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Quantitation of the Nearest-neighbour Effects of Amino Acid Side-Chains that Restrict Conformational Freedom of the Polypeptide Chain using Reversed-Phase Liquid Chromatography of Synthetic Model Peptides with L- and D-amino Acid Substitutions

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Abstract

Side-chain backbone interactions (or “effects”) between nearest neighbours may severely restrict the conformations accessible to a polypeptide chain and thus represent the first step in protein folding. We have quantified nearest-neighbour effects (i to $i + 1$) in peptides through reversed-phase liquid chromatography (RP-HPLC) of model synthetic peptides, where L- and D- amino acids were substituted at the N-terminal end of the peptide sequence, adjacent to a L-Leu residue. These nearest-neighbour effects (expressed as the difference in retention times of L- and D- peptide diastereomers at pH 2 and pH 7) were frequently dramatic, depending on the type of side-chain adjacent to the L-Leu residue, albeit such effects were independent of mobile phase conditions. No nearest-neighbour effects were observed when residue i is adjacent to a Gly residue. Calculation of minimum energy conformations of selected peptides supported the view that, whether a L- or D- amino acid is substituted adjacent to L-Leu, its orientation relative to this bulky Leu side-chain represents the most energetically favourable configuration. We believe that such energetically favourable, and different, configurations of L- and D- peptide diastereomers affect their respective interactions with a hydrophobic stationary phase, which are thus quantified by different RP-HPLC retention times. Side-chain hydrophilicity/hydrophobicity coefficients were generated in the presence of these nearest-neighbour effects and, despite the relative difference in such coefficients generated from peptides substituted with L- or D- amino acids, the relative difference in hydrophilicity/hydrophobicity between different amino acids in the L- or D- series is maintained. Overall, our results demonstrate that such nearest-neighbour effects can clearly restrict conformational space of an amino acid side-chain in a polypeptide chain.

1. Introduction

Although protein folding has been studied for more than 40 years, the exact details remain extremely complex (see reviews in refs. 1-4). The classic experiments by Anfinsen showed that in the presence of a powerful denaturant (8 M urea), ribonuclease had no enzymatic activity or defined structure but the protein could refold and regain its full activity and structure by removal of the denaturant. These results led Anfinsen to conclude that the information needed to specify the complex three-dimensional structure of a protein is contained in its amino acid sequence [5-7]. In order to decipher the problem of protein folding, one must unravel all the

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non-covalent interactions encoded in the primary amino acid sequence that guide the initial hydrophobic collapse in an aqueous environment, subsequently leading to a compact protein fold with well defined secondary structures. The Levinthal Paradox clearly shows that there is insufficient time to search in a random manner the entire conformational space available to a polypeptide chain as an unfolded protein, i.e., the protein must fold through some directed process [8]. The restriction of conformational space could begin with the polypeptide backbone itself. Thus, Ramachandran showed that steric clashes restrict the backbone phi/psi angles of the polypeptide chain [9]. More recently, it has been suggested that the cumulative effects of many local side-chain backbone interactions between each amino acid and its immediate neighbours may severely restrict the conformations accessible to a polypeptide chain [10,11].

Specific pathways for the protein folding process can be initiated by native-like [12] and non-native [13] hydrophobic clusters of non-polar amino acids that maintain residual protein interactions even in experimental denaturing environments [14,15]. Furthermore, the local amphipathicity and hydrophobic clustering pattern of short stretches of amino acid sequence often correlate with their secondary structure, i.e., α -helices and β -sheets [16]. These results suggest that the early stages of protein folding may involve transient formation of secondary structure elements or nucleation sites stabilized by a combination of long range and local hydrophobic interactions. Despite the aforementioned complexity of the protein folding problem, it is clear that both backbone and side-chain forces, e.g., nearest-neighbour effects, contribute to protein folding, although the exact balance between these forces remains unknown and remains a critical parameter in the eventual goal of understanding and predicting the final three-dimensional structure of a protein from polypeptide sequence information alone.

Two major approaches to studying the protein folding problem include the “global” approach where, for instance, the effect of small polypeptide composition changes on overall protein structure and stability (e.g., via site-directed mutagenesis) are examined; and a peptide-based approach where observations of the effect of sequence changes on interactions stabilizing higher orders of conformation (secondary, tertiary, quaternary structure) of synthetic *de novo* designed model peptides are extrapolated to proteins as a whole. The objective of the present study was to demonstrate that we could, via a synthetic peptide-based approach, quantitatively measure nearest-neighbour interactions (or “effects”) in model peptides, since such effects, as noted above, are known to contribute to protein folding and overall protein stability. Thus, nearest-neighbour effects are defined as a restriction of conformational freedom about the peptide bonds between residue i and $i + 1$. It was our hypothesis that these nearest-neighbour effects would be dependent on the type of residue at position i and $i + 1$. In other words, all 20 amino acid side-chains could be substituted adjacent to a Gly residue, with just a hydrogen atom as its side-chain group, and there would be no nearest-neighbour effects between residue i and Gly at the $i + 1$ position. In contrast, substitution of all 20 amino acid side-chains adjacent to a large, bulky and hydrophobic side-chain such as leucine at the $i + 1$ position would show significant nearest-neighbour effects dependent on the residue at position i .

Reversed-phase high-performance liquid chromatography (RP-HPLC) has seen extensive use as a physicochemical model of biological systems, such studies frequently centered on correlating peptide [17-22] or protein [23-30] retention behaviour with polypeptide conformational stability. The assumption with such studies is that the hydrophobic interactions between peptides and proteins with the non-polar stationary phase characteristic of RP-HPLC [31-33] mimics the hydrophobicity and interactions between non-polar residues which are the major driving forces for protein folding and stability. In a previous paper [34], we categorized two types of sequence-dependent effects resulting in deviations from predictable RP-HPLC retention behaviour of peptides: conformational effects resulting in an apparent reduction or enhancement of the overall hydrophobicity of a peptide as a result of its adopting an unique

conformation on interacting with the hydrophobic stationary phase, compared to the hydrophobicity of the peptide if it existed as a random coil; and nearest-neighbour effects resulting in sequence-dependent variability of peptide retention behaviour but independent of a defined secondary structure (β -turn, β -sheet or α -helical). Whilst the former effects have been well studied [34], useful quantification of nearest-neighbour effects on peptide retention behaviour in RP-HPLC has proven more elusive. The present study sets out to remedy this deficiency by observing the RP-HPLC retention behaviour of the model peptide Ac-X-Leu-Gly-Ala-Lys-Gly-Ala-Gly-Val-Gly-amide (where position X is substituted by the 19 L- and D- amino acids and glycine) under several mobile phase conditions. Differences in observed retention times of the L- and D- peptide diastereomers, due to the presence of leucine adjacent to the substitution site, have enabled us to make an initial quantification of nearest-neighbour effects on peptide retention behaviour and should also prove a useful first step in extrapolating such values to the protein folding problem.

2. Experimental

2.1. Materials

Reagent-grade phosphoric acid (H_3PO_4) was obtained from Caledon Laboratories (Georgetown, Ontario, Canada). TFA was obtained from Hydrocarbon Products (River Edge, NJ, USA); NaCl and NaClO_4 were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was obtained from Fisher Scientific (Pittsburgh, PA, USA). Fluoroxyloxymethylcarbonyl (Fmoc) amino acids and resins were obtained from Novabiochem (San Diego, CA, USA). De-ionized water was purified by an E-pure water filtration device from Barnstead/Thermolyne (Dubuque, IA, USA).

2.2. Instrumentation

RP-HPLC runs were carried out on an Agilent 1100 Series liquid chromatograph from Agilent Technologies (Little Falls, DE, USA).

