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Relationship of Sidechain Hydrophobicity and α-Helical Propensity on the Stability of the Single-stranded Amphipathic α-Helix

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The aim of the present investigation is to determine the effect of α-helical propensity and sidechain hydrophobicity on the stability of amphipathic α-helices. Accordingly, a series of 18-residue amphipathic α-helical peptides has been synthesized as a model system where all 20 amino acid residues were substituted on the hydrophobic face of the amphipathic α-helix. In these experiments, all three parameters (sidechain hydrophobicity, α-helical propensity and helix stability) were measured on the same set of peptide analogues. For these peptide analogues that differ by only one amino acid residue, there was a 0.96 kcal/mole difference in α-helical propensity between the most (Ala) and the least (Gly) α-helical analogue, a 12.1-minute difference between the most (Phe) and the least (Asp) retentive analogue on the reversed-phase column, and a 32.3°C difference in melting temperatures between the most (Leu) and the least (Asp) stable analogue. The results show that the hydrophobicity and α-helical propensity of an amino acid sidechain are not correlated with each other, but each contributes to the stability of the amphipathic α-helix. More importantly, the combined effects of α-helical propensity and sidechain hydrophobicity at a ratio of about 2:1 had optimal correlation with α-helix stability. These results suggest that both α-helical propensity and sidechain hydrophobicity should be taken into consideration in the design of α-helical proteins with the desired stability.

Keywords–α-Helix; α-helical propensity; hydrophobicity; HPLC; thermal stability

Abbreviations

CD, circular dichroism spectroscopy; HPLC, high performance liquid chromatography; NPASA, nonpolar accessible surface area; TEAP, triethylammonium phosphate; TFE, 1,1,1-trifluoroethanol.

INTRODUCTION

Sidechain hydrophobicity has long been suspected to contribute to α-helical propensity of amino acids [1–4] and to stabilize the α-helix [5]. Most recently, Blaber *et al.* [2] reported an apparent linear dependence of α-helix propensity on the buried surface area of each amino acid residue and inferred that hydrophobic stabilization contributed substantially to α-helix propensity. However, some doubts about the validity of such correlation had been raised [6] because of the relatively small number of samples used in the study, the relative clustering of surface area estimates, and because the buried surface areas of the bulky residues Phe and Trp did not appear to correspond to their α-helical propensities. In general, there is no clear evidence to prove that sidechain hydrophobicity contributes to α-helical propensity and attempts to correlate stability to amino acid compositions has generally failed [7]. It should also be noted that only about one-third of the residues in a helix contribute to the packing of the apolar core,

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whereas every residue in a helix contributes its conformational preference [8].

The major problem in correlating sidechain hydrophobicity with α-helical propensity and/or α-helix stability is that these parameters are often measured using different protein model systems. The net free energy of stabilization of a protein is generally small, and the differences in stabilities between analogues that only differ by one amino acid residue are even smaller. Comparison of data is very difficult because the small differences in free energies can potentially be masked by inherent differences between model systems, or even from differences in assumptions made during data analysis. Recently, we have determined the α-helical propensities of the 20 naturally occurring amino acids using a 'host–guest' peptide model system [9, 10], which had also been used to determine the hydrophobicity of amino acid sidechains at pH 2 [11]. We have chosen an amphipathic α-helical model system because approximately 50% of α-helices in proteins are amphipathic [12]. Since this feature is often associated with folding and function of the protein, the α-helical domains in proteins represent a good site for mutations in structure and function studies. In addition, the hydrophobic face of the amphipathic α-helix had been shown to be more sensitive to amino acid substitutions than the hydrophilic face [10]. In this study, we used the same peptide analogues to investigate the effects of the hydrophobicity of amino acid sidechains on the formation and stabilization of the α-helical structure.

MATERIALS AND METHODS

Peptide Synthesis, Purification and Characterization

Peptide synthesis was carried out by solid-phase peptide synthesis using standard *t*-Boc chemistry on an Applied Biosystems peptide synthesizer Model 430 (Foster City, CA, USA). The peptides were purified mainly by semipreparative reversed-phase HPLC and their identity and purity were determined by analytical reversed-phase HPLC, amino acid analysis and mass spectrometry. Peptide characterization was carried out by employing CD spectroscopy, size-exclusion chromatography, sedimentation equilibrium experiments and NMR spectroscopy as reported previously [9–11].

