J. Mol. Biol. (1994) **241**, 706–713

Helix-stabilizing Interaction Between Tyrosine and Leucine or Valine when the Spacing is $i, i + 4$

S. Padmanabhan and Robert L. Baldwin

Department of Biochemistry
Beckman Center, Stanford University Medical School, Stanford, CA 94305-5307, U.S.A.

A helix-stabilizing interaction between tyrosine and leucine or valine has been found in alanine-based peptide helices when the spacing is $i, i + 4$. Control peptides have identical compositions but an $i, i + 3$ spacing. This is, to our knowledge, the first report of a helix-stabilizing interaction between two non-polar side-chains in an isolated helix. The results explain why, in an earlier study, leucine was found to have a helix propensity similar to that of alanine in an alanine-based peptide, whereas later work from another laboratory and our own has shown that alanine is markedly more helix-stabilizing than leucine in alanine-based peptides. The change in helix content resulting from the $i, i + 4$ Tyr–Leu interaction is comparable to the changes seen for other specific interactions between pairs of side-chains, such as ion-pair or Phe · His^+ interactions.

Keywords: α-helix stability; non-polar interactions; peptide helices; side-chain interactions

1. Introduction

The hydrophobic interaction that results from exclusion of water when non-polar surfaces are brought into contact is thought by many workers to be the dominant interaction driving protein folding (Nozaki & Tanford, 1971; Chothia, 1974; Dill, 1990). The suggestion has often been made that hydrophobic interactions involving non-polar side-chains may contribute to the stability of individual α-helices (Lotan *et al.*, 1966; Richards & Richmond, 1978; Dill *et al.*, 1993) but it has not yet been demonstrated that two non-polar side-chains can make a specific interaction in an isolated helix to increase the stability of that helix. One reason why it has been difficult to demonstrate such an effect is the loss of conformational entropy that occurs when two side-chains are fixed in a specific conformation. Estimates of side-chain conformational entropy for amino acids in an α-helix are given by Creamer & Rose (1992).

Our first experiments indicating the presence of a specific interaction between tyrosine and valine spaced $i, i + 4$ were made for a different purpose. We were studying the helix contents and NMR spectra of alanine-based peptides each containing a single Ala → Val substitution at a different position in the helix. Because valine is a helix-breaking residue (Padmanabhan *et al.*, 1990), we expected to find a decrease in helix content at all positions of the substitution, with the largest decrease occurring near the center of the peptide (position 9) because the "frayed-end" effect is smallest at the center (Chakrabartty *et al.*, 1991). To our surprise, the peptide with valine at position 5 proved to have a higher helix content than the one with valine at position 4. (Position 4 is closer to the end of the helix and the frayed-end effect should be larger at 4 than 5.) A possible explanation was the presence of an interaction between tyrosine (position 1) and valine (position 5).

This interpretation also suggested a possible explanation for an earlier puzzling result. We had found that a peptide containing three Ala → Leu substitutions has nearly the same helix content as the alanine reference peptide (Padmanabhan *et al.*, 1990), indicating that leucine and alanine have similar helix propensities in this reference peptide. Park *et al.* (1993) have found, however, that alanine has a markedly higher helix propensity than leucine in an alanine-based peptide and this conclusion has been confirmed in our laboratory using a different reference peptide (Chakrabartty *et al.*, 1994). The earlier peptide studied by us that gave the anomalous result contained tyrosine at position 1 and leucine at position 5. Thus, the earlier result might be explained by a tyrosine–leucine interaction when the spacing is $i, i + 4$. Tyrosine at the N or C terminus is used widely in studies of peptide helices to give an accurate determination of peptide concentration, for use in calculating mean residue ellipticity (Marqusee *et al.*, 1989). Thus, the possible occurrence of a helix-stabilizing interaction involving N-terminal tyrosine spaced $i, i + 4$ from leucine or valine is important for the correct evaluation of peptide helix studies, as well as for understanding the

Correspondence: Robert L. Baldwin, Department of Biochemistry, Beckman Center, Stanford University Medical School, Stanford, CA 94305-5307, U.S.A.

0022-2836/94/350706-08 $08.00/0

mechanism of protein folding. One aim of this study is to find out if a helix-stabilizing Tyr–Leu ($i, i + 4$) interaction led to an earlier incorrect evaluation by us of the relative helix propensities of leucine and alanine.

A study of this problem is reported here. The tyrosine–leucine pair is studied both with tyrosine at the N terminus and in the interior of the helix, because of possible complications from N-cap interactions (Chakrabartty *et al.*, 1993*a*). Both orientations of the tyrosine–leucine pair are investigated, with tyrosine either N or C-terminal to leucine. The design of our experiments is to compare the helix contents of two peptides with identical compositions, with tyrosine at the same position in each peptide, and with the spacing between tyrosine and leucine or valine fixed at $i, i + 4$ in one peptide and $i, i + 3$ in the other. The first peptides made to study this problem showed that the helix content is significantly higher when the Tyr-Leu spacing is $i, i + 4$ as compared to $i, i + 3$, and so peptides with an $i, i + 3$ spacing were adopted as controls. The position of the tyrosine residue is held constant in each pair of peptides for two reasons: (1) tyrosine has a low helix propensity in alanine-based peptides (Chakrabartty *et al.*, 1994) and therefore its position affects the overall helix content *via* the frayed-end effect (Chakrabartty *et al.*, 1991), and (2) the tyrosine side-chain contributes significantly to the CD spectrum at 222 nm when the peptide is helical (Chakrabartty *et al.*, 1993*b*). For the latter reason, the relative helix contents of the two peptides were checked by an independent method, hydrogen exchange kinetics (Rohl *et al.*, 1992).

