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Effect of the α-amino group on peptide retention behaviour in reversed-phase chromatography

Determination of the pK_a values of the α-amino group of 19 different N-terminal amino acid residues

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ABSTRACT

We have examined the contribution of the α-amino group to retention behaviour for peptides in reversed-phase chromatography using two series of peptide analogues, one containing an N^{α}-acetylated terminal and the other containing an α-amino group (non-acetylated). The effect of the α-amino group, at pH 2, on the hydrophobicity of the side-chain of the N-terminal residue was obtained by referencing the retention time of the acetylated or non-acetylated peptide to the retention time of a glycine analogue. It was shown that the presence of an α-amino group could decrease or increase the hydrophobicity of the side-chain of the N-terminal residue with respect to the hydrophobicity of the side-chain in the absence of an α-amino group. The effect was also shown to be sequence dependent, with respect to the N-terminal residue. Increasing pH was shown to increase retention time dramatically for the non-acetylated analogues, through the deprotonation of the α-amino group. By separating pairs of acetylated/non-acetylated analogues over the pH range 2–9, it was possible to determine the pK_a of the α-amino group, where it was shown that the pK_a was dependent on two probable factors: (1) the inherent hydrophobicity of the stationary phase; and (2) the amino acid substituted in the N-terminal position. Interestingly, the pK_a values determined were very similar to that found in proteins. It was also possible to determine the pK_a values of some of the substituted amino acids containing ionizable side-chains. This study shows that, in order to understand fully the retention behaviour of peptides containing an α-amino group in reversed-phase chromatography, one must incorporate an α-amino group contribution and its effect on the hydrophobicity of the side-chain of the N-terminal residue.

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INTRODUCTION

It was initially recognized by several research groups, using a wide variety of peptides, that the chromatographic behaviour of peptides in reversed-phase liquid chromatography (RPLC) could be correlated with amino acid composition [1–3]. These groups determined sets of coefficients for predicting peptide retention using computer-calculated regression analysis of retention data. This laboratory has been active for several years in attempting to correlate peptide retention behaviour in RPLC with peptide structure through the use of model synthetic peptide analogues. This minimilistic approach is the method of choice since it allows for a systematic reduction in the number of variables that affect retention behaviour. We have shown that the major contributing factor to peptide retention times on RPLC is amino acid composition [4–7], although factors such as peptide chain length [4,8] and the presence of preferred binding domains [4,9] are also pertinent. Such work has practical relevance not only in determining the best approach to the optimization of peptide separations, but also in deducing the presence of amphipathic α-helical structure in peptides based upon their retention data [4,9]. In addition, we have shown that the effects on peptide retention of ion-pairing reagents and their concentration of these reagents in the mobile phase, are predictable [10]. Indeed the research in this area, carried out in this laboratory, has led to the development of a commercially-available computer-based teaching and research program, ProDigest-LC [4,11,12], which simulates peptide elution profiles on the major modes of high-performance liquid chromatography (HPLC) employed for peptide separations (size-exclusion, ion-exchange and reversed-phase chromatography).

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

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

EXPERIMENTAL

Materials

HPLC-grade water and acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ, USA). ACS-grade orthophosphoric acid and triethylamine (TEA, redistilled before use) was obtained from Anachemia (Toronto, Canada). Trifluoroacetic acid (TFA), 99 + %, was obtained from Aldrich (Milwaukee, WI, USA). Sodium perchlorate ($NaClO_4$) was obtained from BDH (Poole, U.K.).

Instrumentation

The HPLC system consisted of either a Varian Vista Series 5000 chromatograph (Varian, Walnut Creek, CA, USA) or an HP1090 liquid chromatograph (Hewlett-Packard, Avondale, PA, USA), coupled to an HP1040A detection system, HP9000 series 300 computer, HP9133

disc drive, HP2225A Thinkjet printer and HP7460A plotter.

Columns

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

Peptide synthesis

Peptides were synthesized using an Applied Biosystems (Foster City, CA, USA) 430A peptide synthesizer using the following protocol. The peptides were synthesized on co-poly (styrene–1% divinylbenzene) benzhydrylamine–hydrochloride resin (0.92 mmol/g resin). All amino acids were protected at the α-amino position with the *tert.*-butoxycarbonyl (Boc) group and the following side-chain protecting groups were used: [Arg(4-toluenesulfonyl), Asp(cyclohexyl), Cys(4-methylbenzyl), Glu(benzyl), His(2,4-dinitrophenyl), Lys(2-chlorobenzyloxycarbonyl), Ser(benzyl), Thr(benzyl), Trp(formyl), Tyr(2-bromobenzyloxycarbonyl)]. All amino acids were single coupled as preformed symmetrical anhydrides (with the exception of Arg, Asn and Gln which were coupled as the HOBT active ester) in dichloromethane. Boc groups were removed at each cycle with an 80-s reaction with TFA–dichloromethane (33:67, v/v), followed by a second reaction with TFA–dichloromethane (50:50, v/v) for 18.5 min. Neutralizations were carried out using 10% DIEA–DMF (v/v). N-terminal residues were acetylated using acetic anhydride–dichloromethane (25:75, v/v) for 10 min. Prior to cleavage the DNP group of His was removed by treating the peptide resin with a solution of 2-mercaptoethanol (20%) and diisopropylethylamine (10%) in dimethylformamide for 2 h. The peptides were cleaved from the resin by treatment with anhydrous hydrogen fluoride (20 ml/g resin) containing 10% anisole and 2% 1, 2-ethanedithiol for 1 h at −4°C. After cleavage from the resin, the formyl group of Trp was removed by treating the peptide with piperidine–water (2:98, v/v). The resulting deprotected peptide solution was freeze dried. Peptides were determined to be pure by HPLC and mass determination (using a BioIon 20 plasma desorption time-of-flight mass spectrometer).

