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Tests for helix-stabilizing interactions between various nonpolar side chains in alanine-based peptides

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Abstract

Straight-chain, non-natural, nonpolar amino acids norleucine, norvaline, and α-amino-*n*-butyric acid at various spacings do not interact with themselves to stabilize helix formation in alanine-based peptides, but do interact with a Tyr spaced *i*, *i* + 4 to stabilize alanine helices, similar to the helix-stabilizing *i*, *i* + 4 Tyr–Leu and Tyr–Val interactions reported earlier (Padmanabhan S, Baldwin RL, 1994, *J Mol Biol 241*:706–713). Leu spaced *i*, *i* + 4 from another Leu is measurably helix-stabilizing relative to the corresponding *i*, *i* + 3 pair, but less so than for *i*, *i* + 4 Val–Leu, Ile–Leu, or Phe–Leu pairs (relative to the corresponding *i*, *i* + 3 pairs) when Leu is C-terminal to the other nonpolar amino acid. Our results indicate that limited side-chain flexibility in an α-helix strongly favors the interaction between 2 nonpolar residues to stabilize an isolated α-helix.

Keywords: α-helix stability; nonpolar interactions; peptide helices; side-chain conformation; side-chain interactions

The dominant interaction that determines the protein stability and folding pathways is generally believed to be the hydrophobic interaction that results from exclusion of water when nonpolar surfaces are brought into contact (Kauzmann, 1959; Nozaki & Tanford, 1971; Chothia, 1974; Dill, 1990). Initiation of protein folding is thought to involve a collapse or compaction of the unstructured protein driven by hydrophobic interactions that occur simultaneously or in tandem with the formation of secondary structure (Baldwin, 1989; Dill, 1990; Dill et al., 1993), and it has been suggested that hydrophobic interactions involving nonpolar side chains contribute to the stability and formation of individual α-helices (Lotan et al., 1966; Richards & Richmond, 1978; Dill et al., 1993). Only recently, however, has a specific interaction been demonstrated between 2 nonpolar side chains that stabilizes an isolated helix: interaction between the nonpolar residues Tyr and Leu (or Val) spaced *i*, *i* + 4 enhances the formation of isolated α-helices, and the strength of this *i*, *i* + 4 Tyr–Leu interaction can be rationalized in terms of the preferred side-chain conformations of Tyr, Leu, and Val (Padmanabhan & Baldwin, 1994). The major obstacle to forming a helix-stabilizing interaction between 2 nonpolar side chains is expected to be the loss of side-chain conformational entropy when the 2 side chains are fixed in the interacting conformation. This may explain why it has been difficult to demonstrate such interactions. In this respect, tyrosine is a particularly favorable amino acid for forming nonpolar interactions in helices because its side-chain rotamers are restricted in helices (McGregor et al., 1987) and it has a large nonpolar surface area (Chothia, 1974).

In the present study, using the alanine-based peptide system (Marqusee et al., 1989; Padmanabhan et al., 1990), we examine further how nonpolar–nonpolar interactions that affect helix formation depend on side-chain conformation and on side-chain hydrophobicity. The allowed side-chain rotamers in an α-helix are more restricted for the β-branched Val, Ile, and the aromatic Phe and Tyr than for the straight-chain Leu (MacGregor et al., 1987), and Leu, like the non-natural straight chain amino acids norleucine (Nle), norvaline (Nval), and α-amino-*n*-butyric acid (Abu) is a relatively good helix former (Lyu et al., 1991; Padmanabhan & Baldwin, 1991). The side-chain hydrophobicities calculated from the solvent-accessible nonpolar surface area increase from Abu to Nval to Nle, and from Val to Ile and Leu. We first examine if there are any interactions that affect helix formation between the straight-chain residues Nle, Nval, and Abu with themselves or with the more conformationally restricted Tyr. We then investigate if Leu interacts with another Leu to affect helix formation and compare it with the interactions of Leu with the conformationally more restricted Val, Ile, and Phe.