2. Materials and Methods

(a) *Peptide synthesis and purification*

Peptides were synthesized on a Milligen 9050 automated synthesizer using the pentafluorophenyl esters of Fmoc (9-fluorenylmethoxycarbonyl) amino acids (Milligen) on PAL-MBHA (5-(4-Fmoc-aminomethyl-3,5-dimethoxy-phenoxy)-valeric acid-*p*-methylbenzhydrylamine) resin (Milligen) or on Rink (4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy) resin from Advanced ChemTech. Peptides were acetylated at the N terminus using acetic anhydride and then cleaved from the resin with 95:5 trifluoroacetic acid:anisole mixture for two to four hours to yield peptides amidated at the C terminus. The pure peptides were obtained by reverse phase fast protein liquid chromatography (f.p.l.c.-Pharmacia) using a C_{18} resin with gradients of 10 to 40% acetonitrile: water (0.1% trifluoroacetic acid) as described earlier (Padmanabhan & Baldwin, 1991). Peptide identity was confirmed using fast atom bombardment (FAB) mass spectrometry.

(b) *Circular dichroism (CD) measurements*

CD measurements were made on an Aviv 60DS spectropolarimeter equipped with a Hewlett-Packard 89100A temperature control unit. The spectropolarimeter was calibrated with (+)-10-camphorsulfonic acid (Chen & Yang, 1977). CD spectra of peptides at 0°C, 0.1 M KF (pH 7.0; 1 mM potassium phosphate), 40 to 50 μM peptide, were obtained using a 1 mm pathlength cuvette, 0.2 nm step size, 1 s average time and averaged over four scans. Ellipticity measurements at 222 nm, reported as mean molar residue ellipticity ($[\theta]_{222}$ *in deg cm*^{2} *dmol*$^{-1}$) were measured in 1 M NaCl (pH 7.0) and "CD buffer" (our standard buffer for measuring ellipticity *versus* pH: 1 mM each of sodium citrate, sodium phosphate, sodium borate), using 10 mm pathlength cuvettes (10 to 30 μM peptide). Peptide concentrations were determined from the tyrosine absorbance at 275 nm in 6 M guanidium hydrochloride (Brandts & Kaplan, 1973). Trifluoroethanol (TFE) titrations were carried out at 0°C, 0.1 M NaCl (pH 7.0), using the procedure described by Nelson & Kallenbach (1988). The values of $-[\theta]_{222}$ reported here were tested for concentration dependence in the range 10 to 120 μM and were found to be independent of peptide concentration. Helix content was calculated from $[\theta]_{222}$ by using +640 for 0% helix, −34,100 for 100% helix, 17 residues (Scholtz *et al.*, 1991). It has been shown that the Tyr side-chain can contribute as much as +4400 to the $[\theta]_{222}$ of a peptide that is about 80% helical (Chakrabartty *et al.*, 1993*b*), but the dependence of this contribution on helicity of the peptide or the position of Tyr remains to be examined. For this reason we have not corrected for the contribution to $[\theta]_{222}$ by Tyr. The helix contents reported in this paper that are determined from $[\theta]_{222}$ are therefore only approximate.

(c) *NMR experiments*

One-dimensional ^{1}H-NMR spectra of peptide solutions were obtained on a GE GN-Omega 500 NMR spectrometer operating at a ^{1}H frequency of 500.13 MHz. Spectra were recorded using a spectral width of 6000 Hz 16 scans in 16 K data points and a pulse width of 8 μs in (1) 1 M NaCl, ^2H_2O, CD buffer (pH 7.0) at 3°C (using pre-cooled nitrogen) or variable temperature as indicated; (2) 40% (by volume) deuterated trifluorethanol (TFE-d_3), 60% ^2H_2O, containing CD buffer (pH 7.0) at 3°C; (3) 6 M urea, ^2H_2O, CD buffer (pH 7.0) at 3°C. For deuterated NMR samples in urea, peptide and ultrapure urea were dissolved in ^2H_2O, CD buffer, lyophilized extensively and the NMR sample was made up in the requisite amount of ^2H_2O to yield a final urea concentration of 6 M. Chemical shifts corresponding to random coil values obtained for the unstructured peptide acetyl-YKAAVAK-amide (see Table 1) and in 6 M urea solutions are essentially identical. Chemical shifts reported here are those obtained for deuterated samples and referenced to 3-(trimethylsilyl)-propionate (TSP†).

Amide ^{1}H exchange data were collected as follows. The peptide (1 to 2 mM) was first dissolved in H_2O with the pH adjusted to around 2.30 and then lyophilized. The lyophilized peptide was then dissolved at the start of the exchange-out experiment ($t = 0$) in ^2H_2O containing 1 M NaCl and CD buffer at pH* 2.30, all of which were pre-cooled in ice. Multiple one-dimensional spectra were then obtained at 3°C, with a 5000 or 6000 Hz spectral width, 8 μs pulse width, 4096 complex points, 64 scans and a 5 ms recycle delay. The total acquisition time per spectrum was under 1 minute. Spectra were processed using FELIX on a Silicon Graphics Personal Iris computer. A 1 Hz

† Abbreviations used: TSP, 3-(trimethylsilyl)propionate; RELAY, relayed two-dimensional correlated spectroscopy; ppm, parts per million.

line-broadening exponential window function was used prior to Fourier transformation and the total NH intensities were normalized to the intensity of a non-exchanging aromatic tyrosine resonance. All reported pH* values were determined at room temperature after the acquisition of NMR exchange-out spectra and are not corrected for isotope effects (hence pH*). Deviations from the reference pH* of 2.30 were normalized by correcting the time axis according to the equation described previously by Rohl *et al.* (1992):

$$\text{time*} = \text{time}[10^{2.30}/10^{\text{experimental pH*}}], \quad (1)$$

time* being the corrected time. This allows comparison of exchange behavior at a standard pH*.