2.3. Columns

RP-HPLC runs at pH 2 were carried out on a Kromasil C_{18} column (150 mm x 2.1 mm I.D.; 5- μm particle size; 100-Å pore size) from Hichrom, Berkshire, UK; pH 7 runs were carried out on a Zorbax Eclipse XDB- C_8 column (150 mm x 2.1 mm I.D.; 5- μm particle size; 80-Å pore size) from Agilent Technologies, this latter column being designed for excellent chemical and thermal stability at neutral pH values.

2.4. Peptide synthesis and purification

Peptide synthesis was carried out by solid-phase peptide synthesis methodology using a 4-(2', 4'-dimethoxyphenyl-Fmoc-amino methyl)-phenoxyacetamido-norleucyl-MBHA resin with conventional Fmoc chemistry.

2.5. Purification of crude peptides

Crude peptides were purified by RP-HPLC on the Kromasil C_{18} column using a linear AB gradient (1% B/min) at a flow-rate of 0.3 ml/min, where Eluent A is 0.2% aq. TFA and Eluent B is 0.2% TFA in acetonitrile. The correct masses of the peptides were confirmed by electrospray mass spectrometry using a Mariner Biospectrometry Workstation mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA).

2.6 Analytical RP-HPLC of synthetic model peptides

Linear AB gradient (0.25% acetonitrile/min) at a flow-rate of 0.3 ml/min and a temperature of 25°C.

Mobile phase 1: Eluent A is 20 mM aq. H_3PO_4 , pH 2, and Eluent B is 20 mM H_3PO_4 in acetonitrile, denoted pH 2/ H_3PO_4 system.

Mobile phase 2: Eluent A is 20 mM aq. TFA, pH 2, and Eluent B is 20 mM TFA in acetonitrile, denoted pH 2/TFA system.

Mobile phase 3: Eluent A is 10 mM aq. NaH_2PO_4 , adjusted to pH 7 with NaOH, and Eluent B is Eluent A containing 50% acetonitrile, denoted pH 7/no salt system.

Mobile phase 4: same as mobile phase 3 but also both eluents containing 50 mM NaCl, denoted pH 7/NaCl system.

Mobile phase 5: same as mobile phase 3 but also both eluents containing 50 mM NaClO_4 , denoted pH 7/ NaClO_4 system.

2.7. Circular dichroism spectroscopy

Circular dichroism spectroscopy was carried out at 20°C on a Jasco J-810 spectropolarimeter with constant N_2 flushing (Jasco Inc., Easton, MD). Rectangular quartz cells (1.0 mm path length) were used and an average of six scans reported. Results were subsequently expressed as mean residue molar ellipticity $[\theta]$ ($\text{deg}^* \text{cm}^2 \text{dmol}^{-1}$) as calculated in the following manner:

$[\theta] = \theta_{\text{obs}} \times \text{MRW} / [10 \times \text{path length (cm)} \times \text{concentration (mg/ml)}]$ where MRW is the mean residue weight (molecular mass of the peptide divided by the number of residues).

The peptides were prepared as stock solutions in benign buffer (50 mM sodium chloride, 10 mM sodium phosphate pH 7) at ~ 1 mg/ml (~ 1250 μM). Exact peptide concentrations were determined by amino acid analysis in triplicate. For spectra scans, the peptides were diluted to ~ 0.2 mg/ml (~ 250 μM) in either benign buffer or trifluoroethanol (TFE) (50% v/v) and ellipticity was measured from 195-250 nm.

2.8 Calculation of conformational energetics of peptides

Energy calculations were performed on dipeptides representing the two N-terminal residues of model peptides, blocked with N-methylamide at the C-terminal end. This approximation is used since the C-terminal portion of the model peptide sequence is designed to be flexible and have negligible structure; hence, only the conformational behaviour of the first two residues should be important in controlling separation during RP-HPLC. The calculations determine the intrinsic conformational preference of the dipeptides which can then be modulated by interactions with either the solvent or the stationary phase or both if the peptide sits at the interface of these two phases. The conformation of such N-terminal dipeptides is determined mainly by the psi angle of residue 1 and the phi angle of residue 2. These two angles were thus used to create a psi, phi map similar to the phi, psi map of Ramachandran [9]. The psi, phi maps were computed using the Consistent Valence Force Field (CVFF) potential [35], the psi and phi angles being restrained using a forcing function of 3.49 kcal/mol/degree. The angles were incremented by 10 degrees from -180 to +170. At each point, all degrees of freedom were minimized to create a flexible geometry map. All calculations were done in vacuo to determine the intrinsic conformational properties of the dipeptides. For each dipeptide, separate maps were computed for the two main chi1 values seen in protein structures for the corresponding amino acids: 180 degrees and -56 degrees for L amino acids and the corresponding 56 degrees for D- amino acids. The structures reported are the lowest energy conformations over all maps computed for each dipeptide.

3. Results

3.1. Design of model peptides

The sequence chosen to quantify nearest-neighbour effects on peptide retention behaviour during RP-HPLC was Ac-X-L-G-A-K-G-A-G-V-G-amide, where position X was substituted by the 19 L- and 19 D- amino acids and glycine. This sequence contains four Gly residues spread periodically throughout the sequence to ensure that the peptide has no secondary structure tendencies [36,37]. The N-terminus was acetylated and the C-terminus was amidated to eliminate potential effects of a positively charged α -amino group or negatively charged α -carboxyl group on peptide retention behaviour. A single Lys residue was incorporated into the model peptide sequence to ensure peptide solubility over a wide pH range. The four hydrophobic residues in the peptide sequence (2 Ala, 1 Val, 1 Leu) were distributed throughout the peptide sequence to ensure no clustering of hydrophobes and subsequent creation of a preferred binding domain. The 10-residue length of the model peptide was selected to enable full expression of the overall hydrophilicity/hydrophobicity of the peptide in the absence of any chain-length effects (generally >15 residues) [38] which may diminish the contribution to RP-HPLC retention behaviour of substituted residues.

The substitution site, X, was situated next to a Leu residue since it was hypothesized that such a bulky side-chain would maximize any nearest-neighbour effects on the adjacent substituted side-chain. Further, its neutral, non-polar characteristics would eliminate any concerns of data interpretation complications, in this initial study, due to electrostatic (i.e., positively charged or negatively charged side-chains) or polar interactions (e.g., hydrogen bonding) affecting nearest-neighbour effects on the substituted amino acid. Finally, L- and D- amino acids were substituted at position X since it was hypothesized that, due to the different configuration of such amino acid pairs, subsequent differences in conformational freedom about the X-Leu peptide bond would be reflected in differences in exposure of this substituted residue at position X and, hence, could be measured by variations in observed RP-HPLC retention behaviour. Note that, for the purposes of the present study, only the effect of one adjacent (Leu) residue on peptide retention behaviour is under consideration (i.e., position X is the N-terminal residue) in order to simplify data interpretation in this initial assessment of our RP-HPLC/peptide model system. Also note that Gly was at position 3 to prevent any nearest-neighbor interactions with Leu at position 2. Thus, only the interactions or effects caused by the amino acid at position X and the adjacent Leu are being determined.