Results

Peptide design and CD properties

This study uses the alanine-based peptide system described previously that consists mainly of Ala residues, with 4 Lys residues spaced *i*, *i* + 5 present for water solubility, and that shows mo-

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nomeric helix formation in aqueous solutions (Padmanabhan et al., 1990). All peptides are acetylated at the N-terminus and amidated at the C-terminus. A single N-terminal Tyr residue allows accurate determination of peptide concentration from the absorbance at 275 nm in 6 M guanidium hydrochloride (ϵ_{275} = 1,450 M^{-1} cm^{-1}; Brandts & Kaplan, 1973). Sequence-dependent effects on helix formation were studied by making 2 or 3 specific Ala → nonpolar residue substitutions at different spacings in this reference peptide.

The CD spectra of all peptides used in the present study are characteristic of mixtures of helix and coil with 2 minima, one at 222 nm and the other between 203 and 207 nm, and a maximum at 192 nm (Holzwarth & Doty, 1965). Representative CD spectra are shown in Figure 1. An isodichroic point at 203 nm is observed for all peptides except for those containing Phe, where there may be contributions from the the aromatic Phe to the far-UV CD (Manning & Woody, 1989; Chakrabartty et al., 1993). The mean molar residue ellipticity at 222 nm ($-[\theta]_{222}$ deg cm^2 $dmol^{-1}$) for each peptide is directly related to its helix content. The helix contents were measured at 0 °C in aqueous solutions containing 1 M NaCl, pH 7.0, and CD buffer (see Materials and methods). All peptides used in the present study show reversible, concentration-independent thermal transitions demonstrating monomeric helix formation in these conditions (see Marqusee et al., 1989; Padmanabhan et al., 1990).

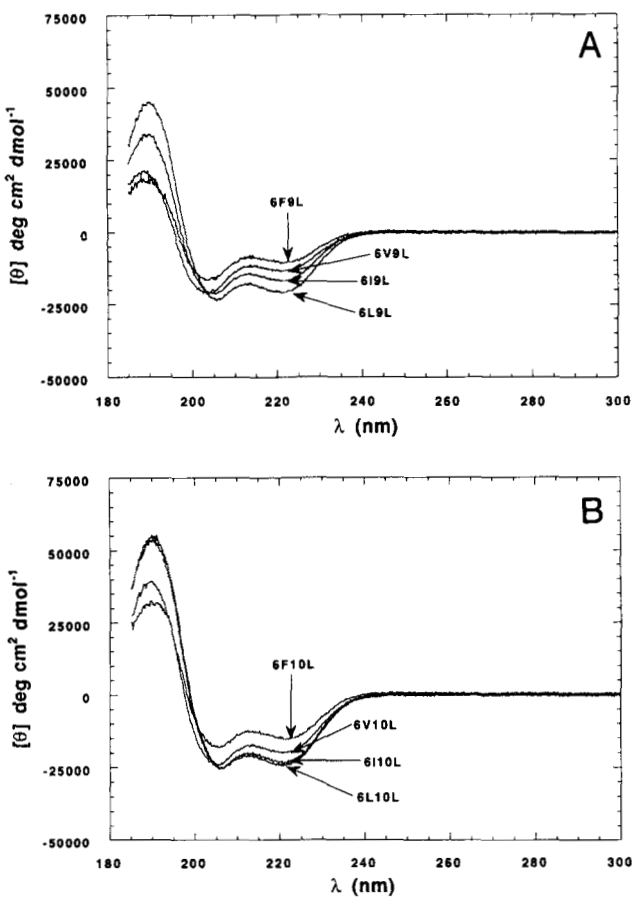

Fig. 1. CD spectra at 0 °C, pH 7.0, 0.1 M KF for peptides **(A)** 6X9L, X = Leu, Val, Ile, and Phe, with i, $i + 3$ X-Leu spacing, and **(B)** 6X10L, X = Leu, Val, Ile, and Phe, with i, $i + 4$ X-Leu spacing. The sequences for these peptides are shown in Table 2.

Isolated α-helices are stabilized by i, i + 4 Tyr-Z interactions (Z = Abu, Nval, or Nle) but not by Z-Z interactions

Table 1 shows 5 sets of alanine-lysine peptides with identical amino acid composition but with different sequences used to investigate Tyr-Z and Z-Z interactions. All have an N-terminal Tyr and 3 Z residues. The names used for the peptides indicate the position of Z in the sequence starting from the N-terminus. Thus, peptides 3Z(5,10,15) have i, $i + 5$ spacing between Z residues at positions 5, 10, and 15. An i, $i + 4$ spacing between the N-terminal Tyr and Z at position 5 is present in peptides 3Z(5,10,15) and peptides 3Z(5,9,15) but is missing in peptides 3Z(4,10,15), 3Z(9,10,15), and 3Z(8,9,10).