The kinetic curves for hydrogen exchange of two pairs of peptides (Figures 1 and 3) were used to give approximate estimates of the helix contents of these peptides as follows. The procedure is basically the same as described by Rohl *et al.* (1992) and their value for the helix nucleation constant ($v^2 = 0.0023$) was used. Each pair of kinetic curves was used to determine three parameters: the average rate constant k_c for exchange from the random coil, which was taken to be the same for both peptides in a pair that differ only in the residue position of a leucine or valine residue, and the average helix propagation parameter $<w>$ was calculated for each peptide. Thus, a Tyr–Leu or Tyr–Val side-chain interaction, when present, contributes to the average value $<w>$. Then the helix content of each peptide was calculated from v^2 and $<w>$ by the Lifson–Roig equation, using the computer program given by J. A. Schellman (Chakrabartty *et al.*, 1991). Note that the comparison between helix content calculated by CD and by exchange kinetics is not expected to be accurate in the case of these peptides, for two reasons. First, each peptide contains a helix-breaking residue, Val or Tyr, at a position inside the helix. In a case like this, analysis of the exchange kinetics by using a single average value for $<w>$ is not an accurate approximation. Second, as mentioned above, each peptide contains a tyrosine residue, whose side-chain contributes substantially to the CD spectrum at 222 nm in a helical peptide (Chakrabartty *et al.*, 1993*b*), the correction for which is not known accurately.

Amide ^{1}H resonances were assigned from absolute value mode RELAY data (Wagner, 1983), obtained using 1 to 2 mM peptide samples in 90% H_2O/10% ^2H_2O containing CD buffer (pH 2.5). Phase cycling was as described by Bax & Drobny (1985). Each RELAY spectrum consisted of 256 blocks of t_1 values with at least 40 transients, and was collected over 1024 data points with a spectral width of 6000 Hz, 1 second recycle delay and a total mixing time of 20 ms. The solvent resonance was suppressed by presaturation.

3. Results

(a) *NMR chemical shifts and helix contents of peptide helices containing a single Ala → Val substitution at various positions*

Table 1 shows the helix contents obtained by CD for peptides with a single Ala → Val substitution at six different residue positions. The αCH and γ_1, γ_2 CH_3 resonances of the single valine residue are resolved in the 1D NMR spectrum and the chemical shifts of these resonances are also shown in Table 1. The highest helix content among the peptides is shown by 1Y5V, the peptide with valine at position 5. The chemical shifts of the Val αCH and γ_1, γ_2 CH_3 resonances also show unusual values in 1Y5V, being more upfield than those found with valine at any other position. The contrast between the chemical shift values of the Val resonances in 1Y5V and the other single valine-containing peptides probably reflects the ring current effect of Tyr1, and thereby the proximity between Tyr at position 1 and Val at position 5.

(b) *Hydrogen exchange measurements of the relative helix contents of peptides 1Y4V and 1Y5V*

To test the conclusion from Table 1 that the helix content of peptide 1Y5V is higher than that of 1Y4V, we measured the hydrogen exchange kinetics of the two peptides by the 1D NMR method of Rohl *et al.* (1992), in which the dry peptide is dissolved at zero time in ^2H_2O containing 1 M NaCl in conditions (pH* 2.30, 3°C) where hydrogen exchange even in unstructured peptides is slow enough to be measured from the change in intensity of the integrated amide proton peak. The presence of helical structure in a peptide is known to slow its exchange kinetics. Rohl *et al.* (1992) found that the kinetic exchange curves for a homologous series of peptides of differing helix contents can be fitted by the Lifson–Roig (Lifson & Roig, 1961) theory of the helix-coil transition using as adjustable parameters only average values for the helix nucleation and propagation parameters and the exchange rate constant in the non-helical, random-

Table 1

Dependence of peptide helix content on the position of Ala → Val substitution

Name	Sequence	$-[\Theta]_{222}$†‡ (deg cm^2 dmol^{-1})	Val ^{1}H chemical shifts (ppm) αCH	$\gamma1, \gamma2$ CH_3
Ref	Acetyl-Y KAAAAKAAAAKAAAAK-Amide	25,000 ± 500	—	—
YKAAVAK	Acetyl-Y KAA V AK-amide	−600 ± 100	4.06	0.95
1Y4V	Acetyl-Y KA V AAKAAAAKAAAAK-amide	18,250 ± 100	3.87	0.97, 1.02
1Y5V	Acetyl-Y KAA V AKAAAAKAAAAK-amide	20,300 ± 600	3.84	0.89, 0.94
1Y9V	Acetyl-Y KAAAAKA V AAKAAAAK-amide	17,200 ± 400	3.87	0.99, 1.05
1Y10V	Acetyl-Y KAAAAKAA V AKAAAAK-amide	16,200 ± 100	3.91	0.99, 1.07
1Y14V	Acetyl-Y KAAAAKAAAAKA V AAK-amide	18,100 ± 250	3.95	0.96, 1.02
1Y15V	Acetyl-Y KAAAAKAAAAKAA V AK-amide	19,490 ± 600	4.01	0.98, 1.04

† 1M NaCl, 1mM Na CD buffer (pH 7.0), 0°C. The mean deviation for at least 3 independent measurements of $-[\Theta]_{222}$ is shown.

‡ The value of $-[\Theta]_{222}$ for 100% helix, obtained by TFE titration, of the reference peptide is 32,200 (± 1100). Thus, the helix content of the reference peptide in this Table is estimated to be 78%.