Determination of α-Helical Propensity

The molar ellipticities of these peptide analogues were determined by CD spectroscopy at 5°C under benign conditions (50 mM phosphate/100 mM KCl, pH 7), as well as in the presence of an α-helix inducing solvent, 1,1,1-trifluoroethanol (50 mM phosphate/100 mM KCl/50% TFE, pH 7). The fraction of peptide folded under benign condition was calculated from the equation $f = (\theta_X - \theta_C)/(\theta_H - \theta_C)$, where θ_X was the observed molar ellipticity of any peptide, while θ_H and θ_C were molar ellipticities of the same peptide in 50% TFE (fully helical form) and 6 M urea (random coil), respectively. The free energy of α-helix formation (ΔG_X) was then calculated from the equation $\Delta G_X = -RT \ln f$ and then normalized against the value for the Gly (ΔG_G) analogue in order to obtain the relative free energy of helix formation ($\Delta\Delta G = \Delta G_X - \Delta G_G$). The Δ values were then taken as the α-helical propensity of each amino acid residue [9].

Determination of Hydrophobicity

The retention time of each peptide analogue was determined on an Aquapore C8 reversed-phase column (4.6 × 220 mm, 300 Å pore size, 7 μm particle size) connected to an Hewlett Packard HPLC, Model 1090A. The samples (≅ 50μg) were injected into the column and eluted at a flow rate of 1 ml/min by employing a linear AB gradient (1%B/min), where buffer A was 100 mM TEAP, pH 7.0, and buffer B was a 1:1 mixture of 200 mM TEAP and acetonitrile. The difference in retention times between each analogue and that of the Gly analogue ($\Delta t_{R(X)} - t_{R(G)}$) was taken as a measure of sidechain hydrophobicity.

TFE Titration

Peptide stock solutions were dissolved in benign buffer (50 mM PO_4/100 mM KCl, pH 7) at about 1 mg/ml. Thirty microlitres of stock peptide solution were mixed with calculated volume of TFE and diluted with water to a final volume of 60 μl. Each peptide solution was then loaded into a 0.02 cm fused silica cell and its ellipticity at 220 nm was measured at 5°C using a Jasco J-500C spectropolarimeter (Jasco, Easton, MD) equipped with a Jasco IF500II interface, an IBM PS/2 running the Jasco DP-500/PS2 system version 1.33a software, and a Lauda (Model RMS) water bath (Brinkmann Instruments, Rexdale, Ontario, Canada) to control the temperature of the cell. The fraction of peptide

folded (f) at each TFE concentration was calculated as shown above, except that θ_X represents the ellipticity of the peptide at a specified TFE concentration.

Temperature Denaturation Studies

Each peptide was dissolved in 30 mM phosphate/50 mM KCl/30% TFE, pH 7.0, to give a peptide concentration of about 0.5 mg/ml. Each solution was loaded into 0.02 cm fused silica cell and its ellipiticity at 220 was measured at different temperatures. The ratio of the ellipticity at a particular temperature (t) relative to that at 5°C $([\theta]_t - \theta_C)/([\theta]_5 - \theta_C)$ was calculated and plotted against temperature in order to obtain the thermal melting profiles. The melting temperature (t_m) was calculated as the temperature at which the α-helix was 50% unfolded $(([\theta]_t - \theta_C)/([\theta]_5 - \theta_C) = 0.5)$ and the values were taken as a measure of α-helix stability.

RESULTS

The α-helical peptide model system used in this study, schematically shown in Figure 1, was designed with the following important criteria taken into consideration [9]: (1) the helix is monomeric and non-interacting; (2) it has well-defined amphipathic faces; (3) alanine has the minimum sidechain that can impart amphipathicity; (4) there is a uniform environment surrounding the substitution site; (5) a central location exists for the substitution site; (6) minimum sidechain interactions occur between alanine residues on the non-polar face and the sidechain of the 'guest' amino acid; and (7) the small size of the peptide maximizes the effects of single amino acid substitutions. Even though alanine has low hydrophobicity this model system provides an amphipathic α-helix with a mean helical hydrophobic moment of 0.59 as calculated by the method of Eisenberg *et al.* [13] and reported by Sereda *et al* [11]. The α-helix was also stabilized on the hydrophilic face by strategically incorporating Lys and Glu residues to promote α-helix stabilizing electrostatic attractions at the $i \rightarrow i+3$ and $i \rightarrow i+4$ positions [14]. Based on this model system the α-helical propensity of the 20 amino acids had previously been determined [9] and are shown in Table 1. The same design criteria are equally important in using these peptide analogues for the determination of the hydrophobicity of amino acid sidechains, as well as in the determination of the stability of these α-helical peptides against temperature denaturation.