3.2. Structural characterization by circular dichroism spectroscopy

To verify that none of the peptides adopted any significant secondary structure in benign conditions (10 mM PO₄ buffer containing 50 mM NaCl, pH 7) and in 50% trifluoroethanol which was used as a mimic of the hydrophobic environment of reversed-phase chromatography, we analyzed peptide structure by circular dichroism spectroscopy. All of the peptides showed random-coil type spectra as expected considering the four Gly residues spread periodically throughout the sequence. Although we could observe small differences in the spectra between X_LL and X_DL peptides, these differences could not be used to interpret any type of defined structure in the presence of the overwhelming random coil structure in the peptides. Our hypothesis was that reversed-phase chromatography would be the most sensitive measure of conformational freedom about the peptide bond between X_LL or X_DL and that these results would be representative of the restriction of conformation space for the folding of a polypeptide chain in aqueous conditions. In other words, the interaction of the peptides with the hydrophobic matrix is a measure of the restriction of conformational space encountered by a polypeptide sequence during the folding event.

3.3. Nearest-neighbour effects on RP-HPLC retention behaviour of model peptides

In a previous paper [39], L- and D- amino acids were substituted at position X of a model peptide with the sequence Ac-X-G-A-K-G-A-G-V-G-L-amide, i.e., with the same overall composition as the model peptide in the present study but with the substitution site now adjacent to a Gly residue. Co-elution of the L- and D- amino acid substituted peptide pairs of these peptides demonstrated that there was complete freedom of rotation about the peptide bond between residue X and Gly. Thus, any variation in retention behaviour of the model peptides in the present study would indeed be due to the substitution site being adjacent to a bulky Leu residue.

The L- and D- amino acid substituted model peptides, as well as the Gly-substituted peptide, were subjected to RP-HPLC under five mobile phase conditions: 20 mM H₃PO₄ or 20 mM TFA at pH 2; and 10 mM PO₄ buffer at pH 7, containing no salt, 50 mM NaCl or 50 mM NaClO₄. Note that a very shallow gradient (0.25% acetonitrile/min) was employed in the peptide separations in order to maximize any differences in retention behaviour of L- and D- peptide diastereomers.

Fig 1 illustrates elution profiles of representative L- and D- amino acid substituted peptide pairs at pH 2 (pH 2/TFA system). The retention data for all peptides under the five mobile phase conditions are shown in Table 1. From Fig. 1 and Table 1 it is clear that, unlike substitution adjacent to a Gly residue [39], the presence of a Leu residue adjacent to the substitution site results in significant separation of L- and D- peptide pairs. In addition, the degree of this separation, as well as the relative order of the L- and D- diastereomers, varies quite dramatically. Such observations are discussed below.

3.3.1. Effect of mobile phase conditions on retention behaviour of peptides—

Table 2 reports the affect of mobile phase additives at pH 2 and pH 7 on retention times of the L- and D- amino acid substituted model peptides. Thus, at pH 2.0, the values shown are the change in peptide retention time between the pH 2/TFA and pH 2/H₃PO₄ mobile phase systems ($\Delta t_R = t_R$ in TFA minus t_R in H₃PO₄), i.e., the effect of replacing an hydrophilic phosphate anion with an hydrophobic trifluoroacetate (TFA⁻) anion. At pH 7, the values shown are the change in peptide time between the pH 7 (no salt) system and either the pH 7/NaCl mobile phase ($\Delta t_R = t_R$ in presence of 50 mM NaCl minus t_R in absence of salt) or the pH 7/NaClO₄ mobile phase ($\Delta t_R = t_R$ in presence of 50 mM NaClO₄ minus t_R in absence of salt). Addition of salts (generally 50-100 mM) to mobile phases over a pH range of ~4 -7 has generally been designed, for silica-based packings, to suppress negatively charged silanol interactions with positively charged solutes [31-33,40-48], with potential selectivity effects tending to be a secondary consideration. However, our laboratory has demonstrated how salt (specifically sodium perchlorate) addition may offer gains in peptide selectivity at low pH [49].

From Table 2, the increase in anionic counterion hydrophobicity (phosphate < TFA⁻) at pH 2 or the addition of salt to the 10 mM NaH₂PO₄ buffer has an essentially equal effect on the retention time of each peptide in a diastereomeric peptide pair, albeit the magnitude of this effect varies depending on the mobile phase and the amino acids and potentially negatively charged (Asp, Glu) side-chains.

At pH 2, the negatively charged anion (phosphate, TFA⁻) of the ion-pairing reagents H₃PO₄ and TFA, respectively, affect peptide retention behaviour via interaction with positively charged groups in the peptide, the more hydrophobic TFA⁻ enhancing peptide retention times over that of the hydrophilic phosphate anion [31-33,50-52]. At pH 2.0, with the exception of Lys, His and Arg, this enhancement of peptide retention time in 20 mM TFA over that of 20 mM H₃PO₄ is due to interaction of the hydrophobic TFA⁻ ion with the single positively charged amino group on the side-chain of the single Lys residue in the remaining 17 peptides. Hence,

for these peptides substituted with neutral (at pH 2.0) side-chains at position X, the effect of the increase in hydrophobicity of the anion on peptide retention time is similar (particularly considering the very shallow nature of the acetonitrile gradient) within a group of peptides (i.e., L- or D- amino acid substituted peptides; the median Δt_R values for the L- and D- diastereomers were 7.9 min + 1.2 and 8.0 min + 1.2, respectively) or similar between diastereomeric peptide pairs (Table 2). However, for the peptides substituted with Lys, His or Arg, the extra positive charge on these peptides resulted in a marked increase in retention time between the hydrophilic H_3PO_4 system and the hydrophobic TFA system (approximately twice the median values for the L- and D- peptide diastereomers noted above).

At pH 7 (Table 2), the addition of 50 mM NaCl to the 10 mM NaH_2PO_4 buffer had, overall, only a relatively small effect on peptide retention time of both the L- and D- series peptides. Again, this effect was relatively similar between L- and D- amino acid substituted peptide pairs and within the L- and D- peptide series, including both those substituted with potentially positively charged (Lys, Arg and His, albeit His is, likely to be completely deprotonated, i.e., neutral, at pH 7) and the potentially negatively charged Asp and Glu amino acids (both of these acidic residues are likely to be completely deprotonated, i.e., negatively charged at pH 7). Such results confirm earlier observations [53] that the chloride ion is relatively ineffective as an ion-pairing reagent. In contrast, the addition of 50 mM $NaClO_4$ to the buffer at pH 7 was much more dramatic than that of 50 mM NaCl, with significant increases in peptide retention time for 18 out of 20 of the peptides for both series of peptides (the only exceptions being the Asp- and Glu-substituted analogs). Such results for $NaClO_4$ are consistent with the highly effective ionpairing properties of the perchlorate (ClO_4^-) anion [53]. In addition, in a similar manner to the pH 2 results where the hydrophobic TFA^- anion enhanced the retention times of the Lys-, His- and Arg-substituted peptides relative to phosphate, the presence of perchlorate at pH 7 generally shows a significant enhancement of the retention times of these peptides over that of the remaining 17 peptides. The retention times of the L- and D- peptide diastereomers are again essentially identical whilst the effect of $NaClO_4$ on retention times within the L- and D- peptide series are similar, albeit there is evidence of some variation which appears to correlate approximately with the hydrophilicity/hydrophobicity of the substituted amino acid; i.e., without including the potentially positively charged (Lys, His, Arg) and the potentially negatively charged (Asp, Glu) residues, the effect of salt addition decreases with decreasing hydrophobicity of the substituted side-chain, e.g., from a value of 13.0 min for the D- Leu-substituted peptide to 8.3 min for D- Asn. A similar, albeit not as clear, effect can also be seen at pH 2. The significantly lesser effect of the presence of $NaClO_4$ on the retention behaviour of the Asp- and Glu- substituted peptides is likely due to the lack of ion-pairing interactions between the positively charged side-chains and the ClO_4^- anion in the presence of the negatively charged side-chains.

