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Journal of Chromatography A, 972 (2002) 61–75

JOURNAL OF CHROMATOGRAPHY A

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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
II. Effect of varying peptide ligand hydrophobicity

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Abstract

The present study represents a continuation of our development of a chromatographic model for studying the hydrophobic interactions which characterize the way a ligand binds to its receptor. We have designed 18-residue amphipathic α-helical peptides (representing the hydrophobic binding domain of a ligand), where the non-polar face interacts with the non-polar face of a reversed-phase stationary phase (representing a receptor protein with a hydrophobic binding pocket). Two series of amphipathic α-helical peptides were subjected to reversed-phase liquid chromatography at pH 2.0, where the ''native'' Ala-face peptide contains seven Ala residues in its non-polar face and the ''native'' Leu-face series contains seven Leu residues in its non-polar face. Mutants of the two series were then prepared by replacing one residue in the centre of the non-polar face in both series of peptides, resulting in amino acid side-chains being exposed to a moderately non-polar environment (Ala series) or a very hydrophobic environment (Leu series) surrounding the substitution site. With this model, we have demonstrated that an increase in non-polarity of the ligand enhances hydrophilicity (decreases hydrophobicity) of all amino acids at the ligand–receptor interface, this effect being dependent on the intrinsic hydrophilicity/hydrophobicity of the side-chain. The addition of salt to the aqueous environment surrounding the binding site of the ligand and receptor was also shown to affect the hydrophilicity/hydrophobicity of amino acids in the binding interface. For the Ala-face mutants, the majority of the non-polar side-chains and the three positively charged residues (Arg, His, Lys) showed significant enhancement of hydrophobicity in the presence of salt; in contrast, in the much more hydrophobic environment of the Leu-face mutants, there was a trend of lesser hydrophobicity enhancement and/or significantly more hydrophilicity enhancement in the presence of salt. Our results should have major implications for the understanding of the hydrophilicity/hydrophobicity of side-chains in varying hydrophobic and aqueous environments.

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

1. Introduction

One of the most interesting developments in recent years of liquid chromatography analysis lies in the employment of reversed-phase liquid chromatog-

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)00356-4

raphy (RP-HPLC) as a physicochemical model of biological systems [1–18]. In addition to this growing role for RP-HPLC as a useful probe of protein structure, 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) [19], since hydrophobic interactions play a key role in the binding of ligands to receptors in biological systems. We then extended our earlier work 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 the non-polar face of a model receptor (represented by reversed-phase packings of different hydrophobicities) as well as the aqueous environment (represented by the RP-HPLC mobile phase) surrounding the ligand–receptor binding site [20].

The present study now examines how the hydrophilicity/hydrophobicity of side-chains is influenced when varying the non-polarity of the ligand environment surrounding the substitution site in the ligand (represented by two series of model amphipathic α-helical peptides), whilst maintaining constant receptor hydrophobicity (represented by a C_8 RP-HPLC packing). In addition, the effect of varying the aqueous environment surrounding the ligand–receptor binding site on side-chain hydrophilicity/hydrophobicity is also evaluated.

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 and purification

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) as previously described [18]. The crude peptides were subsequently purified by RP-HPLC as previously described [20].

2.4. Column

The analytical RP-HPLC column employed to represent the hydrophobic surface of the receptor in the ligand–receptor model was a Zorbax 300SB-C_8 (diisopropyl-*n*-octyl bonded phase) (150×4.6 mm I.D., 5 μm particle size, 300 Å pore size) 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); 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 at room temperature.

3. Results and discussion

3.1. Design of ligand–receptor model system

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 [19], as well as in the companion paper to the present manuscript [20].

Fig. 1(top) shows the sequence of two peptide series, known to have a high potential to form amphipathic α-helices [19,21] and which represent model ligands. Helical net representations of the ''native'' Ala-face peptide, denoted AA (with Ala at all seven non-polar positions), and the ''native''

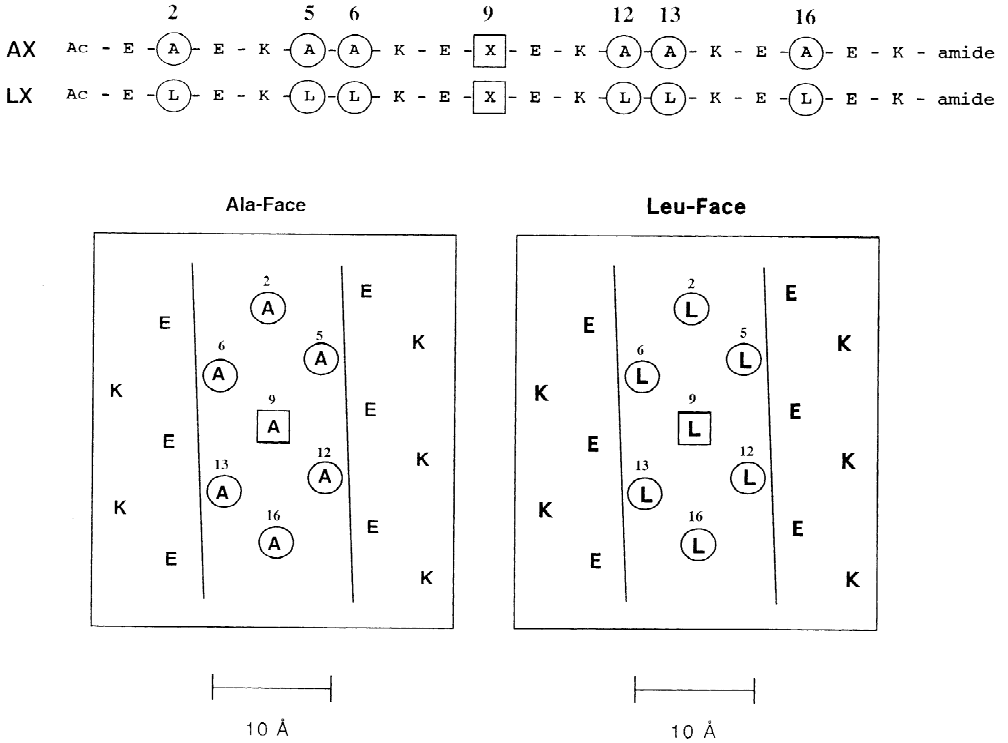

Fig. 1. Design of model synthetic peptides. Top: sequence of mutant peptides, denoted AX and LX series, where the first letter represents amino acid residues used in the hydrophobic face of the peptide and the X represents each of the 20 amino acids (boxed) (single letter code given in the tables) substituted 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'' Ala-face (AA, left) and Leu-face (LL, 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 lines on the α-helical nets represents the hydrophobic face of the peptides.