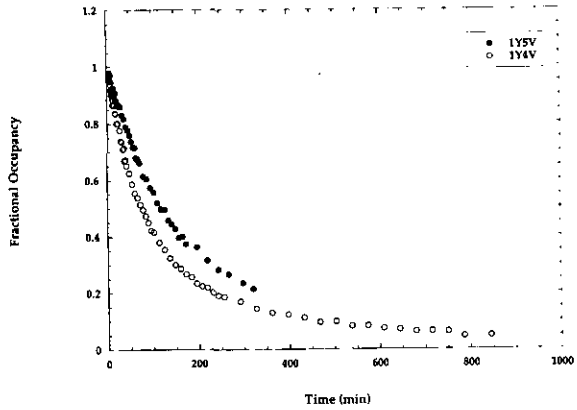

Figure 1. Kinetics of hydrogen exchange for 2 alanine-based peptides each containing a single valine residue either at position 4 (1Y4V) or 5 (1Y5V). Their sequences and properties are given in Table 1. Conditions: 1.0 M NaCl in ^2H_2O (pH* 2.30) 3°C (see Materials and Methods).

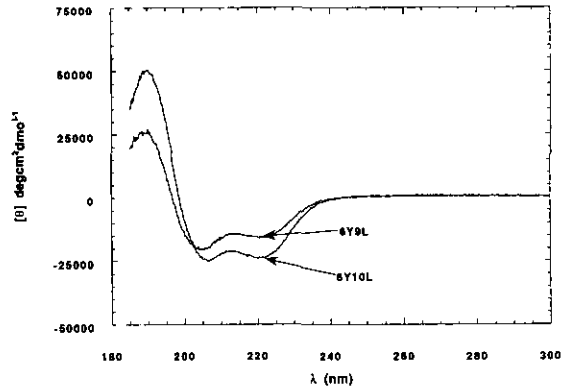

Figure 2. CD spectra of 2 alanine-based peptides containing a single tyrosine and a single leucine residue either at a spacing of $i, i + 4$ (6Y10L) or $i, i + 3$ (6Y9L). Their sequences are given in Table 2. Conditions: 0°C, 0.1 M KF (pH 7.0; 1 mM potassium phosphate) (see Materials and Methods).

coil form. Here we are interested in comparing the relative helix contents of two peptides with identical amino acid compositions and chain lengths. The peptide with the lower helix content should show faster exchange kinetics. Figure 1 shows that the average exchange kinetics of peptide 1Y4V are definitely faster than those of 1Y5V, confirming that peptide 1Y5V has a higher helix content. The helix contents calculated from CD (Table 1) are 60.3% (1Y5V) and 54.4% (1Y4V), difference = 5.9%, while the helix contents calculated from the exchange kinetics are 64.2% (1Y5V) and 49.4% (1Y4V), difference = 14.8%. The lack of quantitative agreement between the two methods is not surprising because each method contains a dubious assumption in the case of these peptides (see Materials and Methods). The qualitative conclusion is, however, the same by both methods: peptide 1Y5V is more helical than 1Y4V, in contrast to expectation based only on the frayed-end effect and the fact that valine is a helix-breaking residue ($s = 0.22$, Chakrabartty, *et al.*, 1994). The value calculated for the average rate constant in the random coil is 0.019 min^{-1}, which is close to the value (0.018 min^{-1}) found by Rohl *et al.* (1992) at 5°C.

(c) *Tests for interaction between tyrosine and leucine spaced* i, i + 4

Table 2 shows the amino acid sequence of the leucine-substituted peptide studied earlier by us (Padmanabhan *et al.*, 1990) and its helix content measured by CD. This peptide, 1Y(5, 10, 15)L, contains tyrosine in position 1 and three leucine residues at positions 5, 10, 15. To test whether there is a helix-stabilizing interaction between Tyr1 and Leu5, the helix content of this peptide was remeasured and a new peptide, 1Y(4, 10, 15)L, was made with Tyr1 and Leu4. There is a substantial decrease in the helix content of the new peptide measured by CD: $-[\theta]_{222}$ drops from 26,000 (±500) for a spacing of $i, i + 4$ to 20,400 (±100) for a spacing of $i, i + 3$ (Table 2).

In order to find out if the effect is dependent on N-cap interactions specific for N-terminal tyrosine, the pair of tyrosine and leucine residues was moved to the interior of the helix: either at positions 6 and 10 ($i, i + 4$ spacing) or at 6 and 9 ($i, i + 3$ spacing) in a second set of 17-residue peptides that contain only a single leucine residue. Figure 2 shows the CD spectra of these two peptides, which indicate that 6Y10L is decidedly more helical than 6Y9L ($-[\theta]_{222}$ = 24,500 (±300) *versus* 17,800 (±200), Table

Table 2
Helix stabilizing interaction between Tyr and Leu 4 residues apart

Peptide	Sequence	Y-L Spacing	$-[\Theta]_{222}$† (deg. cm² dmol⁻¹)
A. *Tyrosine N-terminal*			
1 Y(5, 10, 15)L‡	Ac-**Y**KAA**L**AKAA**L**AKAA**L**AK-NH_2	$i, i + 4$	26,000 ± 500
1 Y(4, 10, 15)L	Ac-**Y**KA**L**AAKAA**L**AKAA**L**AK-NH_2	$i, i + 3$	20,400 ± 100
B. *Tyrosine at an interior position*			
6 Y10L	Ac-AKAAA**Y**KAA**L**AKAAAAK-NH_2	$i, i, + 4$	24,500 ± 300
6 Y9L	Ac-AKAAA**Y**KA**L**AAKAAAAK-NH_2	$i, i + 3$	17,800 ± 200

† The mean residue ellipticity measured at 0°C, 1 M NaCl (pH 7.0) 1 mM CD buffer, peptide concentrations of 15 to 30 μM, 1 cm pathlength cuvette.

‡ This is the peptide studied earlier by Padmanabhan *et al.* (1990). Its value of $-[\Theta]_{222}$ has been re-measured and agrees with the earlier value (26,300).