3.3.2. Evaluation of nearest-neighbour effects by RP-HPLC of model peptides—

Table 3 reports the quantitative effects of nearest-neighbour interactions of the 20 amino acids under the five mobile phase conditions, as defined by the magnitude of the difference in retention times between the L- and D- peptide diastereomers, i.e., $\Delta t_{R(D-L)}$. Interestingly, the Δt_R values are generally very similar for all five mobile phases, i.e., the magnitude of the nearest-neighbor effect is essentially independent of pH and mobile phase conditions. Some exceptions are apparent, notably Glu in the pH 7/ $NaClO_4$ mobile phase (0.1 vs. 0.8-1.4), Asp at pH 7 (-0.7—1.0) versus pH 2 (0), and His in the pH 7/no salt (-4.3) and NaCl (-5.4) systems (versus -3.1—3.3 in other mobile phase systems). However, overall there is a distinct consistency of Δt_R values under all conditions.

Also from Table 3, there is a wide range of $\Delta t_{R(D-L)}$ values for the 20 amino acids, ranging, in the pH 2/ H_3PO_4 system, for example, from + 7.8 min for Phe to -3.1 min for His. This range is quite consistent for all five mobile phases. Significantly, there is a good general correlation

of Δt_R values with relative hydrophilicity/hydrophobicity of the side-chains, i.e., Δt_R becomes generally increasingly more positive with increasing side-chain hydrophobicity and less positive with increasing side-chain hydrophilicity. Note that a positive Δt_R value indicates that the D- amino acid substituted peptide was eluted later than its L-counterpart; in contrast, a negative Δt_R value indicates that the D- amino acid substituted peptide was eluted prior to its L-counterpart.

The varying magnitude of the Δt_R values shown in Table 3 is likely due to a combination of side-chain characteristics (hydrophilicity/hydrophobicity, size and structure) and the final configuration of the L- or D- amino acid when substituted adjacent to a leucine residue. A starting point for speculation would be that, whether a L- or D- amino acid is substituted at position X of the model peptide sequence, the configuration of its side-chain at the end of the polypeptide chain, i.e., its orientation relative to the bulky Leu side-chain at the $i + 1$ position, represents the most energetically favourable configuration. In addition, no interactions are taking place between the substituted amino acid and the adjacent Leu residue. In order to test such speculations, the conformational energetics of selected peptides were studied to determine how the combination of the aforementioned side-chain characteristics and final configuration of the L- or D- amino acid affects the preferred conformation of the peptides when substituted adjacent to a L-Leu residue (see section 2.8 for experimental details). Thus, Fig. 2 shows the minimum energy conformations of dipeptides corresponding to the N-termini of peptides substituted at position X by L-Leu or D- Leu (Fig. 2A), L-Trp or D- Trp (Fig. 2B), L-Ser or D- Ser (Fig. 2C) and L-Thr or D-Thr (Fig. 2D). In addition, Table 4 shows the relative energy (relative to the lowest energy of the map assigned to be zero) of the L-L and D-L dipeptide conformations. This gives the amount of energy needed to make the L-L dipeptide adopt the D-L minimum energy conformation and, similarly, for the D-L dipeptide to adopt the L-L minimum energy conformation.

Thus, for example, when the large, hydrophobic side-chain of D-Leu ($\Delta t_R = 6.7$ min in the pH 2/ H_3PO_4 system) is substituted at position X, it was speculated that the most favourable positioning of this side-chain relative to the adjacent L-Leu may be such that the interaction of the D-Leu side-chain with the hydrophobic stationary is favoured more than with a L-Leu substitution, thus enhancing the apparent hydrophobicity of the D-Leu relative to L-Leu, i.e., the L-amino acid substituted peptide is eluted prior to its D- counterpart. Such a mechanism would also be consistent with the general correlation of side-chain intrinsic hydrophobicity of the non-polar side-chains with their Δt_R values. From Fig. 2A, it is indeed clear that the preferred conformation of the D-Leu substituted peptide puts the two large hydrophobic side-chains on one side of the molecule and able to interact with a non-polar surface such as that characteristic of RP-HPLC. In contrast, for the L-Leu substituted peptide, the hydrophobic side-chains are positioned diagonally opposite to each other and, hence, unable to interact with a planar surface at the same time. Thus, the retention time of the D-Leu substituted peptide would be expected to be greater than that of the L-Leu substituted peptide.

From Table 3, the negative $\Delta t_{R(D-L)}$ values for polar and charged residues may also be explained if it is assumed that the orientation of D-amino acids that favours interaction of hydrophobic side-chains over that of the L-isomers also applies to such highly polar and charged side-chains. For example, favourable orientation of a D-Lys side-chain when adjacent to a Leu residue would bring a positively charged side-chain in proximity to the hydrophobic stationary phase to a greater extent than if a L-Lys residue was substituted, i.e., the overall apparent hydrophobicity of the D-Lys substituted peptide would be less than that of its L-Lys counterpart and, hence, would be eluted earlier.

It is interesting to note that the Δt_R value for Asp (0 min in pH 2/ H_3PO_4 system) became negative, i.e., more polar, at pH 7, following deprotonation (i.e., the acquisition of a negative

charge) at neutral pH. A similar, albeit lesser, trend was also seen for Glu between pH 2 and pH 7, which did not exhibit a negative Δt_R value despite its general decreasing positive Δt_R value.

Such an enhancement in the hydrophilic character of the D-Ser side-chain (a polar hydroxyl group on the β -carbon) relative to the L-Ser could also account for the large negative Δt_R value (-1.6 min) reported in Table 3. In contrast, the positive value for Thr ($\Delta t_R = +1.3$) may be due to masking of the polar hydroxyl group on the β -carbon of the D-Thr side-chain by the methyl group on this same β -carbon, i.e., the hydrophobic characteristics of the D-Thr are dominant, leading to more favoured hydrophobic interactions of this side-chain with the stationary phase relative to L-Thr. Figs. 2C and 2D show, respectively, the preferred conformations of the L-Ser/D-Ser and L-Thr/D-Thr substituted dipeptides. Note that, for the L-Ser substituted dipeptide (Fig. 2C), the non-polar N-terminal methyl group of the N^α-acetyl moiety is on the same side as the adjacent L-Leu side-chain, allowing a strong interaction with a non-polar stationary phase. In contrast, for the D-Ser substituted peptide, the polar hydroxyl group of Ser is on the same side as the adjacent L-Leu side-chain, making this surface a lower affinity interaction with a non-polar stationary phase compared to the L-Ser substituted peptide. In the case of the D-Thr substituted peptide (Fig. 2D), the extra methyl group in the side-chain of Thr provides a non-polar surface for interaction with a non-polar stationary phase. Although this is also true of the L-Thr substituted peptide (Fig. 2D), the amide group of the Thr residue is not hydrogen bonded in this case and is pointing directly towards the non-polar surface. The latter unfavourable situation would explain the reduction in retention time of the L-Thr substituted peptide compared to D-Thr.

Fig. 3 shows a plot of the nearest neighbor effect (Δt_R (D-L)) versus side-chain hydrophobicity of the D-substituted peptides (Δt_R (Gly)). There is an excellent linear correlation ($R = 0.972$) between the observed nearest neighbour effects and side-chain hydrophilicity/hydrophobicity, that is, as the hydrophobicity of the side-chain increases the nearest neighbour effects increase for 17 amino acid side-chains. Similarly, there is an excellent linear correlation ($R = 0.999$) for 3 amino acid side-chains (Trp, Tyr and His) and for these side-chains the nearest neighbour effects are significantly smaller compared to their measured side-chain hydrophobicity relative to the other side-chains.