Leu-face peptide, denoted LL (with Leu at all seven non-polar positions), are shown in Fig. 1(bottom). Alanine is considerably less non-polar than leucine [22], which results 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''. The general denotion of the Ala-face series is AX (Fig. 1, top), with X referring to the central residue at position 9; hence the denotion of the ''native'' peptide with alanine at this position as AA (Fig. 1, bottom left); with glycine at this position, it is denoted AG, etc. The same general terminology was also used for the series of analogues based on leucine (general designation LX); hence the denotation LL for the ''native'' peptide (Fig. 1, bottom right), LG for the substitution of glycine at mutant position 9, etc. Only the analogues substituted at position 9 with the helix-disrupting proline (AP and LP) showed relatively low helical character and, hence, significantly reduced amphipathicity compared to the other analogues [19] and were thus excluded from the study.

On binding to a reversed-phase column, such as the Stablebond [23–27] C_8 column chosen to represent the receptor in the present study, the hydrophobicity of the stationary phase stabilizes the secondary (α-helical) structure [28]. The peptides are eluted as single-stranded amphipathic α-helices during RP-HPLC, interacting with the stationary phase through preferential binding with their hydrophobic faces [19,20]. Thus, 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).

The three mobile phases (mobile phases 1, 2 and 3, described in Experimental) 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 ligand hydrophobicity on hydrophilicity/hydrophobicity of side-chains at the ligand–receptor interface: constant receptor hydrophobicity

Table 1 summarizes the reversed-phase retention behaviour of the AX and LX series of peptide analogues (with the exception of the proline analogues, AP and LP) run with mobile phase 1 (phosphoric acid low ionic strength mobile phase; see Experimental) on the C_8 column. Table 2 summarizes the retention behaviour of the analogues when run with mobile phase 2 (phosphoric acid high ionic strength mobile phase; see Experimental).

The order of amino acid substitutions shown in Tables 1 and 2 (and subsequent tables) was generally based on decreasing retention times of the Ala-face mutants (t_R, AX) under mobile phase 1 conditions, starting with the highest retention time for the isoleucine-substituted analogue (AI; 25.15 min) and ending with the least-retained histidine-substituted analogue (AH; 13.07 min). However, there are several exceptions to this general rule due to a secondary grouping of side-chains into various categories. Thus, Ile and Val (AV; 23.52 min) contain hydrophobic, β-branched aliphatic side-chains; Leu, Cys, Met and Ala (t_R from 25.07 min for AL to 21.07 min for AA) were classed as having hydrophobic aliphatic side-chains; Phe, Trp and Tyr (t_R from 24.51 min for AF to 20.83 min for AY) contain aromatic side-chains; Thr (AT; 18.54 min) and Ser (AS; 17.05 min) contain polar hydroxy groups; Glu (AE; 18.15 min) and Asp (AD; 16.97 min) contain (at pH 2) uncharged carboxyl groups; Gln (AQ; 15.95 min) and Asn (AN; 14.90) contain polar side-chain amide groups; and Arg, Lys and His (t_R from 14.12 min for AR to 13.07 min for AH) all exhibit, at pH 2, a full positive charge on their side-chains. The Gly analogue, AG, represented an internal standard in each mixture of peptides applied to RP-HPLC. As noted previously [20], by grouping the amino acids into these categories, it was hoped to identify any effects which may be common to a group of side-chains with similar characteristics. This order of amino acids was subsequently retained for all tables.

From Tables 1 and 2, the greater retentiveness of the LX series peptides compared to their AX counterparts is quite clear, ranging from 46.90 min (LI) to 31.24 min (LK) in mobile phase 1 (Table 1) and 57.82 min (LI) to 40.74 min (LK) in mobile phase 2 (Table 2) compared to a range of 25.15 min (AI) to 13.07 min (AH) in mobile phase 1 (Table 1) and 33.39 min (AI) to 21.35 min (AK) in mobile phase 2 (Table 2). Such a difference in magnitude of re-

Table 1
Effect of increasing hydrophobicity of environment surrounding the substitution site of the ligand on hydrophilicity/hydrophobicity of side-chains: mobile phase 1 (aqueous 10 m*M* orthophosphoric acid–acetonitrile)

Amino acid substitution		Ala-face mutants[a]		Leu-face mutants[a]		$\Delta\Delta t_R$[d] (min) (Leu-face minus Ala-face)
		t_R, AX (min)	Δt_R, AX−AG (min)[c]	t_R, LX (min)[b]	Δt_R, LX−LG (min)[c]	
Ile	(I)	25.15	+7.39	46.90	+5.21	−2.12
Val	(V)	23.52	+5.76	46.12	+4.43	−1.33
Leu	(L)	25.07	+7.26	46.83	+5.14	−2.12
Met	(M)	23.15	+5.33	44.58	+2.89	−2.44
Cys	(C)	21.37	+3.61	44.31	+2.68	−0.93
Ala	(A)	21.07	+3.31	44.02	+2.33	−0.98
Phe	(F)	24.51	+6.70	45.83	+4.14	−2.56
Trp	(W)	23.75	+5.94	43.46	+1.77	−4.17
Tyr	(Y)	20.83	+3.02	40.54	−1.15	−4.17
Thr	(T)	18.54	+0.78	41.62	0	−0.78
Ser	(S)	17.05	−0.71	40.41	−1.28	−0.57
Glu	(E)	18.15	+0.34	37.87	−3.82	−4.16
Asp	(D)	16.97	−0.84	37.32	−4.37	−3.53
Gln	(Q)	15.95	−1.86	36.44	−5.25	−3.39
Asn	(N)	14.90	−2.91	36.07	−5.62	−2.71
Arg	(R)	14.12	−3.64	32.15	−9.48	−5.84
Lys	(K)	13.27	−4.49	31.24	−10.45	−5.96
His	(H)	13.07	−4.75	31.85	−9.84	−5.09
Gly	(G)	17.76±0.06	–	41.69±0.06	–	–

[a] Sequences of peptides are shown in Fig. 1.
[b] Mobile phase 1 (see Experimental) was used; runs were carried out on the C_8 column described in Experimental; t_R, AX and t_R, LX refer, respectively, to the observed retention times of the Ala-face and Leu-face analogues.
[c] Δt_R refers to the retention time differences between the mutant peptide and the Gly-substituted peptide (i.e., AG or LG).
[d] $\Delta\Delta t_R$ = (Δt_R, LX−LG) minus (Δt_R, AX−AG).

tention times for the two series of amphipathic peptides would be expected considering the much more hydrophobic character of leucine compared to alanine [19,22,29]. Indeed, the magnitude of the retention time difference between a particular pair of peptides in both mobile phases is further evidence that the peptides are interacting with the stationary phase through preferential binding with their hydrophobic faces, e.g. from Table 1 (mobile phase 1), Δt_R, (LI−AI)=46.90−25.15=21.75 min; from Table 2 (mobile phase 2), Δt_R, (LT−AT)=51.48−25.95=25.53 min, etc. In addition, these results also confirm the much greater hydrophobicity of the environment surrounding the substituted side-chain at position 9 of the amphipathic peptide ligands (Fig. 1) of the LX series compared to the AX series.

The retention times of the glycine analogues were now subtracted from the retention times of the other 18 analogues (Δt_R, AX−AG and 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 relative to Gly; thus, the Gly analogues, AG and LG, represent the situation where there is no side-chain present at position 9 (see Fig. 1).