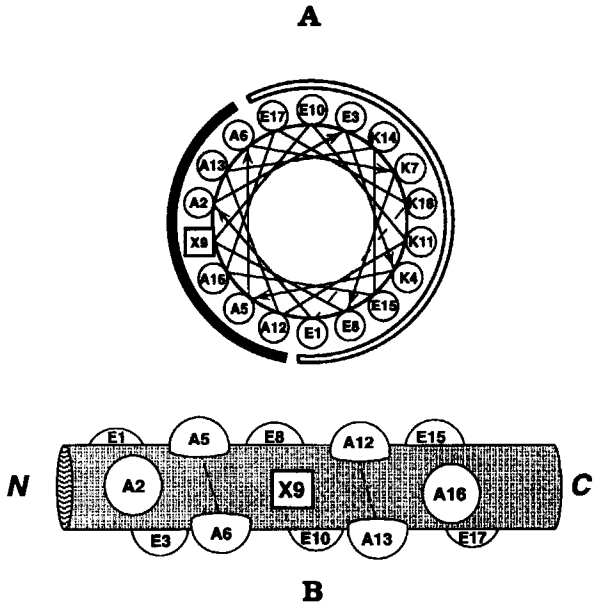

Figure 1 (A) Helical wheel and (B) helical rod representation of the 'host' peptide used in this study. The hydrophobic face (Ala-face) is indicated as a solid arc in A and facing the reader in B. The hydrophilic face is indicated as an open arc. The substitution ('guest') site is at position 9 (boxed) of the hydrophobic face and uniformly surrounded with Ala residues. Standard one-letter designations are used for the amino acid residues.

The retention times in reversed-phase HPLC had previously been suggested as a measure of hydrophobicity of peptide analogues [11, 15]. Considering that the peptide analogues used in this study differed only by one amino acid residue, it was interesting to observe a wide difference in retention times (Table I), a 12.1 min difference between the most retentive (Phe) and the least retentive (Asp) peptides. Thus, the difference in retention times (Δt_R), relative to the Gly peptide, for each peptide analogue is a reliable measure of the differences in the hydrophobicity of the amino acid sidechains.

An excellent correlation ($r = 0.99$, Figure 2A) was observed between the hydrophobicity values at pH 7 and pH 2 [11], when residues with ionizable sidechains at this pH range (Asp, Glu, His) were excluded (the Pro analogue was excluded from all comparisons because of its effect in disrupting the α-helix conformation). This excellent correlation was expected because the same set of peptides was used in these studies. However, it is interesting to find that an excellent correlation ($r = 0.97$, Figure 2B) also exists between the results from this amphipathic α-helical system and those from a random coil peptide

Table 1 α-Helical Propensity (ΔΔG), Sidechain Hydrophobicity (Δt_R) and Helix stability (Δt_m) for Different Peptide Analogues

Amino acid	α-Helical propensity[a] (−ΔΔG, kcal/mole)	Retention time (t_R, min)	Δt_R^b (min)	t_m^c (°C)	Δt_m^d (°C)
Ala	0.96	18.0	3.2	66.3	19.0
Arg	0.90	13.7	− 1.1	64.3	17.0
Leu	0.81	22.4	7.6	73.6	26.3
Lys	0.70	13.0	− 1.8	55.8	8.5
Met	0.67	20.6	5.8	68.2	20.9
Gln	0.61	14.0	− 0.8	59.9	12.6
Ile	0.59	22.5	7.7	71.9	24.6
Trp	0.49	22.4	7.6	63.1	15.8
Phe	0.48	22.6	7.8	65.6	18.3
Tyr	0.43	19.7	4.9	62.2	14.9
Cys	0.43	18.6	3.8	55.9	8.6
Val	0.42	20.7	5.9	65.0	17.7
Asn	0.33	12.6	− 2.2	50.4	3.1
Ser	0.33	14.4	− 0.4	55.0	7.7
His	0.33	15.4	0.6	54.0	6.7
Glu	0.32	12.4	− 2.4	49.2	1.9
Thr	0.28	15.8	1.0	53.8	6.5
Asp	0.21	10.5	− 4.3	41.3	− 6.0
Gly	0	14.8	0	47.3	0
Pro	− 0.83	12.6	− 2.2	n.d.	-

[a]ΔG values from Zhou *et al.* [9] normalized against the value of the Gly analogue.
[b]Difference in retention times between each peptide and that of the Gly analogue in this study.
[c]Measured as the temperature at which half of the α-helix is unfolded (see Materials and methods for details).
[d]Difference in t_m values between each peptide analogue and that of the Gly analogue.

model system [16] at pH 7. The amphipathic α-helical peptides were expected to interact with the reversed-phase column through their non-polar face (preferred binding domain) and bind to the non-polar surface in their helical conformation [11, 15] and, therefore, would be retained in the column longer than the random coil and shorter peptides [16]. However, the high correlation and the closeness of the slope to unity suggest that differences in retention behavior between the two model systems were cancelled when the values were normalized against the retention time of the Gly analogue in each model system. Thus, the retention data in reversed-phase chromatography of random coil peptides and the Ala-face amphipathic α-helical peptides can provide hydrophobicity/hydrophilicity values inherent of amino acid sidechains because of minimal intrachain interactions of sidechains. In other peptide model systems the effect of intrachain interactions may result in differences in the magnitude of sidechain hydrophobicity, but the directional effect on all sidechains is similar [11], that is, the retention times are changed in the same direction.