An interesting anomaly from Table 3 is the Δt_R value for Trp (3.4 min in the pH 2/H₃PO₄ system) which is similar to the moderately hydrophobic side-chain Ala despite its high intrinsic hydrophobicity [39]. However, the observed retention times of both the L-Trp and D-Trp substituted peptides are long (66.5 min and 69.9 min, respectively, in the 20 mM H₃PO₄ system; Table 1), as would be expected for such a hydrophobic side-chain, i.e., it is just the difference in retention time between the two peptides which is unexpectedly small. Fig. 2B shows the preferred conformations of the Trp-substituted dipeptides. From Fig. 2B, the configuration of the side-chain of D-Trp relative to the adjacent L-Leu does suggest a more complementary interaction of these two hydrophobic side-chains with a non-polar surface relative to that obtained with a L-Trp substitution and, hence, producing a greater retention time for the former compared to the latter. However, the interaction of peptides with a non-polar stationary phase is a balance of polar and non-polar interactions. The reduction in retention time difference between the L-Trp and D-Trp substituted peptides is likely due to the high overall hydrophobicity of peptides containing Trp whether in the L or D form. Hence, the interaction of either peptide with the non-polar stationary phase is dominated by the hydrophobic component, with the polar component playing a lesser role. This would imply that the difference in retention times between the L and D peptides will be smaller than might be expected. Additionally, in the D conformation (Fig. 2B), the Trp ring nitrogen is pointing towards the non-polar stationary phase surface and, thus, not making a hydrogen bond. This would reduce the interaction of the D-Trp residue with the stationary phase.

It was demonstrated by our laboratory [54] that a hydrophobic environment enhanced the hydrophilic character of polar/charged side-chains. The orientation of D- Tyr towards the hydrophobic matrix when substituted adjacent to Leu is likely enhancing the hydrophilic nature of its hydroxyl group relative to its L-Tyr counterpart, hence the overall lesser hydrophobicity of the D-Tyr-substituted peptide causing it to be eluted prior to its L-diastereomer ($\Delta t_R = -1.6$ min in the pH 2/H₃PO₄ system).

Interestingly, what these three side-chains (Trp, Tyr and His) have in common at pH 7 is aromaticity and polar hetero-atoms in the rings of His and Trp and the hydroxyl attached to the aromatic ring in the case of Tyr. If there is a difference in solvation-desolvation properties of these side-chains relative to the other 17 side-chains, then the relationship of nearest-neighbour effects and hydrophobicity may be different (Fig. 3).

Fig. 4 now plots the $\Delta t_{R(D-L)}$ values of the 20 amino acids obtained in the pH 2/H₃PO₄ mobile phase versus the pH 2/TFA mobile phase (Fig. 4A) and the $\Delta t_{R(D-L)}$ values at pH 7 in the absence of salt versus those in the presence of 50 mM NaCl or 50 mM NaClO₄ (Fig. 4B). From Fig. 4A, the magnitude of the nearest-neighbour effects at pH 2 is shown to be independent of the relative hydrophobicity of the anion (phosphate < TFA⁻) in the mobile phase (an excellent correlation of 0.997). A similar result is seen at pH 7 (Fig. 4B) where, despite slightly anomalous behaviour of the His value in 50 mM NaCl, the Δt_R values were essentially independent of the effectiveness of the anion (Cl⁻ < ClO₄⁻) in the mobile phase (a correlation of 0.995).

3.3.3. Hydrophilicity/hydrophobicity coefficients of L- and D- amino acids in presence of nearest-neighbour effects—A large number of RP-HPLC-derived hydrophilicity/hydrophobicity coefficients of amino acid side-chains based on RP-HPLC of collections of random peptides [40,55-65] or synthetic model peptides [39,42,54,66] have been published over the past 25 years. Unlike the scales reported by Kovacs et al [39] which were determined in the absence of nearest-neighbor effects (i.e., intrinsic side-chain hydrophilicity/hydrophobicity was measured), these earlier scales were, amongst other factors, influenced by nearest-neighbour effects such as those described in the present study.

In order to illustrate the variation in apparent side-chain hydrophilicity/hydrophobicity which may occur even in a relatively simple model peptide system such as that described in the present study, where amino acid substitutions were made next to a single adjacent Leu residue, hydrophilicity/hydrophobicity coefficients were now determined for both the L- and D- amino acid side-chains in our model peptide. The retention time of the Gly-substituted peptide was used as a reference since Gly has only an hydrogen atom as its side-chain. Thus, the hydrophilicity/hydrophobicity coefficients of the 19 L- side-chains and 19 D- side-chains were generated from the RP- HPLC runs carried out under the five mobile phase conditions from the difference in the retention times (Δt_R) of the X-substituted peptide and the Gly-substituted peptide, i.e., $\Delta t_R = t_R$ X-substituted peptide minus t_R Gly-substituted peptide (Table 5). Thus, from Table 5, side-chains that are more hydrophobic than Gly have positive Δt_R values and side-chains that are more hydrophilic than Gly have negative Δt_R values.

From Table 5, there is a clear difference in the side-chain coefficients obtained from the L- and D- amino acid substituted peptides, despite the similarities in relative hydrophilicity/hydrophobicity within the L- and D- series, i.e., most hydrophobic to most hydrophilic side-chains. In general, side-chains more hydrophobic than that of Gly (i.e., positive Δt_R values) show a greater relative hydrophobicity when substituted as the D- amino acid than the L- amino acid; in contrast, side-chains more hydrophilic than that of Gly (i.e., negative Δt_R values) show a greater relative hydrophilicity when substituted as the D- amino acid than the L- amino acid. Such variations between the L- and D- amino acids are likely due, as discussed previously, to

the different configurations assumed by the side-chains due to the adjacent Leu residue and, hence, their relative exposure to the reversed-phase matrix. Such exceptions to this general observation are apparent: Tyr, despite its moderately high apparent hydrophobicity (Δt_R values of 13.2 min and 14.2 min for D-Tyr and L-Tyr, respectively), shows a higher apparent hydrophobicity for the L- amino acid, likely, as noted above, to the response of its polar hydroxyl group to its environment when in the L- versus D- configuration; Asp shows essentially identical coefficients in the pH 2/H₃PO₄ system and pH 2/TFA system and more apparent hydrophobicity for the L-amino acid relative to the D- isomer in all three pH 7 systems; Gln, unlike in the other four mobile phases, has positive Δt_R values in the pH 7/NaCl system, with L-Gln having a larger apparent hydrophobicity (1.9 min) compared to D- Gln (1.6 min); also, Asn, in a similar manner to Gln, has positive Δt_R values in the pH 7/NaCl system, with values for L-Asn and D-Asn of 1.7 min and 0.7 min, respectively.

Fig. 5 now correlates the hydrophilicity/hydrophobicity coefficients of the L- amino acids (A) and D- amino acids (B) obtained in the pH 2/H₃PO₄ mobile phase versus those obtained in the pH 7/NaClO₄ system. From Fig. 5, 15 out of the 20 coefficients (including Gly) L- and D- amino acids show an excellent correlation ($r = 0.994$ and 0.997 for the L- and D- amino acids, respectively) between the two mobile phase systems. Only the potentially positively charged (Lys, His, Arg) and potentially negatively charged (Asp, Glu) side-chains deviate from these excellent correlations. In the case of the acidic Asp and Glu amino acids, the deprotonation of their side-chains above a pH of ~ 4 and their subsequent assumption of a negative charge makes them considerably more hydrophilic at pH 7 compared to pH 2 (Table 5). In contrast, deprotonation of the His side-chain above a pH value of ~ 6 and ion-pairing of the Lys and Arg side-chains with the negatively charged, highly effective [53] perchlorate anion makes these residues more hydrophobic at pH 7 compared to pH 2 (Table 5). Note that the excellent correlation of the remaining side-chain coefficients (i.e., those with neutral side-chains) indicates that, despite the difference in magnitude of the L- and D- amino acid coefficients derived from the model peptide, these coefficients are independent of pH and change in anionic counterion (phosphate versus perchlorate).