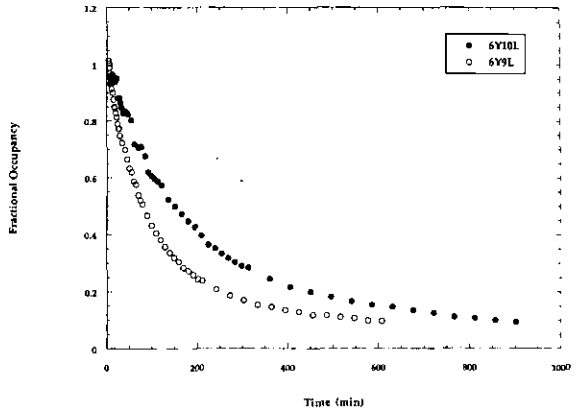

Figure 3. Kinetics of hydrogen exchange shown by peptides 6Y10L and 6Y9L (see Table 2 for their sequences and Figure 2 for their CD spectra). Conditions: 1.0 M NaCl in ^2H_2O (pH* 2.30) 3°C (see Materials and Methods).

2). Both peptides show monomolecular helix formation as shown by the concentration independent and reversible thermal transitions.

This conclusion is tested further by comparing the H-exchange kinetics of the two peptides: peptide 6Y10L exchanges substantially more slowly than 6Y9L (Figure 3). The helix contents of the two peptides calculated from the exchange kinetics are 65.3% (6Y10L) and 43.8% (6Y9L), difference 21.5%. The helix contents calculated by CD are 72.4% and 53.1%, respectively, difference = 19.3%. Thus, the helix contents calculated from exchange kinetics and from CD agree as well as expected for these peptides (see Materials and Methods). The value calculated for the average rate constant in the random coil is $k_c = 0.015$ min^{-1}, which is close to the value (0.018 min^{-1}) reported by Rohl *et al.* (1992) at 5°C.

The ^{1}H chemical shifts in 6Y10L of the Leu αCH (3.92 ppm) and the Leu γ, δ CH_3 (0.78, 0.76 ppm) are considerably more upfield than those for Leu in 6Y9L (4.10 ppm for the αCH and 0.93 and 0.89 ppm for the γ and δ CH_3, respectively), probably because of the ring current effect of Tyr. This suggests the proximity of Tyr and Leu side-chains in 6Y10L, where they are spaced $i,i + 4$. The lower helix contents of the pair of peptides in Table 2B, compared to the pair in Table 2A, probably results from Tyr being inside the helix (position 6) in Table 2B. Tyrosine has a low helix propensity ($s = 0.4$, Chakrabartty *et al.*, 1994) and therefore its position affects the helix content of a peptide through the frayed-end effect.

(d) *Tests for a Tyr–Leu* i, i + 4 *interaction in shorter peptides and in the reverse orientation*

It is useful to test for the presence of a helix-stabilizing interaction in short peptides, where the presence or absence of the interaction may make the difference between observing significant helix formation or not (compare Shoemaker *et al.*, 1985; Forood *et al.*, 1993). Table 3 shows that the change in helix content caused by the presence of the Tyr–Leu $i, i + 4$ interaction in a 12-residue peptide is almost twofold, compared to the control peptide with a Tyr–Leu $i,i + 3$ spacing.

Table 3 also shows a test for the reverse Leu–Tyr interaction in a second pair of 12-residue peptides. The peptide with the $i,i + 4$ spacing has a markedly higher helix content than the one with the $i,i + 3$ spacing, showing that an interaction between tyrosine and leucine also exists in the Leu–Tyr orientation, but the difference in helix content between the $i, i + 4$ and $i, i + 3$ peptides is smaller in the reverse orientation, perhaps because of the difference between Tyr and Leu in their preference for *trans versus gauche plus* χ_1 rotamers (see section (e) below).

(e) *Modeling the Tyr–Leu and Tyr–Val interactions*

Using the graphic molecular modeling program (without energetics), INSIGHT II (Version 2.20, Biosym Technologies, San Diego, CA†), we asked whether the preferred rotamer angles of Tyr and Leu residues in α-helices in proteins are consistent with a Tyr–Leu contact interaction when the spacing is $i, i + 4$. The *trans* (t, 180°) and *gauche plus* (g^+, 300°) conformations about the C^α–C^β bond are equally preferred for leucine, while t is preferred over g^+ by 2:1 for tyrosine; the *gauche minus* (g^-, 60°) conformation is rarely observed for either residue (McGregor *et al.*, 1987). When standard values are used for the helix backbone dihedral angles ($\Phi = -62°$, $\Psi = -41°$; Creighton, 1993), we find that good contact interactions can be made when Tyr is *trans* and Leu is *gauche plus* and the Tyr–Leu spacing is $i,i + 4$, and when Leu is *trans* and Tyr is *gauche plus* and the Leu–Tyr spacing is $i, i + 4$.

† Insight II a registered trademark of Biosym Technologies Inc. 1993.

Table 3

Tests for a Tyr-Leu interaction in shorter peptides and in the reverse orientation

Peptide	Sequence	Y-L or L-Y Spacing	$-[\Theta]_{222}$† (deg. cm^2 dmol^{-1})
3Y7L	Ac-AA<u>Y</u>KAA<u>L</u>AKAAA-NH_2	$i, i + 4$	11,700 ± 300
3 Y6L	Ac-AA<u>Y</u>KA<u>L</u>AAKAAA-NH_2	$i, i + 3$	6500 ± 100
6 L10Y	Ac-AAAKA<u>L</u>AAK<u>Y</u>AA-NH_2	$i, i + 4$	13,600 ± 500
7 L *10* Y	Ac-AAAKAA<u>L</u>AK<u>Y</u>AA-NH_2	$i, i + 3$	10,500 ± 300

† The mean residue ellipticity measured at 0°C, 1 M NaCl (pH 7.0); 1 mM each of sodium phosphate and sodium borate), peptide concentrations of 15 to 30 μM, 1 cm pathlength cuvette.