The conclusions are as follows:

1. Peptides 3Z(5,10,15) and 3Z(5,9,15) have nearly identical helix contents (Table 1), indicating that any (i, $i + 4$) Z-Z interaction between residues 5 and 9 is small for Z = Abu, Nval, or Nle, although the presence of Y1 and Z5 in these peptides allows an (i, $i + 4$) Y-Z interaction that could affect this conclusion.
2. Peptides 3Z(4,10,15), 3Z(9,10,15), and 3Z(8,9,10) have similar helix contents, indicating that the position of a Z residue (apart from its involvement in side-chain interactions) has only a small effect on helix content, and that any Z-Z interactions at these spacings are small. As regards the position dependence, this behavior is expected because a substantial position dependence is found only for strongly helix-breaking residues ($s < 0.5$), according to calculated curves given by Chakrabartty et al. (1991). The s-values of Abu, Nval, and Ile have not been determined using the latest procedures (described by Chakrabartty et al., 1994), but

Table 1. *Dependence of helix content on Z-Z and Y-Z spacing*

Peptide	% Helix[a]			Y-Z spacing
	Z = Abu	Z = Nval	Z = Nle	
3Z(5,10,15)[b]	60	67	74	i, $i + 4$
3Z(5,9,15)[c]	59	69	75	i, $i + 4$
3Z(4,10,15)[d]	9	63	69	i, $i + 3$
3Z(9,10,15)[e]	50	62	64	i, $i + 8$
3Z(8,9,10)[f]	50	62	62	i, $i + 7$

[a] % Helix measured at 0 °C, 1 M NaCl, pH 7.0 (1 mM each of sodium citrate, sodium phosphate, and sodium borate), peptide concentrations of 15–20 μM, 1-cm-pathlength cuvette as described in the Materials and methods. Error in the measurements of % helix was ≤3%. The value of $-[\theta]222$ for 100% helix is taken as −34,100 and the value for 0% helix is +640.

[b] Sequence: Ac-Y KAA Z AKAA Z AKAA Z AK-NH_2.

[c] Sequence: Ac-Y KAA Z AKA Z AAKAA Z AK-NH_2.

[d] Sequence: Ac-Y KA Z AAKAA Z AKAA Z AK-NH_2.

[e] Sequence: Ac-Y KAAAAKA Z Z AKAA Z AK-NH_2.

[f] Sequence: Ac-Y KAAAAK Z Z Z AKAAAAK-NH_2.

from older studies they are known to be well above Ile and below Ala (Lyu et al., 1991; Padmanabhan & Baldwin, 1991). Thus, they are probably well above $s = 0.5$ because for Ile $s = 0.42$ (Chakrabartty et al., 1994).

3. Peptides 3Z(5,10,15) and 3Z(5,9,15) have significantly larger helix contents than peptides 3Z(4,10,15), 3Z(9,10,15), and 3Z(8,9,10). This result indicates that an (i, $i + 4$) Y-Z interaction contributes significantly to helix content, as found earlier for (i, $i + 4$) Y-L and (i, $i + 4$) Y-V (Padmanabhan & Baldwin, 1994). Essentially complete helix formation is found for both peptides 3Z(5,10,15) (with the helix-stabilizing $i, i+4$ Tyr-Z interaction) and peptides 3Z(8,9,10) (without an i, $i + 4$ Tyr-Z interaction) at ≥10 mol % of trifluoroethanol (TFE) (maximum $[\theta]_{222} = -32{,}200 \pm 1{,}100$ deg cm^2 dmol^{-1}; Fig. 2).