In their earlier study, Sereda et al. [19] noted that, when run in mobile phase 3 (TFA mobile phase) on a C_8 column, amino acids with non-polar side-chains exhibited their maximum hydrophobic characteristics in the Ala-face peptides and their minimum hydrophobicity in the Leu-face; conversely, polar and charged side-chains generally exhibited their maximum hydrophilic characteristics in the Leu-face peptides and their minimum hydrophilicity in the Ala-face peptides. Similar results can be seen for both mobile phase 1 (Table 1) and mobile phase 2 (Table 2), i.e., in both the absence and presence of salt. For example, the Δt_R value for the non-polar Ile side-chain is +7.39 min for AI and just +5.21 min

Table 2
Effect on increasing hydrophobicity of environment surrounding the substitution site of the ligand on hydrophilicity/hydrophobicity of side-chains: mobile phase 2 (aqueous 10 m*M* orthophosphoric acid–acetonitrile containing 100 m*M* sodium perchlorate)

Amino acid substitution		Ala-face mutants[a]		Leu-face mutants[a]		$\Delta\Delta t_R$[d] (min) (Leu-face minus Ala-face)
		t_R, AX (min)	Δt_R, AX−AG (min)[c]	t_R, LX (min)[b]	Δt_R, LX−LG (min)[c]	
Ile	(I)	33.39	+8.90	57.82	+6.34	−2.56
Val	(V)	31.62	+7.13	56.79	+5.31	−1.82
Leu	(L)	33.19	+8.71	57.62	+6.22	−2.49
Met	(M)	30.95	+6.48	54.72	+3.32	−3.16
Cys	(C)	29.06	+4.59	54.44	+3.11	−1.48
Ala	(A)	28.04	+3.55	54.11	+2.63	−0.92
Phe	(F)	32.77	+8.29	56.16	+4.76	−3.53
Trp	(W)	31.93	+7.45	52.83	+1.43	−6.02
Tyr	(Y)	28.19	+3.71	49.35	−2.05	−5.76
Thr	(T)	25.95	+1.46	51.48	0	−1.46
Ser	(S)	24.01	−0.48	49.77	−1.71	−1.23
Glu	(E)	24.49	0	46.10	−5.30	−5.30
Asp	(D)	23.27	−1.21	45.87	−5.61	−4.40
Gln	(Q)	22.82	−1.66	44.58	−6.82	−5.16
Asn	(N)	21.42	−3.06	44.55	−6.93	−3.87
Arg	(R)	22.79	−1.70	41.79	−9.54	−7.84
Lys	(K)	21.35	−3.14	40.74	−10.74	−7.60
His	(H)	21.79	−2.68	42.03	−9.45	−6.77
Gly	(G)	24.49±0.04	–	51.48±0.05	–	–

[a] Sequences of peptides are shown in Fig. 1.
[b] Mobile phase 2 (see Experimental) was used; runs were carried out on the C_8 column described in Experimental; t_R, AX and t_R, LX refer, respectively, to the observed retention times of the Ala-face and Leu-face analogues.
[c] Δt_R refers to the retention time differences between the mutant peptide and the Gly-substituted peptide (i.e., AG or LG).
[d] $\Delta\Delta t_R = (\Delta t_R, \text{LX}-\text{LG})$ minus $(\Delta t_R, \text{AX}-\text{AG})$.

for LI in mobile phase 1 (Table 1) and +8.90 min for AI and just +6.34 min for LI in mobile phase 2 (Table 2). In contrast, the Δt_R value for the polar Gln side-chain is −1.86 min for AQ, its hydrophilicity increasing to −5.25 min for LQ in mobile phase 1 (Table 1) and −1.66 min for AQ, its hydrophilicity increasing to −6.82 min for LQ in mobile phase 2 (Table 2). Thus, as noted previously [19], the environment around the mutation site of the peptide ligand may have a profound effect on side-chain hydrophilicity/hydrophobicity. In addition, the interesting observation in Table 1 (mobile phase 1) that Tyr and Glu are hydrophobic relative to Gly in the Ala-face and hydrophilic relative to Gly in the Leu-face was also reported by Sereda et al. [19] when employing mobile phase 3 with a C_8 column. A similar result was also obtained in the present study with mobile phase 2 (Table 2) with the exception that, in this environment, Thr and Gly exhibited identical hydrophilic/hydrophobic characteristics in the Leu-face ligand. The effect on the apparent hydrophilicity/hydrophobicity of the Tyr side-chain is of particular interest here due to its general classification as a non-polar residue (due to its aromatic nature) based on most experimental criteria [30], including high-performance liquid chromatography (HPLC) data [31]. However, in an analogous manner to the behaviour of the polar and charged side-chains in non-polar environments of varying hydrophobicity, it is possible that the polar, hydrophilic character of the phenolic hydroxy group of the Tyr side-chain is minimized in the Ala-face ligand (resulting in the retention behaviour of AY reflecting the non-polar nature of the aromatic benzene ring) and maximized in the Leu-face ligand (resulting in the retention behaviour of LY reflecting the polar nature of the hydroxyl group).

The Δt_R retention times of the Leu-face peptides

(t_R, LX−LG) were now plotted against those of the Ala-face peptides (t_R, AX−AG) for both mobile phase 1 (Table 1) and mobile phase 2 (Table 2). From Fig. 2 there is a good correlation between the sets of data for both mobile phase 1 (Fig. 2A; $r = 0.961$) and mobile phase 2 (Fig. 2C; $r = 0.941$), suggesting that, although the magnitude of the hydrophilicity/hydrophobicity values for the side-chains are different in the Ala- and Leu-face ligand environments, the directional effect on all side-chains is similar when changing the hydrophobicity of the environment surrounding the mutation, i.e., 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. Although not shown, essentially identical results were also obtained for mobile phase 3 (TFA mobile phase).

When the Δt_R values obtained with the Ala-face mutants (Δt_R, AX−AG) are subtracted from the Δt_R values obtained with the Leu-face mutants (Δt_R, LX−LG), another expression (denoted $\Delta\Delta t_R$ in Tables 1 and 2) is obtained. Thus, $\Delta\Delta t_R = (\Delta t_R$, LX−LG) minus ($\Delta t_R$, AX−AG) and represents the effect of increasing ligand hydrophobicity on hydrophilicity/hydrophobicity of side-chains in the substitution site of the ligand. $\Delta\Delta t_R$ values obtained from all three mobile phases (mobile phases 1, 2 and 3; see Experimental) are compared in Fig. 3. From both Fig. 3 and Tables 1 and 2 (mobile phases 1 and 2, respectively), it can be seen that there is a decrease in $\Delta\Delta t_R$ values for all of the 18 amino acids in all three aqueous mobile phases when increasing the hydrophobicity of the environment surrounding the substitution site of the ligand, i.e., the side-chains of all 18 amino acids decrease in hydrophobicity (i.e., increase in hydrophilicity), as expressed by negative $\Delta\Delta t_R$ values, when surrounded by a more hydrophobic ligand environment.

