85
His (H)	−7.27	−5.01	+2.26	−10.79	−11.27	−0.48
Lys (K)	−8.44	−6.30	+2.14	−11.55	−12.62	−1.07

[a] Descriptions of columns can be found in Experimental.
[b] Full descriptions of mobile phase 1 (aqueous 10 m*M* orthophosphoric acid–acetonitrile) and mobile phase 2 (same as mobile phase 1 except for the addition of 100 m*M* sodium perchlorate) can be found in Experimental.
[c] Δt_R refers to the retention time differences between the mutant peptide and the Gly-substituted peptide (LG).
[d] $\Delta\Delta t_R = (\Delta t_R$, LX–LG in mobile phase 2$) - (\Delta t_R$, LX–LG in mobile phase 1).

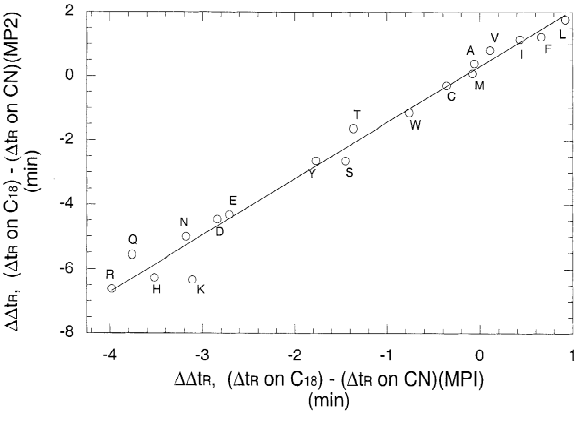

Fig. 4. Plot of $\Delta\Delta t_R$, (Δt_R, [LX–LG] on C_{18} minus Δt_R, [LX–LG] on cyano) in mobile phase 2 (MP2) vs. $\Delta\Delta t_R$ in mobile phase 1 (MP1). Data are taken from Table 1 (MP1) and Table 2 (MP2). The single letter code represents the amino acid substitution at position 9 of the peptide sequence (Fig. 1).

chains, in the two mobile phases considering the generally small differences in $\Delta\Delta t_R$ values. The indication that salt in the mobile phase increases the hydrophobicity disproportionately for these three residues is more dramatically shown in Fig. 5C, where $\Delta\Delta t_R$ (Δt_R in mobile phase 2 minus Δt_R in mobile phase 1) is plotted against side-chain hydrophilicity/hydrophobicity in mobile phase 1 (without salt). The expression $\Delta\Delta t_R$ is a measure of the effect of salt on hydrophilicity/hydrophobicity of the side-chains. Fig. 5C shows that the predicted increase in hydrophilicity for Lys, Arg and His side-chains, based on the observed hydrophilicity/hydrophobicity changes for the other side-chains, does not occur. Instead, a large decrease in hydrophilicity is observed (dotted line denotes predicted region for these residues; arrow indicates the difference between predicted and observed values). These three side-chains are fully protonated at pH 2.0. The increase in