α-Helices are stabilized by i, i + 4 Leu-Val, Leu-Ile, Leu-Phe, and Leu-Leu pairs in both N- to C-terminus orientations

The sequences of peptides used to investigate X-Leu interactions (X = Val, Ile, Phe, or Leu) are shown in Table 2. In peptides 6X9L and 6X10L, X at position 6 is spaced i, $i + 3$ and i, $i + 4$ from Leu at positions 9 and 10, respectively, with X N-terminal to Leu, whereas in peptides 10L13X and 9L13X, X is C-terminal to Leu in i, $i + 3$ and i, $i + 4$ spacing, respectively. In all these peptides (Table 2), both X and Leu are 5 or more residues away from the N-terminal Tyr to avoid i, $i + 4$ Tyr-X interactions (see previous section; also Padmanabhan & Baldwin, 1994). i, $i + 4$ Leu-X spacing was compared with an i, $i + 3$ Leu-X spacing because the i, $i + 4$ interaction is markedly stronger than a possible i, $i + 3$ interaction, as we showed in our earlier study of Tyr-Leu and Tyr-Val interactions (Padmanabhan & Baldwin, 1994) and in the previous section. Further work is needed to determine if the interactions for i, $i + 3$ and other spacings, if any, are measurable.

Table 2 shows that peptides 6X10L have greater helix contents than peptides 6X9L, and the helix contents for peptides 9L13X are greater than for peptides 10L13X (X = Leu, or Val, Ile, Phe). Thus i, $i + 4$ X-Leu interactions are helix stabilizing in both N- to -C orientations of X and Leu. For the N- to -C X-Leu orientation, i, $i + 4$ Val-Leu, Ile-Leu, and Phe-Leu are more helix stabilizing than i, $i + 4$ Leu-Leu (all relative to the corresponding i, $i + 3$ pair) but, for the Leu-X N- to -C orientation, all 4 pairs of interacting nonpolar residues show similar interaction strengths. Curiously, the helix content is uniformly greater when X is C-terminal to L compared with X being N-terminal to L. We have no explanation for this effect. The measurements of the interaction between Phe and another residue are subject to some uncertainty because of the possible contribution of the aromatic Phe side chain to the CD spectrum at 222 nm (Chakrabartty et al., 1993). Similar to the behavior described in the previous section for peptides with and without i, $i + 4$ Tyr-Z interactions, all the peptides, including those with Phe, reach the same maximum value of $-[\theta]_{222} = 32{,}000 \pm 1{,}200$ deg cm^2 dmol^{-1}, as the amount of the organic, helix-stabilizing solvent TFE increases to ≥10 mol % (30 volume %) in 0.1 M NaCl, pH 7.0, at 0 °C (Fig. 3). This demonstrates that the interaction of the Phe aromatic band with the helix does not contribute strongly to $-[\theta]_{222}$ in TFE.

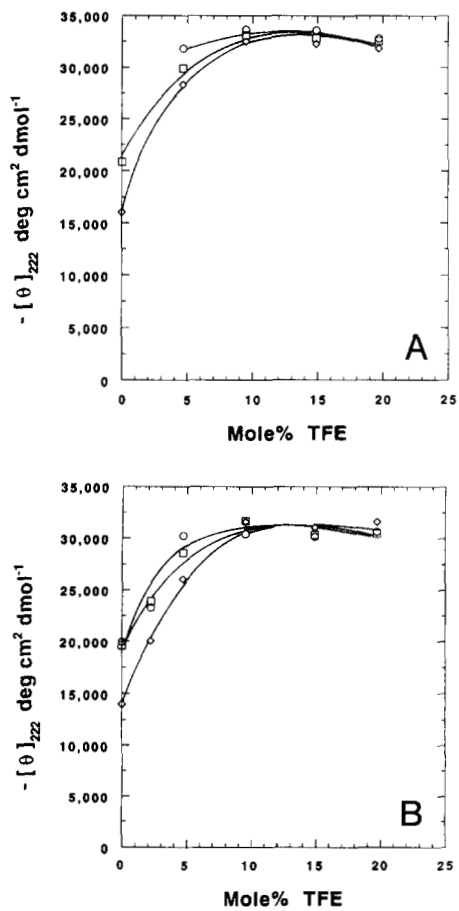

Fig. 2. TFE titrations for peptides 3Z(5,10,15) and 3Z(8,9,10) at 0 °C, pH 7.0, 0.1 M NaCl. Z = Abu (◇), Nval (□), Nle (○). **A:** 3Z(5,10,15). **B:** 3Z(8,9,10). The lines are drawn to aid viewing and have no theoretical significance.