An interesting observation from Figs. 2 and 3 is that the $\Delta\Delta t_R$ values for the side-chains appear to be approximately grouped into three sets of amino acids: thus, group 1, comprised of Ile, Val, Leu, Met, Phe, Cys, Ala, Thr and Ser, exhibit a low to moderate effect of increasing ligand hydrophobicity; group 2, comprised of Trp, Tyr, Glu, Asp, Gln and

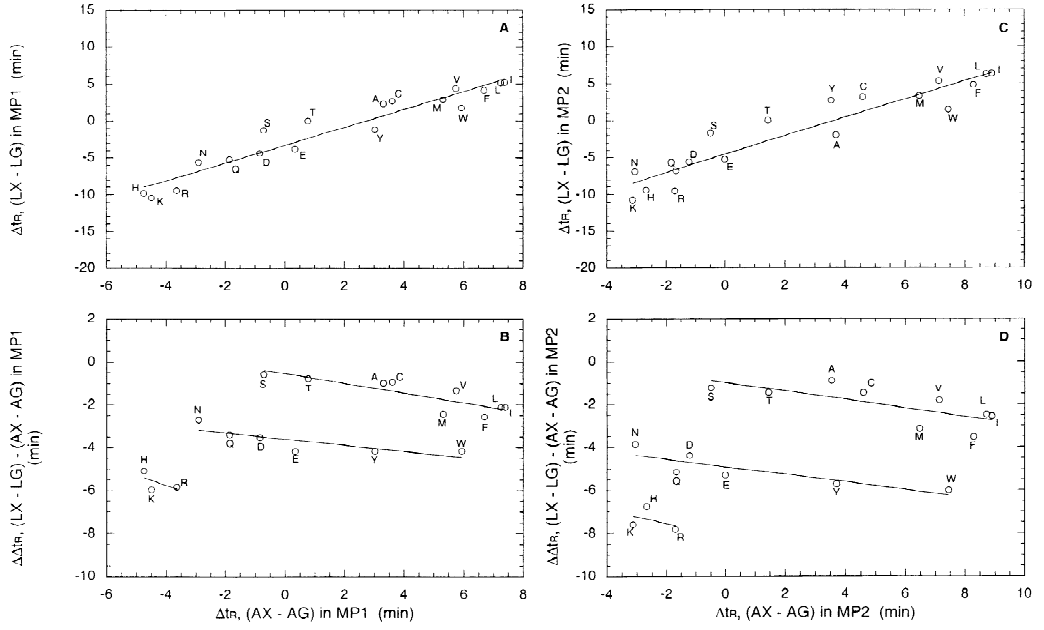

Fig. 2. Plots of Δt_R, (LX−LG) (A) and $\Delta\Delta t_R$, (Δt_R, [LX−LG]−Δt_R, [AX−AG]) (B) versus Δt_R, (AX−AG) in mobile phase 1 (MP1); Δt_R, (LX−LG) (C) and $\Delta\Delta t_R$, (Δt_R [LX−LG]−Δt_R, [AX−AG]) (D) versus Δt_R, (AX−AG) in mobile phase 2 (MP2). Data are taken from Table 1 (mobile phase 1) and Table 2 (mobile phase 2). The single-letter code represents the amino acid substitution at position 9 of the peptide sequence (see Fig. 1).

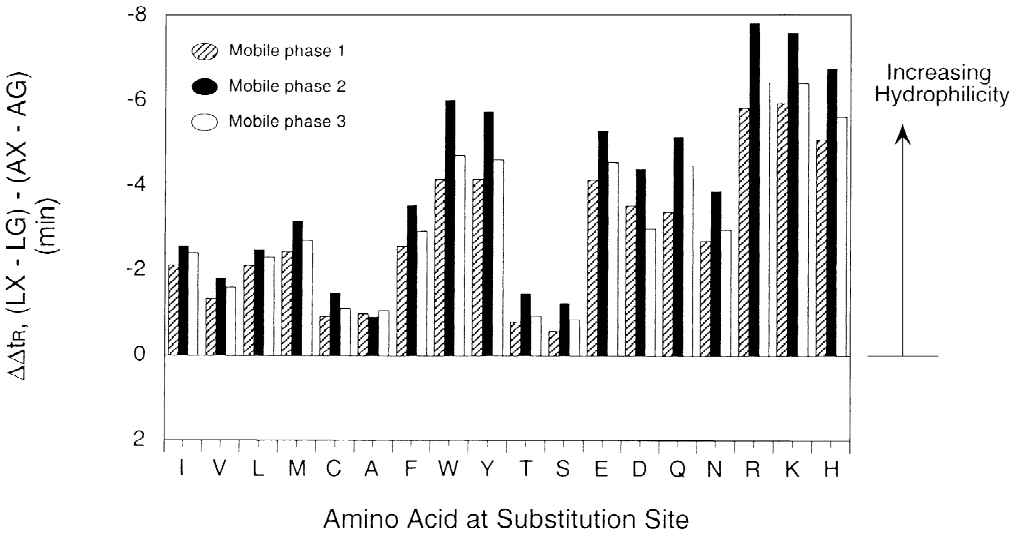

Fig. 3. Effect of varying ligand hydrophobicity on hydrophilicity/hydrophobicity of side-chains substituted in the non-polar face of model amphipathic α-helical peptide ligands. Mobile phases 1, 2 and 3 are described in Experimental. $\Delta\Delta t_R = \Delta t_R$, (LX−LG) minus Δt_R, (AX−AG) data shown in Tables 1 and 2 for mobile phases 1 and 2, respectively. The single letter code represents the amino acid substitution at position 9 of the peptide sequence (Fig. 1).

Asn, exhibit a mid-range effect of increasing ligand hydrophobicity; and group 3, comprised of the positively charged Arg, Lys and His, exhibit the largest effect of increasing ligand hydrophobicity. Within these groups, the $\Delta\Delta t_R$ values appear to be generally dependent on the hydrophilicity/hydrophobicity of the side-chains, albeit in an inverse fashion, i.e., the less hydrophilic (more hydrophobic) the side-chain within the group, the greater the enhancement of hydrophilicity (i.e., the more negative the $\Delta\Delta t_R$ value). This is most easily visualized in Fig. 3 by looking at the effects of ligand hydrophobicity on side-chain hydrophilicity/hydrophobicity in mobile phase 2 (Table 2) as the aqueous environment. Thus, from group 1, $\Delta\Delta t_R$ values range from −1.23 min (Ser) to −2.56 min (Ile), these two side-chains exhibiting Δt_R, AX−AG values of −0.48 min and +8.90 min, respectively; from group 2, $\Delta\Delta t_R$ values range from −3.87 min (Asn) to −6.02 min (Trp), these two side-chains exhibiting Δt_R, AX−AG values of −3.06 min and +7.45 min, respectively; finally, from group 3, $\Delta\Delta t_R$ values range from −6.77 min (His) to −7.84 min (Arg), these two side-chains exhibiting Δt_R, AX−AG values of −2.68 min and −1.70 min, respectively (Table 2). This grouping of the side-chains is highlighted dramatically in Fig. 2B (mobile phase 1) and Fig. 2D (mobile phase 2), where the $\Delta\Delta t_R$ values shown in Tables 1 and 2 are plotted against Δt_R, AX−AG. The three groupings noted above for Fig. 3 are considerably more apparent when presented in this fashion, as are the reasonable correlations between side-chain hydrophilicity/hydrophobicity values (Δt_R, AX−AG) and the magnitude ($\Delta\Delta t_R$) of the effect of increasing ligand hydrophobicity within these groups of side-chains.