Fig. 6 compares the L- and D- amino acid coefficients obtained at pH 2 in the pH 2/H₃PO₄ (A) and pH 2/TFA (B) mobile phase systems. From Fig. 6, the good correlations for all 20 amino acids in both mobile phase systems ($r = 0.982$ and 0.976 , for Panels A and B, respectively) indicates that, despite some small selectivity variations, the side-chain coefficients of the L- and D- amino acids are essentially independent of the nature of the anion in the RP-HPLC mobile phase at pH 2, i.e., the hydrophilic phosphate anion versus the hydrophobic TFA⁻ anion.

Finally, Fig. 7 compares the L- and D- amino acid coefficients obtained at pH 7 in the pH 7/ no salt (A), pH 7/NaCl (B) and pH 7/NaClO₄ (C) mobile phase systems. From Fig. 8, the generally good correlations ($r = 0.977$, 0.972 and 0.979 , for Panels A, B and C, respectively) for all 20 amino acids demonstrates that the side-chain coefficients of the L- and D- amino acids are generally independent of the absence (Panel A) of added anion or the presence and nature of the anion in the RP-HPLC mobile phase at pH 7, i.e., the ineffective (at ion-pairing) Cl⁻ anion (Panel B) versus the effective perchlorate anion (Panel C) [53].

4. Conclusions

Nearest-neighbour effects in peptides have been quantified by RP-HPLC of model synthetic peptides, where L- and D- amino acids were substituted at the N-terminal end of the peptide sequence, adjacent to a Leu residue. Differences in retention times of L- and D- peptide diastereomers represented nearest-neighbour effects at pH 2 and pH 7. These nearest-neighbour effects are quite dramatic (differences in retention time between L- and D- peptide diastereomers ranged from approximately +8 min for Phe to -4 min for His (Table 3), depending

on the type of side-chain adjacent to the Leu residue, and generally independent of mobile phase conditions (pH, ion-pairing reagent and salt). In addition, side-chain hydrophilicity/hydrophobicity coefficients were generated in the presence of these nearest-neighbour effects. Despite the quantitative differences between the L- and D- amino acid side-chain coefficients created by amino substitution adjacent to a L-Leu residue, the relative difference in hydrophilicity/hydrophobicity between different amino acid side-chains in the L- or D- series is maintained, resulting in excellent observed correlations. Such nearest-neighbour effects can clearly restrict conformational space of an amino acid side-chain when substituted adjacent to a bulky residue such as Leu. We believe this initial study represents an important first step in extrapolating nearest- neighbour values to the protein folding process.

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Fig 1.

Demonstration of nearest-neighbor effects in a model peptide system. Column: Kromasil C18. Conditions: linear AB gradient (0.25% CH₃CN/min, starting from 2% CH₃CN) at a flow-rate of 0.3 ml/min, where eluent A is 20 mM aq. TFA and eluent B is 20 mM TFA in CH₃CN; temperature, 25°C. Panels A, B and C are representative RP-HPLC elution profiles at pH 2.0 of peptides of the same sequence (Ac-X-L-G-A-K-G-A-G-V-G-amide), where position X contains L- or D- amino acid substitution adjacent to a Leu residue. The peptides are denoted by the three letter code of the substituted amino acid residues, e.g. D-Lys or L-Lys.

Fig 2.

The minimum in vacuo energy conformation of the following peptides: (A) Ac-L-Leu-L-Leu-NMe and Ac-D-Leu-L-Leu-NMe; (B) Ac-L-Trp-L-Leu-NMe and Ac-D-Trp-L-Leu-NMe; (C) Ac-L-Ser-L-Leu-NMe and Ac-D-Ser-L-Leu-NMe; (D) Ac-L-Thr-L-Leu-NMe and Ac-D-Thr-L-Leu-NMe. The hydrophobic groups (side-chains of Leu and Trp, methyl group of Thr side-chain and methyl group of the N-acetyl blocking group) are coloured purple. Other atoms are coloured as follows: oxygen, red; nitrogen, blue; hydrogen, white; carbon, grey.

Fig 3.

Plot of nearest neighbour effects ($\Delta t_{R(D-L)}$) versus side-chain hydrophobicity of the D-substituted peptides ($\Delta t_{R(Gly)}$) in 10 mM NaH_2PO_4 + 50 mM NaCl mobile phase, pH 7. Closed circles denote data used in the correlation plot ($y = 0.2555x - 0.230$, correlation coefficient $r = 0.972$) and open circles denote data used in correlation plot ($y = 0.2156x - 4.038$, correlation coefficient $r = 0.999$).

Fig 4.

Comparison of nearest-neighbour effects in different mobile phase conditions. Plot of $\Delta t_{R(D-L)}$ of peptides in 20 mM H_3PO_4 (pH 2) versus 20 mM TFA (pH 2) (**A**) and 10 mM NaH_2PO_4 (pH 7) versus 10 mM NaH_2PO_4 (pH 7) with 50 mM NaCl or 50 mM $NaClO_4$ (**B**). Column: Kromasil C18, pH 2; Zorbax XDB C8, pH 7. Conditions: linear AB gradient (0.25% CH_3CN/min) at a flow-rate of 0.3 ml/min. Panel A: eluent A is 20 mM aq. H_3PO_4 or 20 mM TFA, pH 2 and eluent B is the respective eluent A in CH_3CN ; Panel B: eluent A is 10 mM aq. NaH_2PO_4 , pH 7, containing no salt, 50 mM NaCl or 50 mM $NaClO_4$ and eluent B is the respective eluent A containing 50% CH_3CN ; temperature, 25°C. Data are taken from Table 3. The single letter code represents the L- amino acid substitutions at position X of the peptide

sequence shown in Section 3.1. Closed circles denote data used in the correlation plot ($y = 0.9905x + 0.0599$, correlation coefficient $r = 0.997$ and $y = 1.0604x + 0.053$, correlation coefficient $r = 0.995$ for plots in panels A and B, respectively).

Fig 5.

Plot of Hydrophilicity/Hydrophobicity Coefficients ($\Delta t_{R(Gly)}$) determined at pH 2, 20 mM H_3PO_4 versus pH 7 10 mM NaH_2PO_4 plus 50 mM NaClO_4 ; panel A, L- diastereomers; panel B, D- diastereomers. Column: Kromasil C18, pH 2; Zorbax XDB C8, pH 7. Conditions: linear AB gradient (0.25% $\text{CH}_3\text{CN}/\text{min}$) at a flow-rate of 0.3 ml/min, where eluent A is 20 mM aq. H_3PO_4 , pH2 or 10 mM aq. NaH_2PO_4 , pH 7 and eluent B is 20 mM H_3PO_4 in CH_3CN , pH 2 or 10 mM NaH_2PO_4 in 50% CH_3CN , pH 7; temperature 25°C. Data are taken from Table 5. The single letter code represents the L- amino acid substitutions at position X of the peptide sequence shown in Section 3.1. Closed circles denote data used in the correlation plot ($y = 1.0783x + 0.4136$, correlation coefficient $r = 0.994$ and $y = 1.0575x + 0.3138$, correlation

coefficient $r = 0.997$ for plots in panels A and B, respectively); open circles represent results from positively charged substitutions.