RESULTS AND DISCUSSION

Model synthetic peptides

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

Ac–Xxx–(Leu–Gly–Ala–Lys–Gly–Ala–Gly–Val–Gly)–amide (series 1)
H–Xxx–(Leu–Gly–Ala–Lys–Gly–Ala–Gly–Val–Gly)–amide (series 2)
Ac–(Leu–Gly–Ala–Lys–Gly–Ala–Gly–Val–Gly)–amide (core sequence)

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

The core sequence Ac–(Leu–Gly–Ala–Lys–Gly–Ala–Gly–Val–Gly)–amide was chosen since it lacks any ability to form an amphipathic helix, a preferred binding domain, known to cause deviations from predicted retention behavior [9]. A 10-residue length for the peptide analogues was chosen for two reasons: (1) this size of

peptide represents an average sized fragment that may be obtained from a proteolytic digest of a protein; and (2) the effect of chain length on the retention behaviour of a peptide of this size will be small [8].

Residues in the core sequence represent an overall hydrophobicity, as defined by Guo *et al.* [6], which results in a peptide that is eluted within, or near to, a 15–40% acetonitrile range during linear A–B gradient elution, the region where the best resolution may generally be obtained [15]. The presence of a lysine residue at position five of the peptide analogues ensures sufficient peptide solubility.

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

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

From Fig. 1, which plots retention time *versus* pH for a number of analogues, it can be seen that over the pH range tested, pH has a very small effect on the retention behaviour for the majority of acetylated analogues (left panel), as would be expected. The interesting exception is the Leu analogue, which shows a distinct drop in the retention time as the pH is raised. In the pH range of 2 to 6.8, the elution order of the Leu and Ile analogues (the side-chains of these res-