Concerning the groupings of the amino acids shown in Fig. 2B and D, it should be noted that, with the clear exceptions of Thr and Ser, the amino acids making up a specific group are related in their properties. Thus, group 3 is comprised of the positively charged basic residues His, Lys and Arg, i.e., the most hydrophilic of the amino acids at pH 2.0. In an analogous manner to the effect of increasing receptor hydrophobicity on polar and charged side-chains, where hydrophilicity is maximized in the more non-polar environment of the more hydrophobic receptor [19], the effect of increasing ligand hydrophobicity on side-chain hydrophilicity is greatest for these positively charged side-chains. Group 2 is comprised of amino acids which exhibit polar, uncharged characteristics (the acidic amino acids,

Glu and Asp, are protonated, i.e., uncharged, at pH 2) including, due to its phenolic hydroxyl group (see above), Tyr. Although Trp appears to be exhibiting anomalous behaviour here since, in a similar manner to Tyr, it is generally classified as hydrophobic based on retention behaviour during RP-HPLC, it too (unlike Phe) possesses some polar characteristics (an N–H group) on its side-chain in addition to the innate hydrophobicity of the aromatic ring structure. Clearly, such characteristics are maximized in the presence of the extremely hydrophobic environment of the Leu-face ligand. Although not of as great a magnitude as the effect of increasing ligand hydrophobicity on the positively charged side-chains of His, Lys and Arg, the increase in hydrophilicity of the group 2 amino acids with polar, uncharged characteristics remains significant. Within this group, the inverse relationship between side-chain hydrophilicity/hydrophobicity (as expressed by Δt_R, AX−AG values) is quite clear in Fig. 2B and D with the least hydrophilic (Trp, $\Delta t_R = +5.94$ min in mobile phase 1; Table 1, Fig. 2A) showing the greatest change in hydrophilicity ($\Delta\Delta t_R = -4.17$ min) with increasing ligand hydrophobicity and the most hydrophilic (Asn, $\Delta t_R = -2.19$ min) showing the least change in hydrophilicity ($\Delta\Delta t_R = -2.71$ min). Group 1 amino acids, showing the least overall effect on side-chain hydrophilicity of increasing ligand hydrophobicity, is comprised of the non-polar side-chains of Ala, Cys, Met, Val, Leu, Ile and Phe, with the unexpected inclusion of polar Ser and Thr. Again, the inverse relationship between side-chain hydrophilicity/hydrophobicity and relative effect of increasing ligand hydrophobicity is apparent (Fig. 2B and D), with residues of highest hydrophobicity such as Leu, Ile, and Phe showing the greatest change in hydrophobicity (i.e., decreasing hydrophobicity) within this group and the most hydrophilic side-chains (Thr, Ser) showing the least change.

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

The effect of aqueous environment, that is the absence or presence of salt (0.1 *M* sodium perchlorate), on the hydrophobicity of the peptide ligands can be seen by comparing Tables 1 and 2. From Tables 1 and 2, all peptides are eluted later in mobile phase 2 (Table 2) compared to mobile phase 1 (Table 1). In the absence of salt (Table 1), the retention times of the Ala-face ligands range from 13 to 25 min and the Leu-face ligands from 31 to 47 min. In the presence of salt (Table 2), the retention times of the Ala-face ligands range from 21 to 33 min and the Leu-face ligands from 41 to 58 min. Thus, the effect of salt is to increase the retention time of the Ala-face peptide ligands by ~8 min and the Leu-face peptide ligands by ~10 min. This increase in peptide retention times observed on addition of salt to the mobile phase is a result of an increase in affinity of a ligand for its receptor (salt-induced increase in the hydrophobic effect).

Table 3 now summarizes the hydrophilicity/hydrophobicity of each side-chain at the substitution site (Δt_R, LX−LG or Δt_R, AX−AG) in mobile phases 1 and 2. If one examines the hydrophilicity/hydrophobicity of the side-chain at the substitution site in the centre of the hydrophobic face of the peptide ligand (Fig. 1) in the absence and presence of salt, a number of conclusions can be drawn. Thus, the side-chains Ser, Thr, Tyr, Trp, Phe, Ala, Cys, Met, Leu, Val and Ile increase in hydrophobicity in the presence of salt in the context of the Ala-face ligand. Similarly, the side-chains Trp, Phe, Ala, Cys, Met, Leu, Val and Ile increase in hydrophobicity in the presence of salt in the context of the Leu-face ligand. Interestingly, the side-chains Tyr, Ser, Glu, Asp, Gln and Asn increase in hydrophilicity in the presence of salt in the context of the Leu-face ligand and the majority of these side-chains showed little change in hydrophilicity in the context of the Ala-face ligand. Also of note is the effect of salt on the positively charged residues, Arg, Lys and His, at the ligand–receptor interface. Thus, there is an increase in hydrophobicity of these side-chains in the context of the Ala-face ligand but apparently negligible change in hydrophobicity in the context of the Leu-face ligand. However, the latter observation can be rationalized by the expected increase in side-chain hydrophilicity in the presence of salt in the context of the Leu-face ligand being completely counterbalanced by perchlorate counterion neutralization of the positively charged side-chains which increases their

Table 3
Effect on the relative hydrophilicity/hydrophobicity of amino acid side-chains of changing the aqueous environment surrounding the ligand–receptor interface

Amino acid substitution		Leu-face[a]		$\Delta\Delta t_R$ (min)[d] (mobile phase 2 minus mobile phase 1)	Ala-face[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, AX−AG[c] (min)	Mobile phase 2[b] Δt_R, AX−AG[c] (min)	
Ile	(I)	+5.21	+6.34	+1.13	+7.39	+8.90	+1.51
Val	(V)	+4.43	+5.31	+0.88	+5.76	+7.13	+1.37
Leu	(L)	+5.14	+6.22	+1.08	+7.26	+8.71	+1.45
Met	(M)	+2.89	+3.32	+0.43	+5.33	+6.48	+1.15
Cys	(C)	+2.68	+3.11	+0.43	+3.61	+4.59	+0.98
Ala	(A)	+2.33	+2.63	+0.30	+3.31	+3.55	+0.24
Phe	(F)	+4.14	+4.76	+0.62	+6.70	+8.29	+1.59
Trp	(W)	+1.77	+1.43	−0.34	+5.94	+7.45	+1.51
Tyr	(Y)	−1.15	−2.05	−0.90	+3.02	+3.71	+0.69
Thr	(T)	0	0	0	+0.78	+1.46	+0.68
Ser	(S)	−1.28	−1.71	−0.43	−0.71	−0.48	+0.23
Glu	(E)	−3.82	−5.30	−1.48	+0.34	0	−0.34
Asp	(D)	−4.37	−5.61	−1.24	−0.84	−1.21	−0.37
Gln	(Q)	−5.25	−6.82	−1.57	−1.86	−1.66	+0.20
Asn	(N)	−5.62	−6.93	−1.31	−2.91	−3.06	−0.15
Arg	(R)	−9.48	−9.54	−0.06	−3.64	−1.70	+1.94
Lys	(K)	−10.45	−10.74	−0.29	−4.49	−3.14	+1.35
His	(H)	−9.84	−9.45	+0.39	−4.75	−2.68	+2.07