Fig 6.

Plot of Hydrophilicity/Hydrophobicity Coefficients ($\Delta t_{R(Gly)}$) determined at pH 2, comparing D- versus L- diastereomeric pairs. Column: Kromasil C18. Conditions: linear AB gradient (0.25% CH_3CN/min , starting from 2% CH_3CN) at a flow-rate of 0.3 ml/min; panel A, eluent A is 20 mM aq. H_3PO_4 and eluent B is 20 mM H_3PO_4 in CH_3CN ; panel B, eluent A is 20 mM aq. TFA and eluent B is 20 mM TFA in CH_3CN ; temperature 25°C. Data are taken from Table 5. The single letter code represents the L- or D- amino acid substitutions at position X of the peptide sequence shown in Section 3.1. Closed circles denote data used in the correlation plot ($y = 0.7968x - 0.0583$, correlation coefficient $r = 0.982$ and $y = 0.792x + 0.3553$, correlation coefficient $r = 0.976$ for plots in panels A and B, respectively).

Fig 7.

Plot of Hydrophilicity/Hydrophobicity Coefficients ($\Delta t_{R(Gly)}$) determined at pH 7, comparing D- versus L- diastereomeric pairs. Column: Zorbax XDB C8. Conditions: linear AB gradient (0.25% $\text{CH}_3\text{CN}/\text{min}$) at a flow-rate of 0.3 ml/min; panel A, eluent A is 10 mM aq. NaH_2PO_4 and eluent B is 10 mM NaH_2PO_4 in 50% CH_3CN ; panel B, eluent A is 10 mM aq. NaH_2PO_4 with 50 mM NaCl and eluent B is 10 mM NaH_2PO_4 with 50 mM NaCl in 50% CH_3CN ; panel C, eluent A is 10 mM aq. NaH_2PO_4 with 50 mM NaClO_4 and eluent B is 10 mM NaH_2PO_4 with 50 mM NaClO_4 in 50% CH_3CN ; temperature 25°C. Data are taken from Table 5. The single letter code represents the L- or D- amino acid substitutions at position X of the peptide sequence shown in Section 3.1. Closed circles denote data used in the correlation plot ($y =$

$0.7965x - 0.0573$, correlation coefficient $r = 0.977$, $y = 0.7739x + 0.4837$, correlation coefficient $r = 0.972$ and $y = 0.8165x + 0.1331$, correlation coefficient $r = 0.979$ for plots in panels A, B and C respectively).

Table 1

RP-HPLC peptide retention data in various mobile phase conditions at 25°C

Amino Acid Substitution ^a	20 mM H ₃ PO ₄		pH 2 ^b		20 mM TFA		No Salt		pH 7 ^c , 10 mM PO ₄ Buffer		50 mM NaCl		50 mM NaClO ₄	
	t _R (D-)	t _R (L-)	t _R (D-)	t _R (L-)	t _R (D-)	t _R (L-)	t _R (D-)	t _R (L-)	t _R (D-)	t _R (L-)	t _R (D-)	t _R (L-)	t _R (D-)	t _R (L-)
Trp	69.9	66.5	78.4	74.7	71.1	67.9	73.1	69.6	83.3	79.9				
Phe	71.4	63.6	79.0	71.5	72.0	64.5	74.0	65.5	84.4	77.1				
Leu	64.1	57.4	73.2	66.6	66.3	59.9	68.8	62.2	79.3	72.9				
Ile	64.4	56.6	72.9	65.1	65.7	58.4	68.1	60.8	78.3	71.1				
Met	55.1	49.1	63.3	57.3	57.3	51.4	59.3	53.6	68.8	63.2				
Val	54.6	47.9	62.8	55.8	56.8	50.5	58.8	52.2	68.1	61.5				
Tyr	46.6	47.6	54.6	55.5	49.1	50.0	51.8	51.8	58.9	60.2				
Pro	45.4	42.0	54.6	51.3	48.9	45.9	51.0	47.8	59.9	57.2				
Cys	45.7	41.3	53.4	48.8	48.4	44.4	52.6	48.2	58.5	54.2				
Glu	37.7	36.3	44.4	43.4	36.5	35.5	38.6	37.8	40.2	40.1				
Ala	38.4	35.5	46.0	42.8	41.4	38.6	45.4	42.3	51.2	48.2				
Asp	34.7	34.7	41.6	41.6	36.1	37.0	37.9	38.9	37.7	38.4				
Thr	35.6	34.3	42.8	41.4	38.5	37.1	40.4	39.1	47.8	45.1				
Gly	33.3	33.3	40.1	40.1	36.0	36.0	37.6	37.6	45.1	45.1				
Ser	31.6	33.2	38.7	40.1	34.7	36.2	36.4	37.8	43.3	44.6				
Gln	32.8	33.0	39.5	39.9	35.6	35.9	39.2	39.5	44.1	44.1				
Asn	31.9	32.8	38.7	39.7	35.0	35.9	38.3	39.3	43.3	44.1				
Arg	25.8	27.1	40.9	42.2	30.1	31.4	35.3	36.8	48.8	50.5				
His	23.9	27.0	38.3	41.6	28.5	32.8	31.9	37.3	46.1	49.3				
Lys	23.5	25.5	38.4	39.8	30.6	30.0	34.4	33.4	45.5	47.6				

^aThe D- and L- amino acid substitutions at position X in the peptide sequence Ac-X-L-G-A-K-G-A-G-V-G-amide.

^bColumn: Kromasil C18. Conditions: linear AB gradient (0.25% CH₃CN/min, starting from 2% CH₃CN/CH₃CN) at a flow-CN) rate of 0.3 ml/min, where eluent A is 20 mM aq. H₃PO₄ or 20 mM TFA (pH 2) and eluent B is 20 mM H₃PO₄ or 20 mM TFA in CH₃CN; temperature 25 °C. t_R denotes peptide retention time of the D- or L-substituted peptide.

^cColumn: Zorbax XDB C8. Conditions: linear AB gradient (0.25% CH₃CN/min) at a flow-rate of 0.3 ml/min, where eluent A is 10 mM aq. NaH₂PO₄ (pH 7) with or without 50 mM NaCl or 50 mM NaClO₄ and eluent B is the respective eluent A containing 50% CH₃CN; temperature 25°C. t_R denotes peptide retention time of the D- or L-substituted peptide.

Table 2

Effects of Mobile Phase Conditions

Amino Acid Substitution ^a	pH 2 ^b Δ(TFA-H ₃ PO ₄) ^c		pH 7 ^b Δ(NaCl-No Salt) ^d		pH 7 ^b Δ(NaClO ₄ -No Salt) ^e	
	D	L	D	L	D	L
Trp	8.5	8.2	2.0	1.7	12.3	12.0
Phe	7.6	7.9	2.0	1.0	12.5	12.6
Leu	9.1	9.2	2.5	2.3	13.0	13.0
Ile	8.5	8.5	2.3	2.4	12.6	12.7
Met	8.2	8.2	2.0	2.2	11.5	11.8
Val	8.2	7.9	2.0	1.7	11.3	11.0
Tyr	8.0	7.9	1.9	1.8	9.8	10.2
Pro	9.1	9.3	2.1	1.9	11.0	11.3
Cys	7.7	7.5	4.1	3.8	10.1	9.8
Ala	7.6	7.3	4.0	3.7	9.9	9.6
Thr	7.2	7.1	1.9	2.0	9.3	9.2
Gly	6.8	6.8	1.6	1.6	9.1	9.1
Ser	7.1	6.9	1.7	1.6	8.6	8.4
Gln	6.7	6.9	3.5	3.6	8.5	8.5
Asn	6.8	6.9	3.3	3.4	8.3	8.2
Arg	15.1	15.1	5.2	5.4	20.4	17.4
His	14.4	14.6	3.4	4.5	17.6	16.5
Lys	14.9	14.3	3.8	3.4	14.9	17.6
Asp	6.9	6.9	1.8	1.9	1.6	1.4
Glu	6.8	7.1	2.2	2.3	3.7	4.6

^aThe D- and L-amino acid substitutions at position X in the peptide sequence Ac-X-L-G-A-K-G-A-G-V-G-amide.