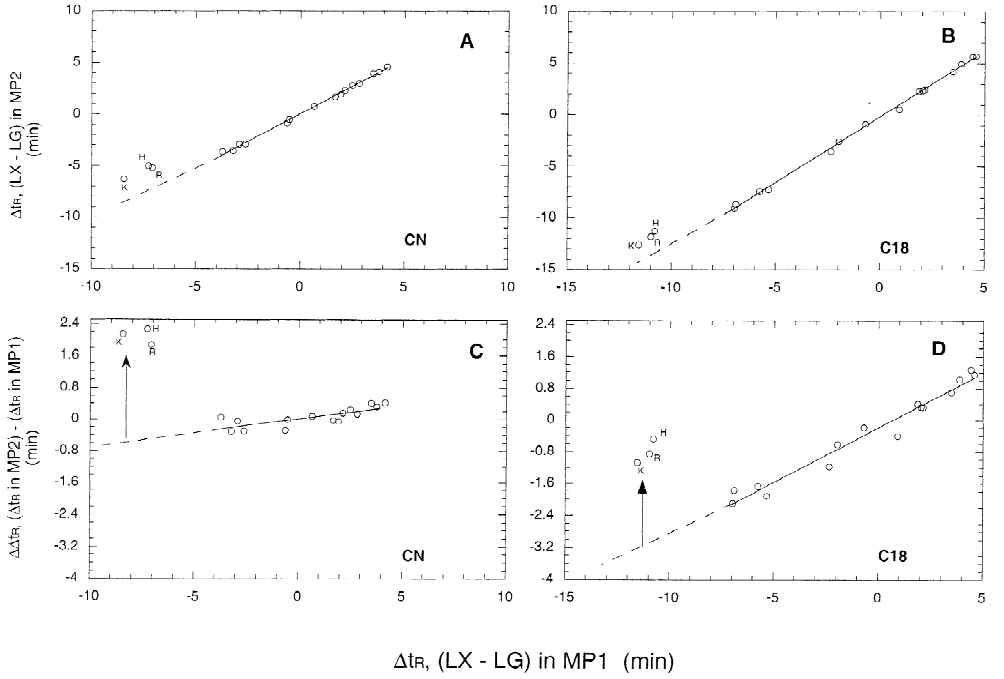

Fig. 5. Plots of Δt_R, (LX–LG) in mobile phase 2 (MP2) vs. Δt_R, (LX–LG) in mobile phase 1 (MP1) for cyano column (A) and C_{18} column (B) and plots of $\Delta\Delta t_R$, (Δt_R, [LX–LG] in mobile phase 2 minus Δt_R, [LX–LG] in mobile phase 1) vs. Δt_R, (LX–LG) in mobile phase 1 (MP1) for cyano column (C) and C_{18} column (D). Data are taken from Table 4. The single letter code represents the amino acid substitution at position 9 of the peptide sequence (Fig. 1).

hydrophobicity in the presence of 100 m*M* sodium perchlorate can be explained by ion-pairing of the ClO_4^- anion with the positively charged side-chains which, hence, neutralize the positive charge and therefore increase side-chain hydrophobicity (i.e. decrease hydrophilicity). These results also suggest that the salt anion is buried with the positively charged side-chain in the ligand–receptor interface when the receptor is of lower hydrophobicity (cyano).

In contrast to the cyano column, the results of the C_{18} column show that, when receptor hydrophobicity is high, adding salt to the mobile phase has significant effects on the hydrophilicity/hydrophobicity of the side-chains (Table 4) (an average $\Delta\Delta t_R$ magnitude of 1.0, compared to just 0.19 for the cyano column, when excluding Arg, Lys and His). The addition of salt to the mobile phase increases the hydrophobicity of the hydrophobic side-chains (Leu, Met, Cys, Ala, Ile, Val, Phe), with the most substantial effects on the most hydrophobic side-chains (Leu, Ile, Val, Phe). These results indicate that the effect of salt in the mobile phase is related to the overall hydrophobicity of the side-chains, i.e. the hydrophobicity values (Δt_R in mobile phase 1) of Leu, Ile, Val and Phe side-chains are 4.42, 4.59, 3.88 and 3.49, respectively, and the addition of salt results in increases of these values ($\Delta\Delta t_R$ in mobile phase 2 minus mobile phase 1) of 1.26, 1.13, 1.02 and 0.70, respectively (Table 4). Conversely, the salt increases the hydrophilicity of the polar and positively charged side-chains (Tyr, Trp, Thr, Ser, Gln, Asn, Glu, Asp, Arg, Lys and His) in this very hydrophobic receptor environment (see $\Delta\Delta t_R$ in mobile phase 2 minus mobile phase 1 values; Table 4). When side-chain hydrophilicity/hydrophobicity in mobile phase 2 is plotted against side-chain hydrophilicity/hydrophobicity in mobile phase 1 (Fig. 5B), there is an excellent correlation ($r=0.990$) for all amino acid side-chains, again with the exception of the positively charged side-chains (Arg, Lys and His). In a similar manner to results obtained on the cyano

column (Fig. 5C), the disproportionate effect of the presence of salt on these three residues is shown more dramatically in Fig. 5D, where $\Delta\Delta t_R$ (Δt_R, mobile phase 2 minus Δt_R, mobile phase 1) is plotted against side-chain hydrophilicity/hydrophobicity in mobile phase 1 (without salt), with the predicted increase in hydrophilicity/hydrophobicity of Lys, Arg and His (denoted by dotted line) again not being observed. As noted above, $\Delta\Delta t_R$ is a measure of the effect of salt on hydrophilicity/hydrophobicity of the side-chains. Again in a similar manner to results obtained on the cyano column, the large decrease in hydrophilicity of Lys, Arg and His (denoted by arrow) can be explained by the ion-pairing of the perchlorate anion with the positively charged side-chains, neutralizing the positive charge and, hence, increasing the hydrophobicity (i.e. decrease hydrophilicity) of the positively charged side-chain/perchlorate ion pair. Interestingly, the difference between predicted and observed changes in hydrophilicity/hydrophobicity is similar on both columns, e.g. for Arg, the difference is ~2.8 min on the cyano column (Fig. 5C) and ~2.5 min on the C_{18} column (Fig. 5D). These results also suggest that the positively charged residues are being buried in the ligand–receptor interface as ion-pairs with perchlorate anions since there are only hydrophobic groups on the receptor (C_{18} column). These results also suggest that burying a positively charged residue of a ligand within the hydrophobic interface of a receptor will require an anion to be paired with the charged side-chain unless the receptor has a negatively charged group in the receptor for compensation of positively charged residues.