[a] Sequences of Leu-face and Ala-face peptide ligands are shown in Fig. 1.
[b] Full descriptions of mobile phase 1 (aqueous 10 m*M* orthophosphoric acid–acetonitrile) and mobile phase 2 (same as mobile phase 1 except 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 peptides (LG, AG).
[d] $\Delta\Delta t_R = (\Delta t_R$, LX−LG in mobile phase 2) minus (Δt_R, LX−LG in mobile phase 1) or (Δt_R, AX−AG in mobile phase 2) minus (Δt_R, AX−AG in mobile phase 1).

hydrophobicity (see Fig. 5B and D below for discussion of this effect).

The effect of 100 m*M* sodium perchlorate on the hydrophilicity/hydrophobicity of the side-chains ($\Delta\Delta t_R$) in the non-polar face of both ligands can 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 or $\Delta\Delta t_R = (\Delta t_R$, AX−AG) in mobile phase 2 minus (Δt_R, AX−AG) in mobile phase 1. Such values allow another way of evaluating the effect of changing or increasing the hydrophobicity of the ligand, 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 ligand. Thus, with the sole exception of Ala, the effect of salt is greatest on hydrophobicity of the side-chains when present in the centre of the Ala-face ligand compared to the Leu-face ligand (Table 3). On the other hand, the effect of salt is greatest on the hydrophilicity of the side-chains (except Ala) when present in the centre of the Leu-face ligand compared to the Ala-face ligand (Table 3). 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 ($r = 0.990$) (Fig. 4), showing that, although the hydrophobicity change of the ligand 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.

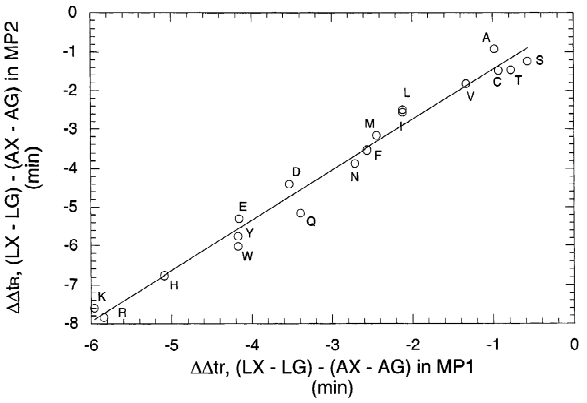

Fig. 4. Plot of $\Delta\Delta t_R$, (Δt_R, [LX−LG] minus Δt_R, [AX−AG]) in mobile phase 2 (MP2) versus $\Delta\Delta t_R$, (Δt_R, [LX−LG] minus Δt_R, [AX−AG]) in mobile phase 1 (MP1). Data are taken from Table 1 (MP1) and Table 2 (MP2). The single letter code represents the amino acid substitution of position 9 of the peptide sequence (Fig. 1).

Fig. 5A shows an excellent correlation ($r = 0.991$) of the hydrophilicity/hydrophobicity of the amino acid side-chains in the substitution site of the Ala-face ligands (Δt_R, AX−AG) in the two mobile phases considering the generally small differences in $\Delta\Delta t_R$ values (Table 3), the only exceptions being the three charged residues, Arg, His and Lys. The indication that salt in the mobile phase influences the hydrophobicity of these three residues disproportionately is more clearly shown in Fig. 5B, 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. 5B 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. Indeed, these three positively charged amino acids are becoming more hydrophobic instead of more hydrophilic (dotted lines denote 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 hydrophobicity in the presence of 100 m*M* sodium perchlorate can be explained by ion-pairing of the

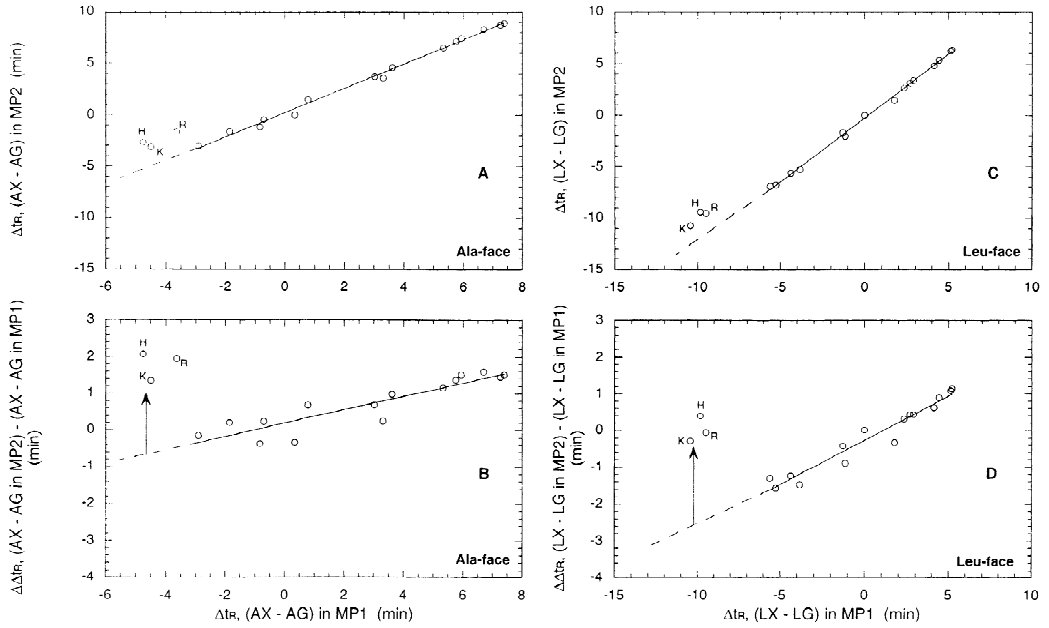

Fig. 5. Plots of Δt_R, (AX−AG) in mobile phase 2 (MP2) (A) and $\Delta\Delta t_R$, (Δt_R, [AX−AG in MP2] minus Δt_R, [AX−AG in MP1]) (B) versus Δt_R, (AX−AG) in mobile phase 1 (MP1); Δt_R, (LX−LG) in MP2 (C) and $\Delta\Delta t_R$, (Δt_R, [LX−LG in MP2] minus Δt_R, [LX−LG in MP1]) (D) versus Δt_R, (LX−LG) in MP1. Data are taken from Table 3. The single letter code represents the amino acid substitution at position 9 of the peptide sequence (Fig. 1).