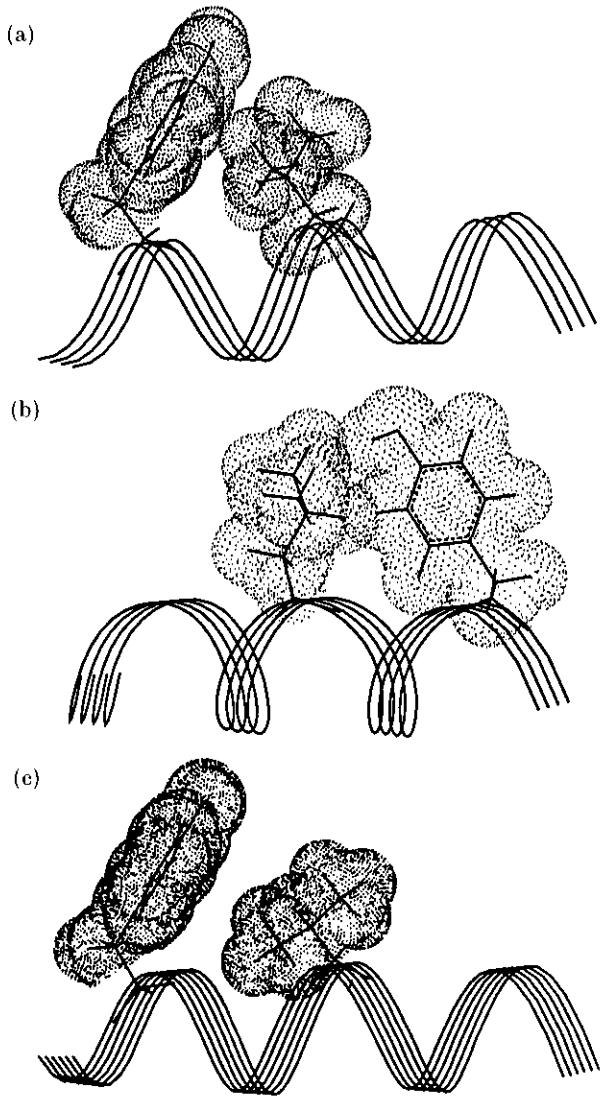

Figure 4. Models of contact interactions made by (a) Tyr and Leu or by (b) Leu and Tyr in a standard helix using the program INSIGHT II (see text). The χ_1 rotamer of Tyr is *trans* (180°) and χ_2 is 77°; the χ_1 rotamer of Leu is *gauche* + (287°) and χ_2 is 179°. χ_1, χ_2 = 190°, 56° for Leu and 287°, 102° for Tyr (see text and Table 4 in McGregor *et al.* (1987)). The definition of the dihedral angles conforms to the IUPAC-IUB (1970) convention, but the χ_1 and χ_2 angles are converted to the range 0° to 360° rather than −180° to +180°, according to definitions described by McGregor *et al.* (1987). The van der Waals surfaces of the Leu and Tyr side-chains are also shown. (c) Possible model of an (i, i + 4) Tyr–Val interaction using standard rotamer angles from McGregor *et al.* (1987): Tyr χ_1 180°; χ_2 77°; Val χ_1 166°.

These models are shown in Figure 4(a), (b), and the values of χ_1, χ_2 are given in the Figure legend.

A corresponding model for the Tyr–Val interaction was built and is shown in Figure 4(c): the angles are given in the Figure legend. Although there is no van der Waals contact between the two residues using these standard rotamer angles for Tyr and Val, exclusion of water between the two residues can nevertheless occur (see Figure 1, Richards, 1977).

4. Discussion

(a) *Nature and properties of the Tyr–Leu interaction*

The evidence presented here for the Tyr–Leu $i, i + 4$ interaction appears straightforward. The contribution of the tyrosine side-chain to the CD spectrum at 222 nm can present a problem when tyrosine is inside an α-helix or even when it is at the N terminus (Chakrabartty *et al.*, 1993*b*), but this effect has been taken into account here by using a control $i, i + 3$ peptide with tyrosine at the same position as in the $i,i + 4$ peptide. Three pairs of peptides give the same result (Tables 2 and 3): the helix content found by CD is substantially higher when the Tyr–Leu spacing is $i, i + 4$ than when it is $i, i + 3$. Slower hydrogen exchange kinetics in the peptide with the $i, i + 4$ spacing (Figure 3) confirm that the difference measured by CD corresponds to a difference in helix content between the two peptides and is not caused merely by a contribution of the Tyr side-chain to the CD signal at 222 nm (Chakrabartty *et al.*, 1993*b*). A smaller, but significant, CD difference between the $i, i + 4$ and $i, i + 3$ spacings is found for the reverse Leu–Tyr orientation (Table 3).

The size of the change in $[\theta]_{222}$ when the $i, i + 4$ Tyr–Leu interaction is present (about −6000 deg cm^2 dmol^{-1}, Tables 2 and 3) is roughly comparable to the change seen in the C-peptide helix from ribonuclease A when either the Glu 2^- · Arg 10^+ ion pair interaction (Fairman *et al.*, 1990) or the Phe 8 · His 12^+ pseudo-H-bond interaction (Shoemaker *et al.*, 1990) is present, or when the latter interaction is present in an alanine-based helix (Armstrong *et al.*, 1993). The change reported here is also roughly comparable to the change seen when the best N-cap amino acid (Asn) is replaced by the worst N-cap residue (Gln) in an alanine-based helix (Chakrabartty *et al.*, 1993*a*). Thus, the non-polar interaction observed when Tyr and Leu are spaced $i,i + 4$ is comparable in strength to other helix-stabilizing interactions reported earlier that involve pairs of specific side-chains. It is possible in principle to determine the standard free energy of the interaction when only a single pair of interacting residues is present in an alanine-based peptide (Scholtz *et al.*, 1993). The Tyr–Leu system is not a favorable one, however, for applying this methodology when the helix content is determined by CD because the Tyr side-chain contributes to the CD spectrum of a helical peptide (Chakrabartty *et al.*, 1993*b*).