^bColumn: pH 2, Kromasil C18; pH 7, Zorbax XDB C8. Conditions: pH 2, shown in Table 1; pH 7, shown in Table 1.

^cDenotes difference in peptide retention time between 20 mM TFA and 20 mM H₃PO₄ (pH2) mobile phase systems shown in Table 1.

^dDenotes difference in peptide retention time between pH 7 mobile phase system containing 50 mM NaCl and pH 7 mobile phase system in the absence of salt (Table 1).

^eDenotes difference in peptide retention time between pH 7 mobile phase system containing 50 mM NaClO₄ and pH 7 mobile phase system in the absence of salt (Table 1).

Table 3
Nearest-Neighbour Effects determined at 25°C by RP-HPLC of model peptides.

Amino Acid Substitution ^a	20 mM H ₃ PO ₄ Δt _{R(D-L)} ^c	pH 2 ^b 20 mM TFA Δt _{R(D-L)}	No Salt Δt _{R(D-L)}	pH 7 ^b , 10 mM PO ₄ Buffer 50 mM NaCl Δt _{R(D-L)}	50 mM NaClO ₄ Δt _{R(D-L)}
Phe	7.8	7.5	7.5	8.5	7.3
Ile	7.8	7.8	7.3	7.3	7.2
Leu	6.7	6.6	6.4	6.6	6.4
Val	6.7	7.0	6.3	6.6	6.6
Met	6.0	6.0	5.9	5.7	5.6
Cys	4.4	4.6	4.0	4.4	4.3
Pro	3.4	3.3	3.0	3.2	2.7
Tyr	3.4	3.7	3.2	3.5	3.4
Ala	2.9	3.2	2.8	3.1	3.0
Glu	1.4	1.0	1.0	0.8	0.1
Thr	1.3	1.4	1.4	1.3	1.5
Asp	0.0	0.0	-0.9	-1.0	-0.7
Gly	0.0	0.0	0.0	0.0	0.0
Gln	-0.2	-0.4	-0.3	-0.3	-0.3
Asn	-0.9	-1.0	-0.9	-1.0	-0.8
Tyr	-1.0	-0.9	-0.9	-0.9	-1.3
Arg	-1.3	-1.3	-1.3	-1.5	-1.6
Lys	-2.0	-1.4	-0.6	-1.0	-2.1
Ser	-1.6	-1.4	-1.5	-1.4	-1.3
His	-3.1	-3.3	-4.3	-5.4	-3.2

^aThe D- and L- amino acid substitutions at position X in the peptide sequence Ac-X-L-G-A-K-G-A-G-V-G-amide.

^bColumn and Conditions are shown in Table 1.

^cΔt_{R(D-L)} R denotes the difference in retention time between D- and L- diastereomers.

Table 4

Energy difference between the map minimum conformation and the nearest minimum energy to the minimum energy conformation of the opposite chirality map

Peptide	Minimum Energy Conformation (psi-1,phi-2)	Nearest Minimum Opposite Chirality (psi-1,phi-2)	Energy Difference (kcal/mol)
L-Leu - L-Leu	90,-90	-70,-90	3.16
D-Leu - L-Leu	-90,-90	70,-90	2.69
L-Ser - L-Leu	80,-90	-20,-90	3.60
D-Ser - L-Leu	-80,-80	80,-80	6.23
L-Thr - L-Leu	70,-90	-100,-90	3.28
D-Thr - L-Leu	-100,-90	20,-90	2.54
L-Trp - L-Leu	100,-90	-80,-90	4.44
D-Trp - L-Leu	-140,-90	70,-90	2.09

The energy differences are computed as the energy at the defined conformation minus the energy of the global map minimum conformation (hence the energy of the global map minimum is zero). This is done independently for each different map.

Table 5
RP-HPLC Hydrophilicity/Hydrophobicity Coefficients in various mobile phases at 25°C

Amino Acid Substitution ^a	20 mM H ₃ PO ₄ pH 2		20 mM TFA		No Salt		pH 7 ^b , 10 mM PO ₄ Buffer 50 mM NaCl		50 mM NaClO ₄	
	Δt_R (D-Gly)	Δt_R (D-Gly)	Δt_R (D-Gly)	Δt_R (D-Gly)	Δt_R (D-Gly)	Δt_R (D-Gly)	Δt_R (D-Gly)	Δt_R (D-Gly)	Δt_R (D-Gly)	Δt_R (D-Gly)
Tyr	36.6	33.2	38.3	34.6	35.0	31.9	35.5	32.1	38.2	34.9
Phe	38.0	30.3	38.9	31.3	35.9	28.5	36.4	27.9	39.3	32.0
Leu	30.8	24.1	33.1	26.4	30.3	23.9	31.2	24.7	34.2	27.9
Ile	31.1	23.3	32.8	25.0	29.7	22.4	30.5	23.2	33.3	26.0
Met	21.8	15.7	23.2	17.2	21.3	15.4	21.7	16.0	23.7	18.1
Val	21.3	14.6	22.7	15.7	20.8	14.5	21.2	14.6	23.0	16.4
Tyr	13.2	14.2	14.5	15.3	13.0	14.0	13.3	14.2	13.8	15.2
Pro	12.1	8.6	14.5	11.2	12.8	9.9	13.4	10.2	14.8	12.1
Cys	12.4	7.9	13.3	8.7	12.4	8.3	15.0	10.6	13.5	9.1
Glu	4.3	3.0	4.3	3.2	0.5	-0.5	1.1	0.3	-4.9	-5.0
Ala	5.0	2.2	5.9	2.7	5.4	2.5	7.8	4.7	6.2	3.1
Asp	1.4	1.4	1.4	1.5	0.1	1.0	0.3	1.3	-7.4	-6.7
Thr	2.2	1.0	2.7	1.3	2.5	1.1	2.8	1.5	2.7	1.2
Gly	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ser	-1.8	-0.2	-1.4	-0.1	-1.3	0.2	-1.2	0.3	-1.8	-0.5
Gln	-0.6	-0.3	-0.6	-0.3	-0.4	-0.1	1.6	1.9	-1.0	-0.6
Asn	-1.5	-0.6	-1.4	-0.5	-1.0	-0.1	0.7	1.7	-1.8	-0.9
Arg	-7.6	-6.2	0.7	2.1	-6.0	-4.7	-2.3	-0.7	5.4	3.8
Lys	-9.4	-6.3	-1.8	1.5	-7.5	-3.2	-5.7	-0.3	1.0	4.2
His	-9.8	-7.9	-1.7	-0.3	-5.4	-6.1	-3.2	-4.2	0.4	2.6

^aThe D- and L-amino acid substitutions at position X in the peptide sequence Ac-X-L-G-A-K-G-A-V-G-amide.

^bColumn: pH 2, Kromasil C18; pH 7, Zorbax XDB C8. Conditions: pH 2, shown in Table 1; pH 7, shown in Table 1.

^c Δt_R (D-Gly) or Δt_R (L-Gly) denotes the retention time, t_R , of the D- or L-amino acid substituted peptide minus the retention time of the Gly substituted peptide.