The validity of the relative retention times as a measure of relative sidechain hydrophobicity is supported by their excellent correlation with thermodynamic scales, such as those calculated from the free energy of transferring acetyl-aminoacyl-amide [17] ($r = 0.96$, Figure 2C) or amino acid [18] ($r = 0.93$, Figure 2D) from an aqueous solution into an organic solvent. Relatively good correlations were also observed with thermodynamic values from other model systems [13, 19, 20]. These results suggest that the hydrophobic surface of the sorbant mimics the hydrophobic environment of an organic solvent or in the protein interior.

To assess the stability of these α-helical peptides, the ideal method would be to conduct temperature denaturation under benign conditions. Unfortunately, under benign conditions the starting α-helical contents were very different for each peptide and, in most cases, the initial structures were less than 50% α-helical [9]. This made it impossible to obtain temperature denaturation curves for all analogues that were comparable to each other and, therefore, alternative conditions had to be explored. Figure 3 shows that for three representative peptides with the highest (Ala), medium (Val) and lowest (Gly) ellipticities, 30% TFE was the minimal concentration required to attain full helical structure. Therefore,

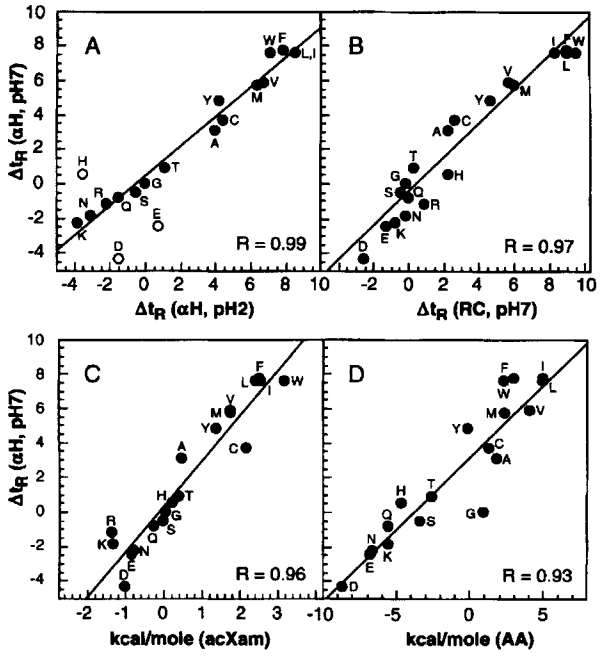

Figure 2 Comparison of the sidechain hydrophobicities obtained from amphipathic α-helical peptides at pH 7 with those obtained from (A) the same peptides at pH 2 [11] and (B) random coil peptides at pH 7 [16]. The numbers in both axes represent the difference in retention times (Δt_R) between each peptide and that of the Gly analogue which are taken as a measure of the relative hydrophobicities of the amino acid sidechain (indicated in single letter designations). The correlation coefficient in A was calculated without the values for the ionizable residues H, D and E. α-H represents the amphipathic α-helical peptides and RC represents random coil peptides. Similar comparison of sidechain hydrophobicities from the relative retention times (Δt_R) of amino acids with thermodynamic scales obtained from the free energy of transfer of (C) acetyl-aminoacyl-amides (ac-X-am, [17] and (D) amino acids (AA, [18]) from aqueous solution to a non-polar solvent.

temperature denaturation studies were conducted in the presence of 30% TFE. While TFE has been extensively used to induce α-helical structure in short peptides that normally exist in random coil conformation in aqueous solutions [5, 21–25], it also promotes the monomeric α-helical form by disrupting the tertiary and quaternary structures [26]. Finally, it mimics the hydrophobic environment that the non-polar face would encounter on interacting with the hydrophobic core in the folded protein.

The temperature denaturation profiles of representative peptide analogues (Figure 4) indicate a gradual unfolding of the α-helical structures, for both amino acid sidechains with the greatest (Ala) and the

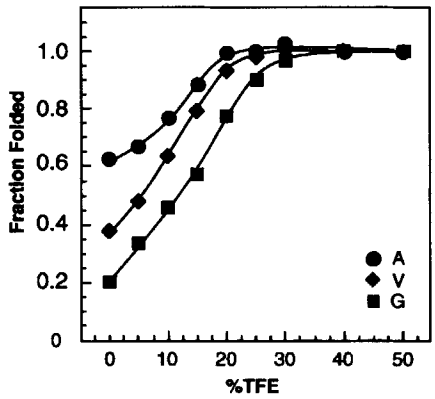

Figure 3 TFE titration of three representative peptide analogues at 5°C. The symbols used are (●) for Ala, (◆) for Val and (■) for Gly.

least (Gly) α-helical propensity (Figure 4). As shown in Table 1 there were also wide variations in the melting temperatures of these peptide analogues, a 32.3°C difference in t_m values between the most stable (Leu) and the least stable (Asp) analogue (Figure 4B). This remarkable variation and well-dispersed distribution in t_m values for a set of peptide analogues that differed in sequence by only one amino acid residue, again indicates that these peptides are good models for helix stability studies.