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 side-chain hydrophilicity/hydrophobicity in the substitution site of the Leu-face ligands (Δt_R, LX−LG) in mobile phase 2 is now plotted against mobile phase 1 (Fig. 5C), there is an excellent correlation ($r = 0.990$) for all amino acids, again with the exception of the positively charged side-chains (Arg, His, Lys). In a similar manner to results obtained with the Ala-face ligands (Fig. 5B), 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, in 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 of Arg, Lys and His (denoted by dotted line) again not being observed, becoming instead more hydrophobic (i.e, less hydrophilic). 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 with the Ala-face ligands, the large decrease in hydrophilicity (increase in hydrophobicity) of Arg, Lys 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 of the positively charged side-chain/perchlorate ion-pair. It is interesting to note that the difference between predicted and observed changes in hydrophilicity/hydrophobicity is similar for both series of peptide ligands, e.g. for Lys, the difference is ~2.2 min for both the Ala-face ligand (Fig. 5B) and the Leu-face ligand (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_8 column). In addition, these results further suggest that burial of a positively charged residue of a ligand with the hydrophobic surface of a receptor will require an anion to be paired with the 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 ligand hydrophobicity is well illustrated in Fig. 6. Thus, for the Ala-face peptides (i.e., substitution of side-chains into the centre of the moderately non-polar face of an amphipathic peptide ligand), the majority of the non-polar side-chains (Ile, Val, Leu, Met, Cys, Phe and Trp; Ala is the single exception) show significant enhancement of hydrophobicity (as expressed by $+\Delta\Delta t_R$ values; Table 3) on addition of salt to the aqueous environment. The positively charged residues (Arg, His, Lys) show a similar enhancement of hydrophobicity due, as noted above, to ion-pairing of the perchlorate anion with positively charged side-chains, thus neutralizing the positive charge and decreasing its inherent hydrophilicity. A somewhat lesser effect of salt is apparent with the polar uncharged side-chains (including Tyr), where generally moderate enhancement of hydrophobicity ($+\Delta\Delta t_R$ values; Tyr, Thr, Ser, Gln) or hydrophilicity ($-\Delta\Delta t_R$ values; Glu, Asp, Asn) is observed.

From Fig. 6, it can be seen that, in the much more hydrophobic environment of the non-polar face of the Leu-face ligands, there is a trend of lesser hydrophobicity enhancement of the side-chains and/or significantly more enhancement of hydrophilicity in the presence of salt compared to the effects observed with the Ala-face ligands. Thus, the non-polar ligands, including Ala, all exhibit hydrophobic enhancement in the presence of salt ($+\Delta\Delta t_R$ values), albeit of smaller magnitude than when substituted in the Ala-face ligand. Interestingly, the three positively charged residues (Arg, Lys, His) exhibit a considerable decrease in hydrophobicity in this environment, significantly greater than for the non-polar residues. Finally, the polar residues (with the exception of Thr) now exhibit a considerable enhancement of hydrophilicity, on addition of salt, in the much more non-polar Leu-face environment. This enhancement includes Tyr and Trp which, as noted previously (Tables 1 and 2), appear to maximize expression of their non-polar (i.e. aromatic) characteristics in the moderately non-polar environment of the Ala-face ligand and their polar (e.g., hydroxyl group of Tyr

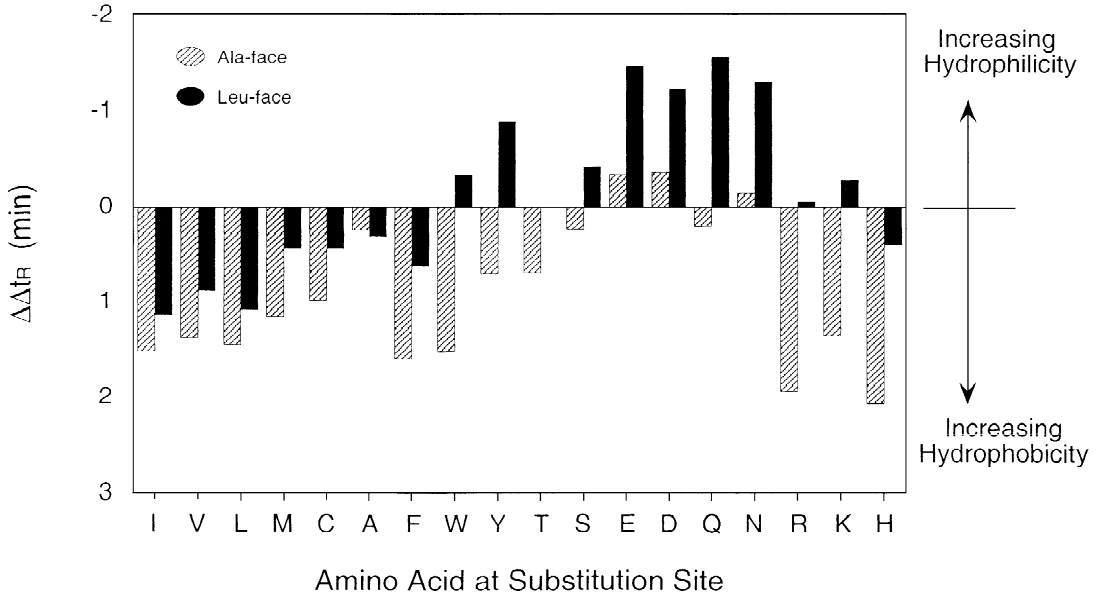

Fig. 6. Effect of salt on hydrophilicity/hydrophobicity of side-chains substituted in the non-polar faces of model amphipathic α-helical ligands. $\Delta\Delta t_R = \Delta t_R$, (AX−AG) in mobile phase 2 minus Δt_R, (AX−AG) in mobile phase 1 (hatched) or Δt_R, (LX−LG) in mobile phase 2 minus Δt_R, (LX−LG) in mobile phase 1 (shaded). Mobile phases 1 and 2 are described in Experimental. Data are taken from Table 3. The single letter code represents the amino acid substitution at position 9 of the peptide sequence (Fig. 1).

and N–H of Trp) in the highly non-polar environment of the Leu-face ligand.

3.4. Comparison of effects of changes in receptor hydrophobicity or ligand hydrophobicity on hydrophilicity/hydrophobicity of side-chains at the ligand–receptor interface

Fig. 7 compares the effect of increasing receptor hydrophobicity (cyano to C_{18} stationary phase) on side-chain hydrophilicity/hydrophobicity at the ligand–receptor interface. Thus, for increasing receptor hydrophobicity, $\Delta\Delta t_R = (\Delta t_R$, LX−LG on C_{18} column) minus (Δt_R, LX−LG on cyano column); for increasing ligand hydrophobicity, $\Delta\Delta t_R = (\Delta t_R$, LX−LG on C_8 column) minus (Δt_R, AX−AG on C_8 column). From Fig. 7, comparisons are made for all three mobile phases. As was described previously (Fig. 3), there is a decrease in $\Delta\Delta t_R$ values for all 18 amino acids in all three aqueous mobile phases when increasing the hydrophobicity of the environment surrounding the substitution site of the ligand, i.e., the side-chains of all 18 amino acids decrease in hydrophobicity, as expressed by negative $\Delta\Delta t_R$ values, when surrounded by a more hydrophobic environment. In contrast, although the overall trend is similar to that when increasing the ligand hydrophobicity, several of the more hydrophobic amino acid side-chains (Ile, Val, Leu, Phe) interestingly became more hydrophobic (i.e., they exhibited positive $\Delta\Delta t_R$ values) as the hydrophobic environment created by the receptor became more hydrophobic.