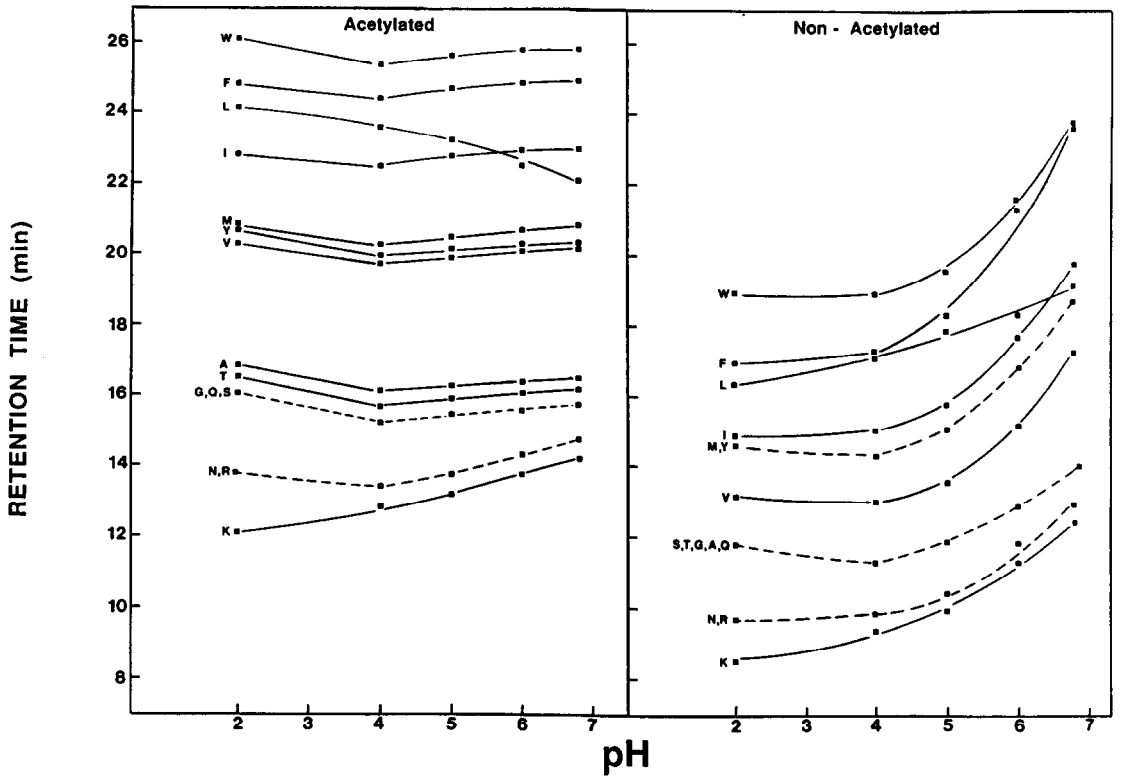

Fig. 1. Plot of retention time of acetylated (left) and non-acetylated (right) peptide analogues *versus* pH. Column: Pep-S C_{18}/C_2, 250 × 4 mm I.D., 5-μm particle size, 100-Å pore size. Mobile phase: linear A–B gradient elution (2% B/min, equivalent to 1% acetonitrile/min) at a flow-rate of 1 ml/min.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

idues have the same number of carbon atoms, but differ in that Ile is β-branched) is reversed.

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

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

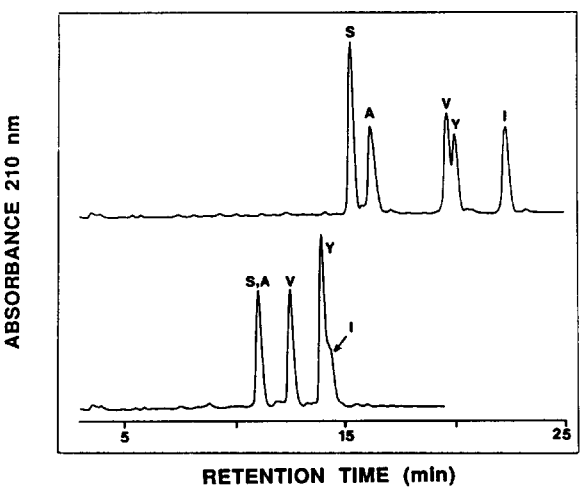

Fig. 2. Effect of the α-amino group on the elution profile of a mixture of five peptide analogues, either acetylated (upper) or non-acetylated (lower), at pH 2. Column as in Fig. 1. Mobile phase: linear A–B gradient elution (2% B/min, equivalent to 1% acetonitrile/min) at a flow-rate of 1 ml/min, using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

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

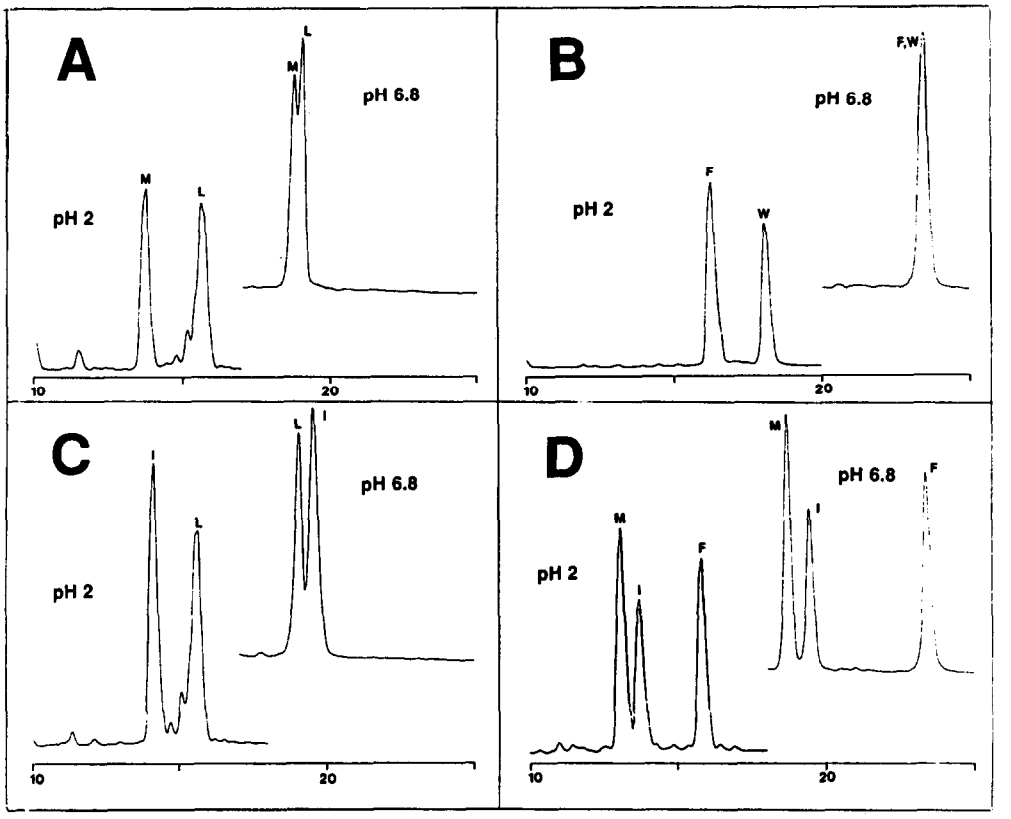

Fig. 3. Effect of pH on reversed-phase chromatographic elution profiles of specific pairs of non-acetylated peptide analogues. Column and mobile phase as in Fig. 1, using pH 2 and pH 4–7 systems. For A–D, see text.