The three sets of values from the same set of peptides were then compared by calculating the correlation coefficients between specific pairs of variables. Figure 5A shows that sidechain hydrophobicity (Δt_R) has good correlation ($r = 0.82$) with α-helix stability (Δt_m), as also evident from the distribution of the amino acids with the more hydrophobic sidechains in the upper right-hand quadrant of the graph, while those with less hydrophobic sidechains are localized in the lower left quadrant. However, other sidechain hydrophobicity scales [13, 17–20] did not correlate with helix stability (Δt_m) values.

The α-helical propensity [9] also had fair correlation with helix stability (Δt_m) of these peptides ($r = 0.74$, Figure 5B), but the existence of this relationship with other model systems is not clear. For example, helix stability (Δt_m) had fair correlations with α-helical propensity values generated from a non-amphipathic α-helix [27] ($r = 0.79$) and from mutagenesis of site 44 of T4 lysozyme [2] ($r = 0.78$), but were not correlated with those from synthetic coiled-coil model system [8] ($r = 0.57$) and mutagenesis of site 32 of barnase [28] ($r = 0.29$). In addition, while the Δt_m values correlated well with the α-helix

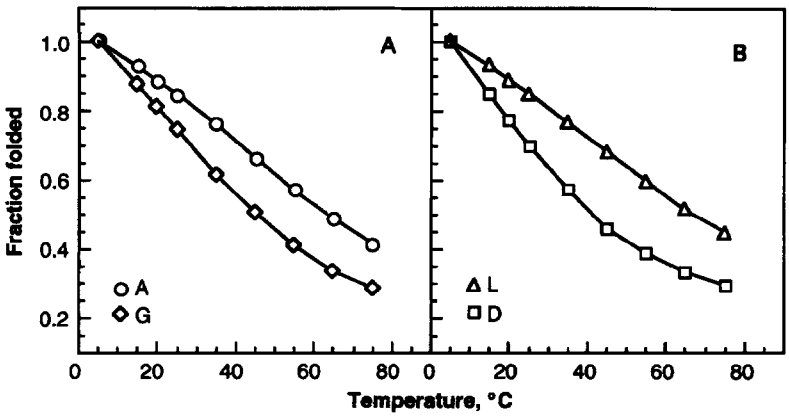

Figure 4 Temperature denaturation profiles of representative peptide analogues. Panel A shows the analogues with the highest (○, Ala) and lowest (◇, Gly) α-helical propensities, and panel B shows the most stable (Δ, Leu) and least stable (□, Asp) peptide analogues. The vertical axis represents the fraction of folded species, calculated as the ratio of the observed molar ellipticity at any temperature relative to the molar ellipticity at 5°C. See Materials and methods for experimental conditions.

propagation parameters of Wojcik *et al.* [29] ($r = 0.85$), essentially no correlation was observed with those of Park *et al.* [30] ($r = 0.45$).

Although either sidechain hydrophobicity (Δt_R) or α-helical propensity ($\Delta\Delta G$) correlates with helix stability (Δt_m), there was no correlation at all between sidechain hydrophobicity and α-helical propensity from this study ($r = 0.31$, Figure 5C). In addition, α-helical propensity did not correlate with sidechain hydrophobicity scales generated from random coil model peptides ($r = 0.32$) [16], the hydrophobicity values calculated by Blaber *et al.* [31] ($r = 0.58$) based on the surface area of buried sidechains [3], or any of the other thermodynamic hydrophobicity scales [13, 17–20].

Since our results indicate that both hydrophobicity (Figure 5A) and α-helical propensity (Figure 5B) individually correlate with α-helix stability, we deemed it necessary to find out if a combination of these two parameters would show a better correlation with α-helix stability. Because of differences in magnitude, the individual values for each set of variables could not be directly added. Therefore, the $\Delta\Delta G$, Δt_R and Δt_m values were normalized to a scale of 0 for the Gly analogue and 100 for the highest values (Table 2). Then different combinations of normalized $\Delta\Delta G$ and Δt_R values were calculated and plotted against Δt_m to obtain their respective correlation coefficients. Figure 6 shows that the best correlation ($r = 0.96$ was observed at around the 2:1 ratio of $\Delta\Delta G : \Delta t_R$ (indicated by an arrow). Calculated based on this ratio, the combined values of α-helical propensity and sidechain hydrophobicity (Table 2) now has excellent correlation with the stability of these peptide analogues (Figure 5D).