Two observations common to the effects of changing ligand hydrophobicity or receptor hydrophobicity are also apparent from Fig. 7: (1) there are enhanced interactions between ligand and receptor with both increasing receptor or ligand hydrophilicity following the addition of 100 m*M* sodium perchlorate (mobile phase 2) to the aqueous environment surrounding the ligand–receptor interface; and (2) there appears to be a general correlation between the effect of increasing receptor or ligand hydrophobicity on side-chain hydrophilicity/hydrophobicity and the underlying polarity/non-polarity of side-chains. However, concerning point (2), despite this general correlation, there are still profound differences be-

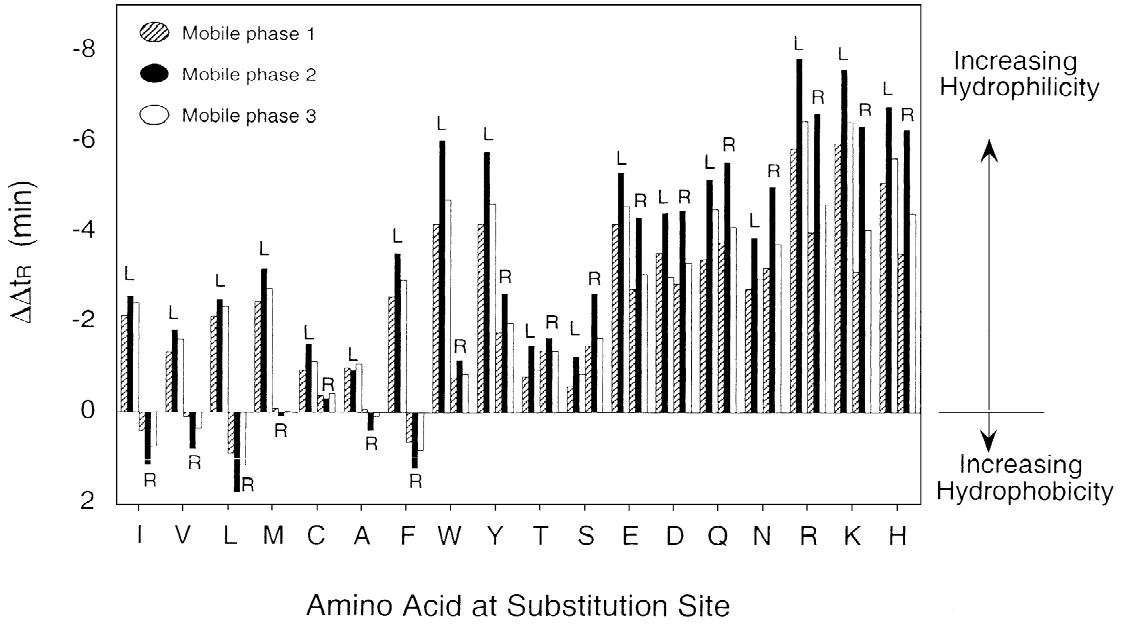

Fig. 7. Comparison of effects of varying receptor hydrophobicity or ligand hydrophobicity on hydrophilicity/hydrophobicity of side-chains substituted in the non-polar face of a model amphipathic α-helical peptide ligand. For each side-chain, the three histograms on the left, denoted by R (receptor), are results obtained from the Leu-face peptide series (denoted LX series; sequence shown in Fig. 1) when increasing the hydrophobicity of the receptor (cyano to C_{18} column); and the three on the right, denoted by L (Ligand), are results obtained from increasing the hydrophobicity surrounding the substitution site of the peptide ligand (Ala-face to Leu-face peptide series, denoted AX and LX series, respectively; sequences are shown in Fig. 1). For the three histograms on the right, denoted by R, $\Delta\Delta t_R = \Delta t_R$, (LX−LG) on the C_{18} column minus Δt_R, (LX−LG) on the cyano column (data not shown); for the three histograms on the left (denoted by L), $\Delta\Delta t_R = \Delta t_R$, (LX−LG) minus Δt_R, (AX−AG) (data for mobile phases 1 and 2 shown in Table 3). The single letter code represents the amino acid substitution at position 9 of the peptide sequence (Fig. 1). Both columns were obtained from Agilent Technologies.

tween the patterns of these effects of increasing receptor or ligand hydrophobicity on side-chain hydrophilicity/hydrophobicity. Thus, it has already been described (Figs. 2B,D and 3) that the effect of increasing ligand hydrophobicity appears to group the side-chains into three sets of amino acids, with the $\Delta\Delta t_R$ values within these three groups appearing generally to correlate in an inverse fashion with the hydrophilicity/hydrophobicity of the side-chains, i.e., the less hydrophilic (more hydrophobic) the side-chain within the group, the greater the enhancement of hydrophilicity (i.e., the more negative the $\Delta\Delta t_R$ value) when increasing ligand hydrophobicity. Conversely, this group effect is not observed in Fig. 7 when increasing receptor hydrophobicity, nor is the inverse correlation noted above for increasing ligand hydrophobicity. Thus, the more hydrophilic the side-chain (as characterized by Δt_R, LX−LG values), the greater the effect of increasing receptor hydrophobicity on the hydrophilicity of the side-chain on the face of the ligand. Indeed, Mant and Hodges [20] reported a clear correlation ($r = 0.995$) between side-chain hydrophilicity/hydrophobicity values for all of the amino acids and the magnitude of the effect of increasing receptor hydrophobicity (cyano to C_{18} column) on these values.

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 elution behaviour of de novo designed model amphipathic α-helical peptides (representing the hydrophobic binding domain of a ligand) on a reversed-phase packing (representing a receptor pro-

tein with a hydrophobic binding pocket). With this model, we have demonstrated how an increase in ligand hydrophobicity, represented by an increase in hydrophobicity of the environment surrounding the substitution site of the ligand, whilst maintaining constant receptor (C_8 column hydrophobicity), enhances hydrophilicity (decreases hydrophobicity) of all amino acids at the ligand–receptor interface. The aqueous environment surrounding the binding site of the receptor and ligand was also shown to affect the hydrophilicity/hydrophobicity of amino acids in the binding interface. Together with a separate paper which examines how the hydrophilicity/hydrophobicity of side-chains is influenced when varying the hydrophobicity of the receptor (i.e., reversed-phase packings of varying hydrophobicity [20], the potential of this RP-HPLC-based ligand–receptor model has been rigorously assessed. Refinements of the model include the preparation and packing of stationary phases of varying functional group and/or ligand density in the laboratory [32–34] in order to enhance the flexibility of the ligand–receptor model still further.

Acknowledgements

This work was supported by a N.I.H. grant to R.S.H. (R01GM 61855).

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