The dependence of the effect of changing the aqueous environment (i.e. addition of salt) around the ligand–receptor interface on receptor hydrophobicity is particularly well illustrated in Fig. 6. Thus, the significant enhancement in hydrophobicity of the positively charged Arg, Lys, and His side-chains in the presence of the cyano column contrasts dramatically with their increase in hydrophilicity in the presence of the much more hydrophobic C_{18} stationary phase. In addition, all of the polar residues

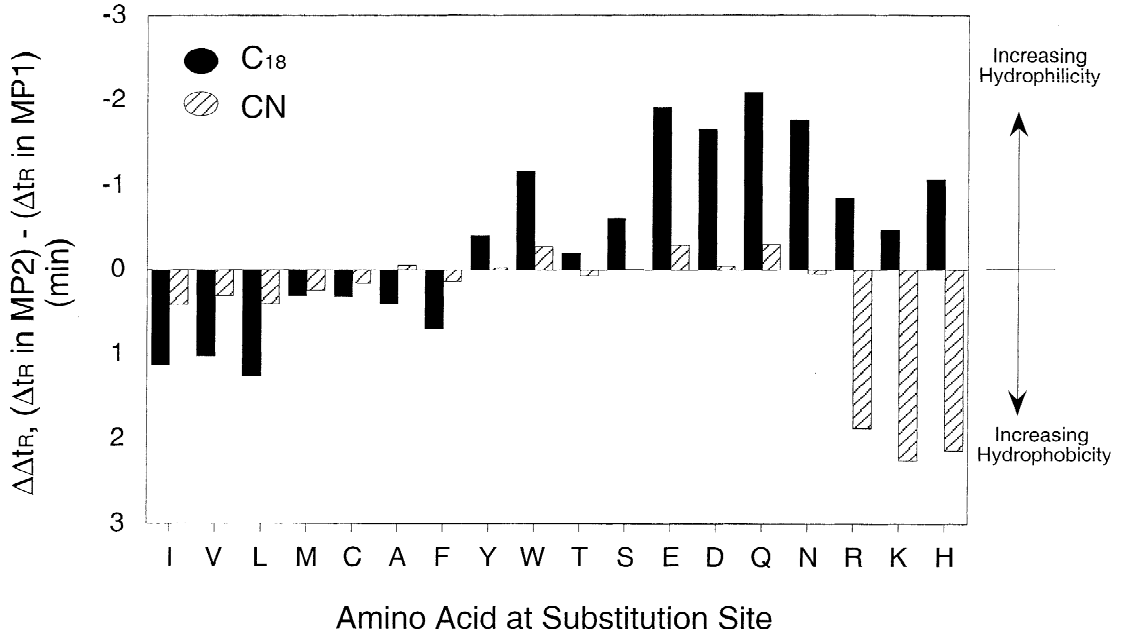

Fig. 6. Effect of salt on hydrophilicity/hydrophobicity of side-chains substituted in the non-polar face of a model amphipathic α-helical ligand. C_{18} and CN (cyano) denote the RP-HPLC columns representing receptors in the ligand–receptor model. $\Delta\Delta t_R = \Delta t_R$, (LX–LG) in mobile phase 2 minus Δt_R, (LX–LG) in mobile phase 1. Mobile phases 1 and 2 are described in Experimental. Data are taken from Table 4. The single letter code represents the amino acid substitution at position 9 of the peptide sequence (Fig. 1).

(including Tyr) exhibit, to a greater or lesser extent, enhancement of the hydrophilic character on the C_{18} column in the presence of salt, particularly when compared to the much lesser hydrophilicity/hydrophobicity changes effected on the cyano column. Finally, enhancement of the hydrophobic character in the presence of salt is clearly more pronounced at the ligand–C_{18} receptor interface compared to the ligand–cyano receptor model.

4. Conclusions

The present study describes the further 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 retention behaviour of de novo designed model amphipathic α-helical peptides (representing the hydrophobic binding domain of a ligand) on reversed-phase packings of varying hydrophobicity (representing a receptor protein with a hydrophobic binding pocket). With this model, we have demonstrated how an increase in receptor hydrophobicity (represented by an increase in column stationary phase hydrophobicity) significantly enhances hydrophilicity of polar amino acid side-chains at the ligand–receptor interface, while moderately enhancing the hydrophobicity of several non-polar side-chains. The aqueous environment surrounding the binding site of the receptor and ligand was also shown to affect profoundly the hydrophilicity/hydrophobicity of amino acids in the binding interface. The present study examines the effects on amino acid side-chain hydrophilicity/hydrophobicity of varying receptor hydrophobicity whilst maintaining a constant environment surrounding the substitution site on the peptide ligand. A separate paper will examine the other side of the equation, i.e. how the hydrophilicity/hydrophobicity of side-chains is influenced when varying the non-polarity of the ligand environment surrounding the substitution site, as well as the aqueous environment surrounding the ligand(s)–receptor interface. Possible refinements of this chromatographic model also include the preparation and packing of stationary phases of varying functional group and/or ligand density in the laboratory [43–45]; the potential for such tailored stationary phases then becomes an important option for enhancing the flexibility of the ligand–receptor model.

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