The nature of the Tyr–Leu interaction is almost certainly exclusion of water from non-polar surface that is buried when the side-chains interact. Van der Waals interactions between two non-polar side-chains are expected to be approximately the same, per unit of surface area, as the van der Waals interactions between a non-polar side-chain and water (Lee, 1991), so that van der Waals interactions alone are not able to drive complex formation between non-polar side-chains. The major obstacle to forming a

helix-stabilizing interaction between two non-polar side-chains is expected to be the loss of side-chain conformational entropy when the two side-chains are fixed in the interacting conformation. In this respect, tyrosine may be a particularly favorable amino acid for forming non-polar interactions in helices because its side-chain rotamers are restricted in helices (McGregor *et al.*, 1987) and it has a large non-polar surface area. Figure 4 shows that models for Tyr–Leu and Leu–Tyr $i, i + 4$ interactions can be built, but an analysis of the energetics of the interaction for different combinations of rotamers remains to be worked out.

It is important to determine the structure of a single helix containing a pair of interacting Tyr, Leu or Leu, Tyr residues. This is not likely to be accomplished readily, since it is difficult to crystallize isolated helices in aqueous solution, and NMR structure determination requires, among other things, that the helix content of the peptide be close to 100%.

(b) *Non-polar side-chain interactions within helices in protein folding*

Hydrophobic interactions between non-polar side-chains are believed to be the major factor responsible for docking two helices in a protein (Richmond & Richards, 1978; Chothia *et al.*, 1981). Whether or not hydrophobic interactions between non-polar side-chains are also important in stabilizing isolated α-helices is more debatable. Richards & Richmond (1978) investigated the question by determining the amount of buried non-polar surface area belonging to various amino acid side-chains in helices in myoglobin, as well as in a polyalanine helix. They concluded that the amount of buried non-polar surface area when the helix is formed is fairly constant among the various amino acids, either in the helices of myoglobin or in a polyalanine helix. The question was examined experimentally by Blaber *et al.* (1993) who measured X-ray structures and thermal stabilities for a series of mutants with various amino acids at a solvent-exposed site (residue 44) in a helix of bacteriophage T4 lysozyme, and who concluded that the amount of non-polar surface buried when the helix is formed is a significant determinant of the helix propensity. The effect on stability of folding of substituting glycine for alanine at different positions in the two helices of barnase was compared to the change in buried non-polar surface area by Serrano *et al.* (1992), who concluded that the hydrophobic interaction is of major importance here.

A set of 144 protein structures in the Protein Data Base was surveyed for Tyr–Leu and Leu–Tyr $i, i + 4$ pairs in protein helices (T. Klingler, personal communication). This was done using the program Iditis (Oxford Molecular Group) written by S. P. Gardener and J. M. Thornton. The results indicate that the statistical significance of Tyr–Leu and Leu–Tyr $i, i + 4$ occurrences is marginal (data not shown). This conclusion is not surprising, because non-polar side-chains commonly occur on the hydrophobic faces of helices that become buried during folding, and the resulting tertiary interactions involving non-polar side-chains are energetically more important than the interactions that can occur in isolated helices. Nevertheless, hydrophobic contacts within single units of secondary structure, either α-helices or β-sheets, could be important at early stages of folding in directing the folding pathway towards the correct native structure (Dill *et al.*, 1993).

We thank Tod Klingler for help in searching the Protein Data Base, Avi Chakrabartty for his discussion, Stewart Loh for help with Insight II, Carol Rohl for help with the hydrogen exchange calculations, and the Mass Spectrometry Facility, University of California at San Francisco (supported by NIH Grant RR 01614) for verification of peptide identity. S.P. was a Fellow of the Arthritis Foundation. Our work was supported by NIH Grant GM 31475.

References

Armstrong, K. M., Fairman, R. & Baldwin, R. L. (1993). The $(i, i + 4)$ Phe-His interaction studied in an alanine-based α-helix. *J. Mol. Biol.* **230**, 284–291.

Bax, A. & Drobny, G. (1985). Optimization of two-dimensional homonuclear relayed coherence transfer NMR spectroscopy. *J. Magn. Reson.* **61**, 306–320.

Blaber, M., Zhang, X. & Matthews, B. W. (1993). Structural basis of amino acid α-helix propensity. *Science*, **260**, 1637–1640.

Brandts, J. F. & Kaplan, K. J. (1973). Derivative spectroscopy applied to tyrosyl chromophores. Studies on ribonuclease, lima bean inhibitors, insulin and pancreatic trypsin inhibitor. *Biochemistry*, **12**, 2011–2024.

Chakrabartty, A., Schellman, J. A. & Baldwin, R. L. (1991). Large differences in the helix propensities of alanine and glycine. *Nature (London)*, **351**, 586–588.

Chakrabartty, A., Doig, A. J. & Baldwin, R. L. (1993*a*). Helix capping propensities in peptides parallel those in proteins. *Proc. Nat. Acad. Sci., U.S.A.* **90**, 11332–11336.

Chakrabartty, A., Kortemme, T., Padmanabhan, S. & Baldwin, R. L. (1993*b*). Aromatic sidechain contribution to far ultraviolet circular dichroism of helical peptides and its effect on the measurement of helix propensities. *Biochemistry*, **32**, 5560–5565.

Chakrabartty, A., Kortemme, T., & Baldwin, R. L. (1994). Helix propensities of the amino acids measured in alanine-based peptides without helix-stabilizing side-chain interactions. *Protein Sci.* **3**, 843–852.

Chen, G. C. & Yang, J. T. (1977). Two point calibration of circular dichrometer with d-10-camphorsulfonic acid. *Anal. Letters*, **10**, 1195–1207.