DISCUSSION

It has been suggested recently that the stability of the α-helix is determined both by the α-helical propensities of the amino acids and by interactions between sidechains [32]. However, the contribution of sidechain hydrophobicity on the formation and stabilization of the α-helix has been difficult to assess because estimates of these parameters were often obtained from different model systems. On one hand, sidechain hydrophobicity had been estimated from either the relative solubilities or partition coefficients of model compounds between two solvent systems [13, 17–20, 33] or from the retention times of different peptide analogues on a reversed-phase HPLC column [11, 16]. On the other hand, the α-helical propensity of amino acids has often been determined on the basis of the relative stabilities of a variety of peptide [8, 9, 27, 32] and protein [2, 28, 31] model systems. Therefore, the uniqueness of this study is that all three parameters (α-helical propensity, sidechain hydrophobicity and stability) were determined from one set of model peptides in order to make direct comparisons of the results.

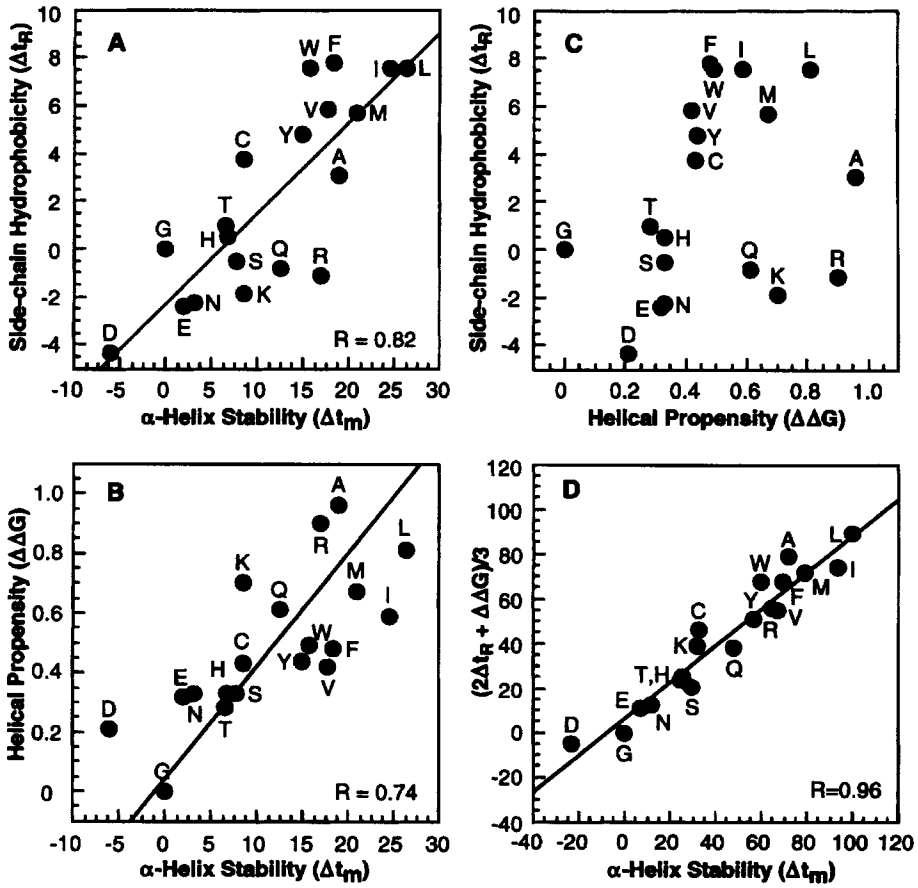

Figure 5 Plots showing the relationships between the three parameters being studied. Plot A shows a good correlation between sidechain hydrophobicity (Δt_R) and α-helix stability (Δt_m). Plot B shows a fair correlation between α-helical propensity and α-helix stability. Plot C shows no correlation (no regression line drawn) between sidechain hydrophobicity (Δt_R) and α-helical propensity ($-\Delta\Delta G$). Plot D shows excellent correlation between the normalized α-helix stability and the combined values of the normalized α-helical propensity ($\Delta\Delta G$) and sidechain hydrophobicity (Δt_R) values (2:1 ratio) (Table II).

The results of this study, summarized in Figure 7, show that while sidechain hydrophobicity and α-helical propensity individually promote α-helix stability only to a certain extent, their combined effect (Table 2) is a better indicator of stability for peptides designed with predisposed structure and stability. This suggests a cooperative effect of α-helical propensity and the hydrophobicity of amino acid sidechains in nucleating and stabilizing the amphipathic α-helix during protein folding. This observation is not surprising because when an α-helix forms the sidechain atoms of one residue can be in contact with the backbone or sidechain atoms of residues in the next turn of the helix and may, therefore, be partially removed from contact with the solvent [3]. The methyl groups of alanine residues constitute the minimum functional group that can impart amphipathicity of the α-helix and, in our model peptides, minimal interactions are expected between these methyl groups and the sidechain of the 'guest' amino acid [2, 8, 32]. Nevertheless, the methylene group at the β-position of the sidechain of all 'guest' amino acids (except Gly) is probably uniformly shielded by the methyl groups of alanine in the $i-3, i-4, i+3$ and $i+4$ positions. Since the sidechains of all other 'guest' amino acids (with the exception of Gly) are larger than that of Ala, they are expected to 'stick out' and be exposed to the solvent. Since solvation by water is the predominant factor in hydrophobicity [34], any stabilization or destabilization of the α-helical structure of these peptides can be attributed mainly to the interactions between water and the