H–Ile analogues are not baseline resolved; whereas, this is achieved at pH 6.8. Also from Fig. 3D, the H–Phe analogue is much better separated from the other two analogues at pH 6.8 (Δt for the Phe/Ile pair = 3.9 min) than at pH2 (Δt for the Phe/Ile pair = 2.1 min). Fig. 3C shows the inversion of elution order of the non-acetylated analogue Ile/Leu pair at pH 6.8 and pH 2 (it should be noted that this effect is also seen with the acetylated Ile/Leu pair; Fig. 1, left panel).

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

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

$$a = t_{R_{H\text{-}Gly}} - t_{R_{Ac\text{-}Gly}} \quad (1)$$

$$H = t_{R_{Ac\text{-}Xxx}} - t_{R_{Ac\text{-}Gly}} \quad (2)$$

$$h = [t_{R_{H\text{-}Xxx}} - t_{R_{Ac\text{-}Gly}}] - a \quad (3)$$

$$s = h - H \quad (4)$$

The effect of the α-amino group on the retention behaviour of each of the peptide analogues at pH 2 was determined using eqn. 1, where a is the difference between the retention time of the α-amino glycine analogue, $t_{R_{H-Gly}}$, and that of the acetylated glycine analogue, $t_{R_{Ac-Gly}}$. From eq. 2, the retention time of the acetylated analogue, $t_{R_{Ac-Xxx}}$, minus the retention time of the acetylated glycine analogue, $t_{R_{Ac-Gly}}$, defines the hydrophobicity, H, of the side-chain in the absence of an α-amino group at pH 2. The retention time difference between the α-amino analogue, $t_{R_{H-Xxx}}$, and the acetylated glycine analogue, $t_{R_{Ac-Gly}}$, is a combination of two effects: the α-amino group contribution, a, as defined by eqn. 1, plus the hydrophobicity of the side-chain of the N-terminal residue, h, in the presence of an α-amino group. As a result, the hydrophobicity of the side-chain of the N-terminal residue in the presence of a free α-amino group may be calculated from eqn. 3. It should be noted that, in subtracting the retention time of the acetylated glycine analogue from either the acetylated or non-acetylated analogues, the effect seen will be due to the side-chain of each substituted amino acid. The effect of the α-amino group on the side-chain of the N-terminal residue, s, may therefore be calculated from the difference between the values of h and H (eqn. 4), *i.e.*, the hydrophobicity, h, of the side-chain in the presence of an α-amino group minus the hydrophobicity, H, of the side-chain in the absence of an α-amino group will provide a value for the effect of the α-amino group on the hydrophobicity of the side-chain. The calculated values obtained from retention time data are summarized in Table I.

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

The hydrophobicities of the side-chains in the

TABLE I

EFFECT OF THE α-AMINO GROUP ON HYDROPHOBICITY OF THE SIDE-CHAIN OF THE N-TERMINAL RESIDUE

Column and mobile phase as in Fig. 5, using the pH 2 system.

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

[a] Amino acid represents the substitution made in the N-terminal position of each peptide analogue, as described under *Model synthetic peptides*.

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

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

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

presence of an α-amino group (h) are also shown in Table I. For example, in the presence of an α-amino group, the hydrophobicity of the Trp side-chain is decreased from 10.1 min to 9.3 min, suggesting that the presence of the fully positively charged α-amino group (at pH 2) caused the reduction in hydrophobicity of the Trp side-chain, resulting in the 0.8 min decrease in the retention time for this peptide sequence. The presence of an α-amino group also results in decreased hydrophobicities for other hydrophobic residues, *e.g.*, Phe was reduced from 8.8 to 5.5 min and Leu was reduced from 7.5 to 4.6 min. Alternatively, the presence of a charged α-amino group causes the hydrophilicity of the side-chain of several hydrophilic residues (*e.g.*, Lys, His, Arg) to decrease, *e.g.*, a decrease in side-chain hydrophilicity is seen for Lys, where the negative contribution to the retention time of this peptide sequence results in a decrease from −3.5 min to −1.6 min. It should be noted that similar values of H and h were obtained when these series of peptide analogues were separated by linear A–B gradient elution using a C_8 silica-based column (data not shown).