Chothia, C. H. (1974). Hydrophobic bonding and accessible surface area in proteins. *Nature (London)*, **248**, 338–339.

Chothia, C., Levitt, M. & Richardson, D. (1981). Helix to helix packing in proteins. *J. Mol. Biol.* **145**, 215–250.

Creamer, T. P. & Rose, G. D. (1992). Side-chain entropy opposes α-helix formation but rationalizes experimentally determined helix-forming propensities. *Proc. Nat. Acad. Sci., U.S.A.* **89**, 5937–5941.

Creighton, T. E. (1993). In *Proteins: Structures and Molecular Properties*, p. 183, W. H. Freeman & Co.

Dill, K. A. (1990). Dominant forces in protein folding. *Biochemistry*, **29**, 7133–7155.

Dill, K. A., Fiebig, K. M. & Chan, H. S. (1993). Cooperativity in protein folding kinetics. *Proc. Nat. Acad. Sci., U.S.A.* **90**, 1942–1946.

Fairman, R., Shoemaker, K. R., York, E. J., Stewart, J. M. & Baldwin, R. L. (1990). The Glu 2^- · Arg 10^+ sidechain interaction in the C-peptide helix of ribonuclease A. *Biophys. Chem.* **37**, 107–119.

Forood, B., Feliciano, E. J. & Nambiar, K. P. (1993). Stabilization of α-helical structures in short peptides via end capping. *Proc. Nat. Acad. Sci., U.S.A.* **90**, 939–842.

IUPAC-IUB Commission on Biochemical Nomenclature (1970). *J. Mol. Biol.* **52**, 1–17.

Lee, B. (1991). Solvent reorganization contribution to the transfer thermodynamics of small molecules. *Biopolymers*, **31**, 993–1008.

Lifson, S. & Roig, A. (1961). On the theory of helix-coil transition in polypeptides. *J. Chem. Phys.* **34**, 1963–1974.

Lotan, N., Yaron, A. & Berger, A. (1966). The stabilization of the α-helix in aqueous solution by hydrophobic sidechain interaction. *Biopolymers*, **4**, 365–368.

Marqusee, S., Robbins, V. & Baldwin, R. L. (1989). Unusually stable helix formation in short alanine-based peptides. *Proc. Nat. Acad. Sci., U.S.A.* **86**, 5286–5290.

McGregor, M. J., Islam, S. A. & Sternberg, M. J. E. (1987). Analysis of the relationship between side-chain conformation and secondary structure in globular proteins. *J. Mol. Biol.* **198**, 295–310.

Nelson, J. W. & Kallenbach, N. R. (1988). Stabilization of the ribonuclease S-peptide α-helix by trifluoroethanol. *Proteins: Struct. Funct. Genet.* **1**, 211–217.

Nozaki, Y. & Tanford, C. (1971). The solubility of amino acids and two glycine peptides in aqueous ethanol and dioxane solutions. *J. Biol. Chem.* **246**, 2211–2217.

Padmanabhan, S. & Baldwin, R. L. (1991). Straight chain non-polar amino acids are good helix formers in water. *J. Mol. Biol.* **219**, 135–137.

Padmanabhan, S., Marqusee, S., Ridgeway, T., Laue, T. M. & Baldwin, R. L. (1990). Relative helix-forming tendencies of non-polar amino acids. *Nature (London)*, **344**, 268–270.

Park, S.-H., Shalongo, W. & Stellwagen, E. (1993). Residue parameters obtained from dichroic analysis of peptides of defined sequence. *Biochemistry*, **32**, 7048–7053.

Richards, F. M. (1977). Areas, volumes, packing and protein structure. *Annu. Rev. Biophys. Bioeng.* **6**, 151–176.

Richards, F. M. & Richmond, T. (1978). Solvent, interfaces and protein structure. In *Molecular Interactions and Activity in Proteins*, pp. 23–45, vol. 60, CIBA Foundation Symposium.

Richmond, T. J. & Richards, F. M. (1978). Packing of α-helices: geometrical constraints and contact areas. *J. Mol. Biol.* **119**, 537–555.

Rohl, C. A., Scholtz, J. M., York, E. J., Stewart, J. M. & Baldwin, R. L. (1992). Kinetics of amide proton exchange in helical peptides of varying chain lengths. Interpretation by the Lifson–Roig equation. *Biochemistry*, **31**, 1263–1269.

Scholtz, J. M., Qian, H., York, E. J., Stewart, J. M. & Baldwin, R. L. (1991). Parameters of helix-coil transition theory for alanine-based peptides of varying chain lengths in water. *Biopolymers*, **31**, 1463–1470.

Scholtz, J. M., Qian, H., Robbins, V. H. & Baldwin, R. L. (1993). The energetics of ion-pair and hydrogen-bonding interactions in a helical peptide. *Biochemistry*, **32**, 9668–9676.

Serrano, L., Neira, J.-L., Sancho, J. & Fersht, A. R. (1992). Effect of alanine versus glycine in α-helices on protein stability. *Nature (London)*, **356**, 453–455.

Shoemaker, K. R., Kim, P. S., Brems, D. N., Marqusee, S., York, E. J., Chaiken, I. M., Stewart, J. M. & Baldwin, R. L. (1985). Nature of the charged-group effect on the stability of the C-peptide helix. *Proc. Nat. Acad. Sci., U.S.A.* **82**, 2349–2353.

Shoemaker, K. R., Fairman, R., Schultz, D. A., Robertson, A. D., York, E. J., Stewart, J. M. & Baldwin, R. L. (1990). Sidechain interactions in the C-peptide helix: Phe 8 · His 12^+. *Biopolymers*, **29**, 1–11.

Wagner, G. (1983). Two-dimensional relayed coherence transfer spectroscopy of a protein. *J. Magn. Reson.* **55**, 151–156.

Edited by P. E. Wright

(Received 18 November 1993; accepted 6 June 1994)