Table 2 Composite Helix-forming Parameters Ranked from the Highest to the Lowest Values and Compared with the Normalized Values of the Differences in α-Helical Propensity ($\Delta\Delta G$), Sidechain Hydrophobicity (Δt_R) and Helix Stability (Δt_m)

Amino acid	α-Helical propensity[a] ($\Delta\Delta G$)	Sidechain hydrophobicity[b] (Δt_R)	Composite helix factor[c] (rank) $(2\Delta\Delta G + \Delta t_R)/3$	Helix stability[d] (Δt_m)
Ala	100	41	80 (2)	72
Arg	94	− 14	58 (7)	65
Leu	84	97	89 (1)	100
Lys	73	− 23	41 (11)	32
Met	70	74	71 (4)	79
Gln	64	− 10	39 (12)	48
Ile	61	99	74 (3)	94
Trp	51	97	67 (5)	60
Phe	50	100	67 (5)	70
Tyr	45	63	50 (9)	57
Cys	45	49	46 (10)	33
Val	44	76	54 (8)	67
Asn	34	− 28	13 (16)	12
Ser	34	− 5	21 (15)	29
His	34	8	25 (13)	25
Glu	33	− 31	12 (17)	7
Thr	29	13	23 (14)	25
Asp	22	− 55	− 4 (19)	− 23
Gly	0	0	0 (18)	0

[a]Calculated as $(\Delta\Delta G_X / -0.96) \times 100$, where $\Delta\Delta G_X$ is the α-helical propensity of any peptide analogue relative to the Gly analogue and 0.96 is the α-helical propensity of the Ala analogue.
[b]Calculated as $(\Delta t_{RX}/7.8) \times 100$, where 7.8 is the relative retention time of the Phe analogue and Δt_{RX} is the retention time of a particular peptide analogue relative to the Gly analogue.
[c]These values represent the composite contributions from α-helical propensity and sidechain hydrophobicity calculated on a 2:1 basis.
[d]Calculated as $(\Delta t_{mx}/26.3) \times 100$, where 26.3 is the relative melting temperature of the Leu analogue and Δt_{mx} is the melting temperature of any peptide analogue relative to the Gly analogue. The values for $\Delta\Delta G_X$, Δt_{mx} and Δt_{RX} were obtained from Table 1 with the highest value in each parameter set normalized to 100 and the value for the Gly analogue taken as zero.

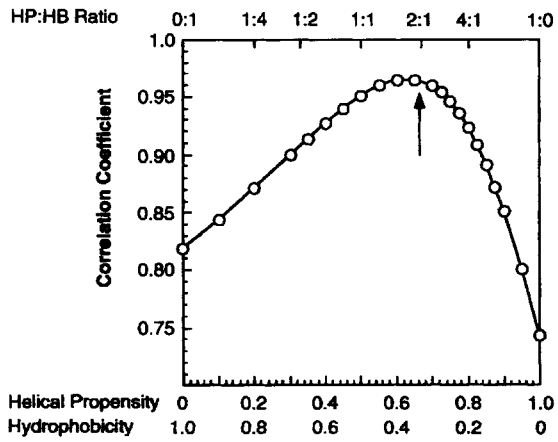

Figure 6 Plot of correlation coefficient between different combinations of α-helical propensity (HP) and sidechain hydrophobicity (HB) with peptide stability. The arrow indicates the optimum correlation at HP:HB ratio of 2:1.

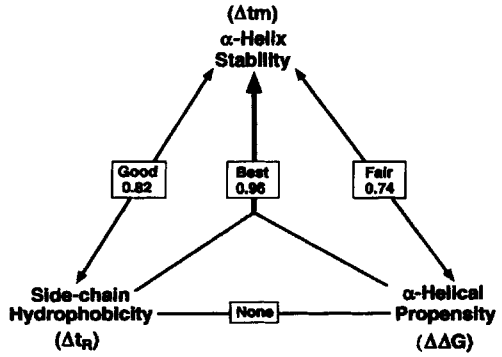

Figure 7 Schematic representation of the relationship between sidechain by hydrophobicity (Δt_R), melting temperature (Δt_m) and α-helical propensity ($\Delta\Delta G$). The numbers in the boxes represent their respective correlation coefficients. The correlation coefficient of 0.96 was calculated at the combined ratio of α-helical propensity and sidechain hydrophobicity values of 2:1.