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

The results shown in Figs. 4 and 5 are further evidence that the α-amino group does affect the

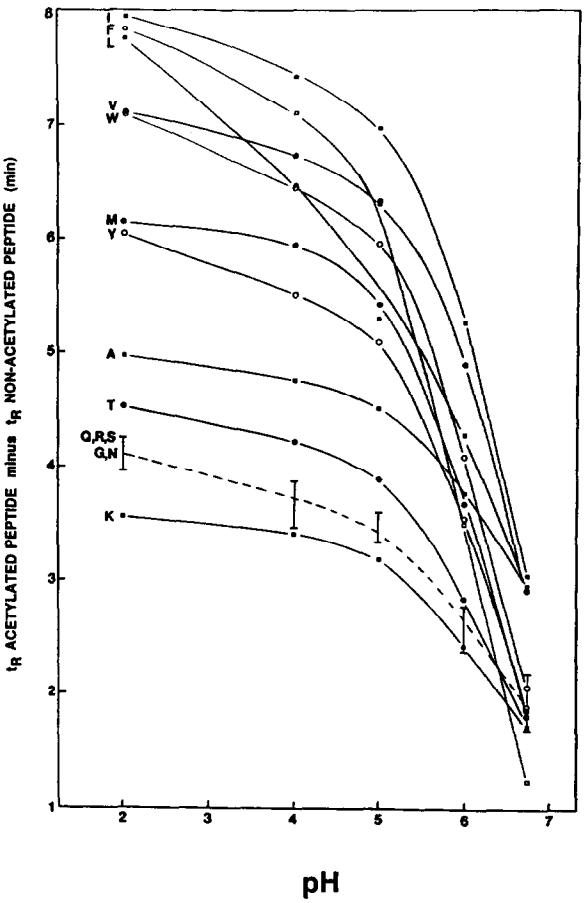

Fig. 4. Plot of retention time of acetylated peptide analogue minus retention time of non-acetylated peptide analogue *versus* pH. Column and mobile phase as in Fig. 1, using pH 2 and pH 4–7 systems. Dotted lines denote an average retention time difference *versus* pH profile of peptide analogues whose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

hydrophobicity of the side-chain of the N-terminal residue. If, at pH 2, one assumes that the α-amino group did not affect the hydrophobicity of the side-chain, then one would expect that the retention time difference between the acetylated analogue and the non-acetylated analogue (*i.e.*, $t_{R_{Ac-Xxx}} - t_{R_{H-Xxx}}$) would be the same for all peptide analogues, essentially, this difference would be equal to the contribution of the α-amino group. From Fig. 5A, it can be seen that the difference between each acetylated/non-acetylated analogue pair exhibits a unique value. For example, $t_{R_{Ac-Ile}}$ minus $t_{R_{H-Ile}}$ has a value

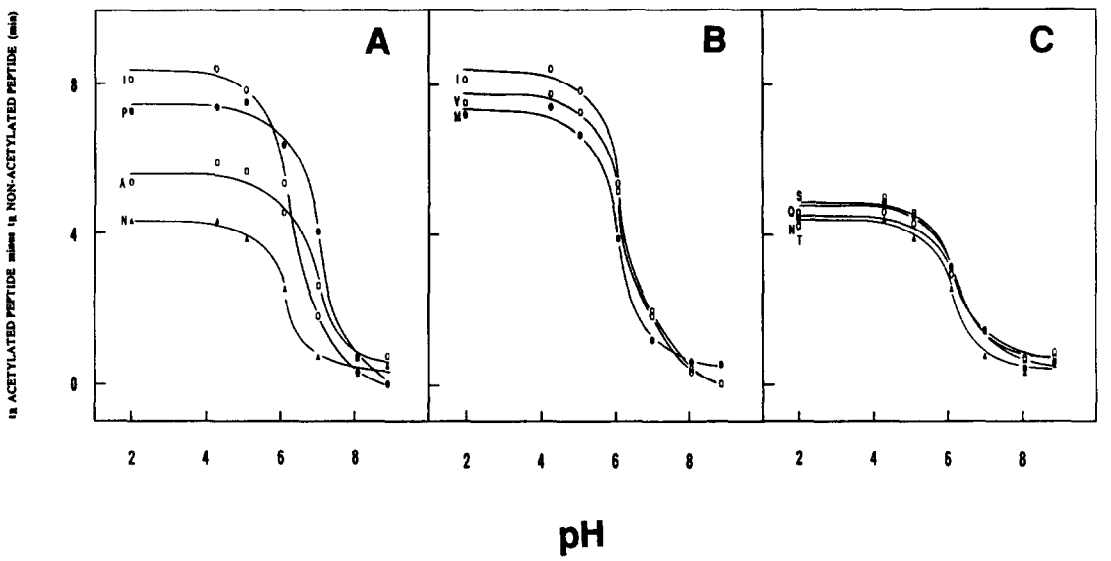

Fig. 5. Plot of retention time of acetylated peptide analogue minus the retention time of the non-acetylated peptide analogue *versus* pH. Column: PLRP-S (250 × 4.6 mm I.D., 5 μm, 100 Å). Mobile phase: linear A–B gradient elution (2% B/min equivalent to 1% acetonitrile/min) at a flow-rate of 1 ml/min.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

that is slightly greater than 8; whereas, $t_{R_{Ac-Asn}}$ minus $t_{R_{H-Asn}}$ has a value that is slightly greater than 4. This further supports the concept that the α-amino group affects the hydrophobicity of the N-terminal residue and that it does so in a residue dependent manner.

Titration of the α-amino group of the peptide analogues

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

Retention time data for the acetylated and the non-acetylated analogues generated for producing Fig. 1, were used to produce Fig. 4, which plots retention times of acetylated analogues minus the retention times of the corresponding non-acetylated analogues *versus* pH. These plots represent partial titration curves for the α-amino group. From Fig. 4, it can be seen that each analogue, or group of analogues, exhibits an unique profile, suggesting that the α-amino group is being deprotonated at different rates depending on the N-terminal substitution. For example, the Leu analogue shows a very dramatic retention time difference compared to the Lys analogue, where the retention time difference is considerably smaller. This plot suggested that, if the pH range of the plot could be extended to higher pH values, the pK_a of the α-amino group for each analogue could be determined. In order to extend this range, acetylated and non-acetylated analogues were chromatographed on a poylstyrene column, PLRP-S, using linear AB gradient elution (1% acetonitrile/min at a flow-rate of 1 ml/min) over a pH range of 2 to 9. The retention time data from these runs is summarized in Fig. 5, which shows the α-amino group titration curves for selected analogues and the pK_a values obtained from these data are presented in Table II. Fig.

TABLE II

pK_a VALUES OF THE α-AMINO GROUPS OF PEPTIDE ANALOGUES CONTAINING 19 DIFFERENT N-TERMINAL AMINO ACID RESIDUES

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

[a] Amino acid denotes the substitution made in the N-terminal position of each peptide analogue as described under *Model synthetic peptides*.

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

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

5A illustrates the large range of pK_a values exhibited by the peptide analogues, *e.g.*, Asn has a pK_a value of 6.1; in contrast, Pro has a pK_a of 7.1. Fig. 5B and C show that for some analogues, the pK_a values may be very similar, *e.g.*, the values for Ile and Val are 6.4 and 6.5, respectively, and those of Ser and Gln are 6.3 and 6.4, respectively. It should also be noted that, at pH 9, all analogues approach a similar retention time difference [*i.e.*, $t_{R_{Ac-Xxx}} - t_{R_{H-Xxx}}$], suggesting that the deprotonated α-amino group has a similar contribution to retention behaviour as that of an N^{α}-acetylated terminal.

From Table II, it can be seen that the range of pK_a values of the α-amino groups obtained in this study varies from 7.1 (Pro) to 6.0 (Phe); in contrast, the pK_a values for the free amino acids vary from 9.0 to 10.6 [16], *i.e.*, the pK_a values obtained in this study are considerably lower than those for free amino acids in solution. The pK_a for the α-amino group in some proteins may be much lower than that found for free amino acids in solution, *e.g.*, in human CO-hemoglobin (α-chain), the value is 6.72 [17] and in bovine pancreatic ribonuclease A the value is 8.14 [18] representing pK_a values that are on the order of 1–2 pH units lower than that found for the free amino acids. It has been previously reported that an increasingly hydrophobic environment may affect the ionization of a potentially ionizable group as evidenced by the decreased dissociation of the α-carboxyl group of glycine (*e.g.*, increased pK_a from 2.35 to 3.96) and the increased dissociation of the α-amino group of glycine (*e.g.*, decreased pK_a from 9.78 to 7.42) with increasing percentage of organic solvent in aqueous solutions of this amino acid [19]. These examples taken together suggest that the environment can have a major effect on the dissociation of an ionizable group. From Table II, it can be seen that the pK_a of the α-amino group determined in this study is reasonably similar to the pK_a values found in some proteins (*i.e.*, 6.0 to 7.1 for this study as opposed to 6.72 to 8.14 found in proteins). Due to the inherently hydrophobic nature of the stationary phase that was used to separate these peptide analogues and the fact that a hydrophobic environment could affect the pK_a of an α-amino group, this suggests that the hydrophobic stationary phase may be a reasonable mimic for the hydrophobic environment created by proteins. It has also been previously reported, with simple organic molecules, that substitutions on the carbon atom adjacent to an ionizable group (*e.g.*, COOH) may alter the ionization of that group, *e.g.*, the carboxyl group of ethanoic acid, H_3C-CO_2H, where the α-carbon contains three hydrogen atoms, has a pK_a of 5.55; whereas that of 2,2-diethylbutanoic acid, $(CH_3CH_2)_3-C-CO_2H$, where the α-carbon has three ethyl groups attached to it, has a pK_a of 6.44 [19]. Creighton [19], refers to this effect as a steric effect. In the