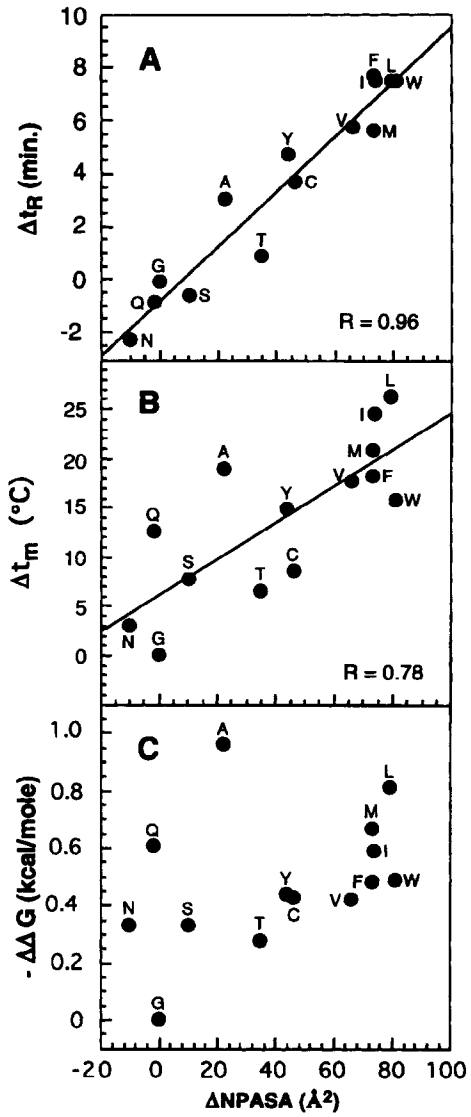

Figure 8 Plots of the relationship between the relative nonpolar accessible surface area (ΔNPASA) and (A) sidechain hydrophobicity (Δt_R), (B) peptide stability (Δt_m) and (C) α-helical propensity (ΔΔG).

varying sizes of the exposed hydrophobic surface of amino acid sidechains. This is consistent with the excellent correlation ($r = 0.96$, Figure 8A) observed between sidechain hydrophobicity (Δt_R) and the relative non-polar accessible surface area calculated by Sereda *et al.* [11] from 14 of the same peptide analogues used in this study (excluded were 5 peptide analogues containing the charged residues Asp, Glu, His, Lys and Arg). A good correlation ($r = 0.83$) has also been reported between the surface areas of all amino acid sidechains and their free energy of transfer from vapour to cyclohexane [18]. In turn, the hydrophobicity of the exposed sidechains contribute to helix stabilization as shown by the fair correlation between Δt_m and ΔNPASA ($r = 0.78$, Figure 8B). It should be recalled that the rest of the stability contribution comes from its inherent α-helical propensity.

This interpretation is consistent with the view that sidechain hydrophobicity is an important force in protein folding [35]. The helix–coil transition has been shown to be affected by both entropy and enthalpy [36], but at low temperatures the hydrophobic effect is believed to be entropic [35, 37]. While sidechain entropy favours the unfolded state of the polypeptide backbone, unfolding the α-helix would result in an unfavourable solvent exposure of the hydrophobic sidechains of the 'guest' amino acid, as well as the methyl groups of the surrounding alanine residues. The increase in non-polar surface area that results from the unfolding of hydrophobic sidechains of the non-polar face is expected to result in a large loss of entropy of the 'ordering' of water molecules that would be in contact with the hydrophobic surface [34, 35, 37]. Because of the proportionately large gain in entropy of water upon folding of the more hydrophobic sidechains, the more hydrophobic peptide analogues attain a more stable folded (α-helical) structure. Additional stabilization may also come from the overall increase in hydrophobicity on the non-polar face which, even in the absence of van der Waals contacts, restricts access of water molecules to and prevents solvation of the peptide backbone.

The lack of correlation between ΔNPASA and α-helical propensity (Figure 8C) is consistent with the lack of correlation between sidechain hydrophobicity and α-helical propensity (Figure 5C). Contrary to previous suggestions [1–4], these results suggest that sidechain hydrophobicity plays a lesser role in α-helix formation, but is more important in stabilizing the α-helix once formed. These results stress the importance of taking into consideration both α-helical propensity and sidechain hydrophobicity values in the design of α-helical peptides, and the composite helix factor in Table 2 should provide at least a semi-quantitative measure of their combined effects.

Finally, this study has also demonstrated an important application of reversed-phase HPLC technology in studying molecular structures. Traditionally, reversed-phase HPLC has been almost exclusively used in purification protocols. However, these results support our previous suggestion that peptide and protein molecules can interact with the reversed-phase column in specific folded forms (α-

helical secondary structures) [15] and, therefore, the hydrophobicity of reversed-phase chromatography can be used to mimic protein–ligand interactions involving α-helical conformations [11].

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