present study, the pK_a values for the Ala and Leu substituted analogues were 6.8 and 6.3, respectively. This decrease in pK_a could be explained either by a steric effect or simply an increase in the hydrophobicity of the amino acid side-chain between Ala and Leu. It has been previously reported by Cantor and Schimmel [20] that electrostatic interactions can also have a significant effect on the pK_a of the α-amino group. For example, when titrating the α-amino group of the amino acid Ala (where there are favourable electrostatic interactions between the α-amino group and the α-carboxyl group) the pK_a value is 9.69; in contrast when titrating $(Ala)_4$ (where this same electrostatic interaction is no longer significant), the pK_a value is 7.94, suggesting that a favourable interaction results in a decreased dissociation of the α-amino group [20]. In the present study, the same effect is seen when comparing the N-terminal residue of the peptide analogues containing an acidic side-chain as opposed to those containing a basic side-chain. Basic residues result in a lower pK_a (*e.g.*, Arg = 6.1, Lys = 6.2) than acidic residues (*e.g.*, Glu = 6.6, Asp = 6.8), possibly through unfavourable (repulsive) electrostatic interactions between the positively charged side-chain of the basic residue and the α-amino group, resulting in increased dissociation of the α-amino group (*i.e.*, a lower pK_a); in contrast, there is the potential for favourable (attractive) electrostatic interactions between the negatively charged side-chain of an acidic residue and the α-amino group, resulting in decreased dissociation of the α-amino group (*i.e.*, a higher pK_a). From Table II, it can be seen that a range of pK_a values (6.0 to 7.1) was obtained for the analogues tested, suggesting that the pK_a is dependent on the N-terminal residue. This dependency is consistent with the pK_a of the α-amino group of substituted dipeptides of glycine (*e.g.*, the pK_a for Asp–Gly = 9.07, Pro–Gly = 8.97, Gly–Gly = 8.13, Ser–Gly = 7.33 and Asn–Gly = 7.25) [21] which suggests that the pK_a is dependent on the N-terminal residue. These data taken together suggest that the pK_a values obtained in this study are dependent on three factors: (1) the hydrophobicity of the environment which stems from the hydrophobicity of the stationary phase (2) the hydrophobicity of the N-terminal residue and (3) the charge on the side-chain of the N-terminal residue.

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

Fig. 6 plots retention times of acetylated analogues minus the retention time of the acetylated core peptide *versus* pH. This plot is effectively a titration curve of residues with ionizable side-chains. From Fig. 6A, it can be seen that, for these analogues, with neutral amino acids at the N-terminal, increasing pH has little effect on retention behaviour. This would be expected, since these substitutions do not contain ionizable side-chains (with the exception of Tyr) and the data presented in this plot is consistent with the plot in Fig. 1, *i.e.*, the plot in Fig. 6 is an alternate way of representing the effect of pH on the acetylated analogues, with the exception that the pH range has been extended to pH 9.

Fig. 6B allows one to observe the titration of ionizable side-chains of the N-terminal residue. The pK_a values obtained from this plot are presented in Table III. From this table, it can be seen that the pK_a value for the side-chain of an acidic residue can be significantly higher in proteins than for the pK_a in free amino acids. For example, the pK_a of Asp is 3.65 for the free amino acid; in contrast, in some proteins this value may be as high as 6.7 or 6.8 (Asp in the catalytic site of the serine proteases α-lytic protease and trypsin respectively) or 10 (Asp 96 in the membrane bound protein bacteriorhodopsin [29]). From the crystallographic structure of serine proteases, the Asp residue in this site is known to be in a hydrophobic environment which would tend to raise the pK_a with respect to the free amino acid [22]. Also, the pK_a of Glu is 4.25 for the free amino acid; in contrast, for example, it may be as high as 6.0 in lysozyme (*e.g.*, Glu 35). From the crystal structure of lysozyme, it is known that Glu 35 is in a hydrophobic environment and in addition, the ionization of this Glu is thought to be affected by the ionization of Asp 52, resulting in a pK_a that is much higher than that of the free amino acid [24]. In addition, the pK_a of Glu 35 may be

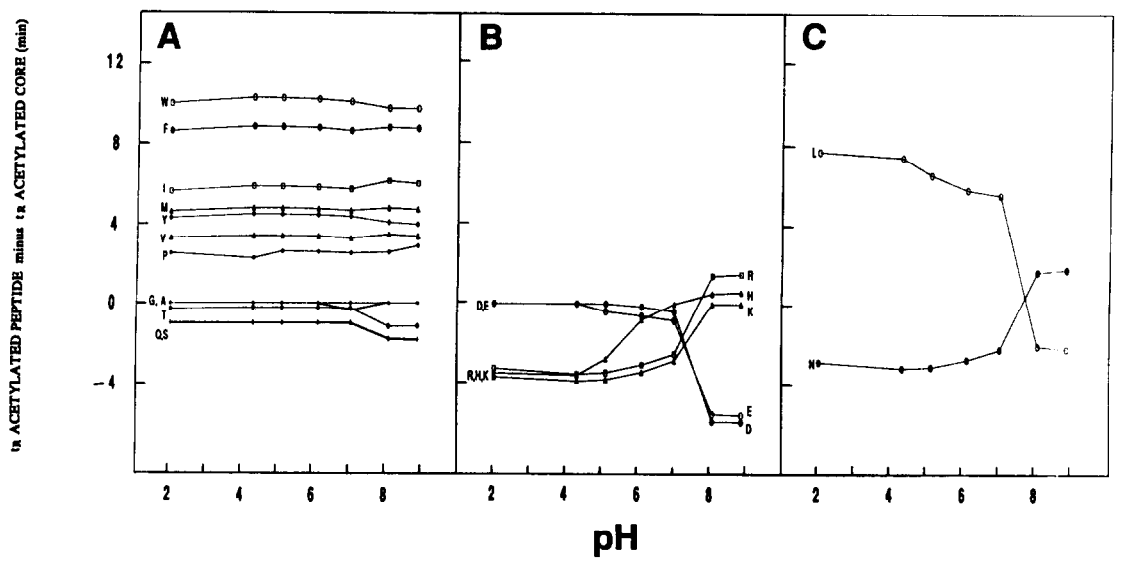

Fig. 6. Plot of retention time of acetylated peptide analogue minus the retention time of the acetylated core (Ac–Leu–Gly–Ala–Lys–Gly–Ala–Gly–Val–Gly–amide) *versus* pH. Column and mobile phase as in Fig. 5.

TABLE III

pK_a VALUES OF THE FUNCTIONAL SIDE-CHAINS OF FIVE N-TERMINAL AMINO ACID RESIDUES

Peptide analogue[a]	This study[b]	Amino acid[c]	Proteins
Asp (D)	7.5	3.65	6.7/6.8[d], 10[d]
Glu (E)	7.4	4.25	6.0, 6.5, 8.0–8.5[e]
Arg (R)	7.3	12.48	11.6–12.6[f]
Lys (K)	7.4	10.79	9.11/5.9[g]
His (H)	5.8	6.0	5.0–8.0[h]

[a] Amino acid denotes the substitution made in the N-terminal position of each peptide analogue as described under *Model synthetic peptides*.

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

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

[d] Asp (D): Asp = 6.7, Asp in catalytic triad of α-lytic protease [22], Asp 102 = 6.8 in trypsin [23] and Asp 96 = 10 in the photosynthetic protein bacteriorhodopsin [29].

[e] Glu (E): Glu 35 in lysozyme; native, enzyme-inhibitor (NAG_3) complex and enzyme–substrate (glycol–chitin) complex respectively in 0.15 *M* KCl [24,25].

[f] Arg (R): Arg = 11.6–12.6, p. 120 [26].

[g] Lys (K): Lys 41 = 9.11 in bovine pancreatic ribonuclease A [18]; Lys = 5.9, Lys at active site of acetoacetate decarboxylase [27].

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

significantly altered when either inhibitor or substrate is bound at the active site (*e.g.*, pK_a is 6.5 and 8.0 to 8.5 for bound inhibitor or substrate, respectively) [25]. In comparison, the pK_a values determined in this study were found to be significantly higher than that for the free amino acid (*e.g.*, Asp = 7.5 and Glu = 7.4) but comparable to those observed in proteins. In the same manner that the stationary phase (or hydrophobic environment of a protein) could potentially cause the value to decrease for the α-amino group, the stationary phase could be responsible for the values obtained for these acidic residues (*i.e.*, a more hydrophobic environment results in decreased dissociation, *i.e.*, a higher pK_a value) and the high values obtained in this study could be a reflection of the very hydrophobic nature of the stationary phase. The high values obtained for the side-chains of the acidic residues could have important implications in the use of cationic pairing agents, where it has been suggested that 0.01 *M* triethylamine acetate at pH 5.5 or 0.01 *M* tetrabutylammonium phosphate at pH 7 [28], be used to effect a separation of peptides/proteins containing acidic residues. With the pK_a of the acidic residues being as high as 7.5/7.4 in RPLC (as determined in this study), there may be no ion-pairing at the lower pH (5.5). From Table

III, it can also be seen that, for residues with basic side-chains, the pK_a in proteins can be lower than that for the free amino acids. For example, the pK_a for Arg is 12.48 in the amino acid; whereas, in proteins this value may be decreased to 11.6. The pK_a value for Lys is 10.79 in the free amino acid; whereas, in proteins this value may be decreased to 9.11 (Lys 41 in bovine pancreatic ribonuclease A, where this reduced value appears to be the result of a neighboring charged Arg residue) [18]. In addition, the pK_a of the ε-amino group of a Lys residue in the active site of the enzyme acetoacetate decarboxylase (the pK_a is only 5.9), may be significantly different than that of the free amino acid [27]. As with the α-amino group, the environment of these side-chains can significantly alter the values for the pK_a and the values obtained in this study may reflect the very hydrophobic nature of the stationary phase. No value for the pK_a was obtained for Tyr and this is probably due to the fact that the pK_a values for this residue in proteins can range from 9.5 to >12 [16], which is out of range for this study.

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

CONCLUSIONS

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

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