Table 2. *Helix-stabilizing interaction between X and Leu spaced i, i + 4*

Peptide	Spacing	N- to -C orientation	% Helix[a] X = Leu	X = Val	X = Ile	X = Phe
6X10L[b]	$i, i + 4$	X-L	76	68	74	58
6X9L[c]	$i, i + 3$	X-L	68	52	61	44
9L13X[d]	$i, i + 4$	L-X	84	71	84	62
10L13X[e]	$i, i + 3$	L-X	72	54	71	50

[a] % Helix measured at 0 °C, 1 M NaCl, pH 7.0 (1 mM each of sodium citrate, sodium phosphate, and sodium borate), peptide concentrations of 15–20 μM, 1-cm-pathlength cuvette as described in the Materials and methods. Error in the measurements of % helix was ≤3%. The value of $-[\theta]_{222}$ for 100% helix is taken as −34,100 and the value for 0% helix is +640.

[b] Sequence: Ac-Y KAAA X KAA L AKAAAAK-NH$_2$.

[c] Sequence: Ac-Y KAAA X KA L AAKAAAAK-NH$_2$.

[d] Sequence: Ac-Y KAAAAKA L AAK X AAAK-NH$_2$.

[e] Sequence: Ac-Y KAAAAKAA L AK X AAAK-NH$_2$.

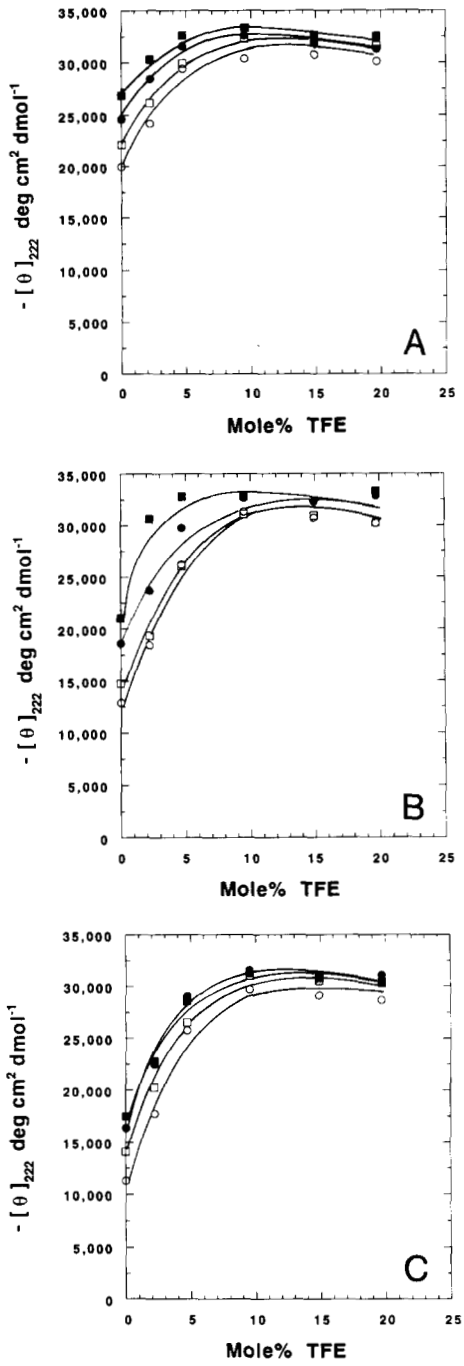

Fig. 3. TFE titrations for peptides 6X9L (○), 6X10L (●), 10L13X (□), and 9X13X (■) at 0 °C, pH 7.0, 0.1 M NaCl. **A:** X = Leu. **B:** X = Val. **C:** X = Phe. The lines are drawn to aid viewing and have no theoretical significance.

Discussion

Stabilization of isolated α-helices by nonpolar residues spaced i, i + 4 depends on allowed side-chain rotamers

The results obtained in the present study demonstrate that any helix-stabilizing interaction between nonpolar residues spaced i, $i + 4$ depends strongly on the limited flexibility of at least 1 partner in the interaction. Straight-chain nonpolar residues spaced i, $i + 4$ either do not stabilize helices (Abu–Abu, Nval–Nval, or Nle–Nle pairs), or else stabilize helices to a small extent (Leu–Leu pairs). Helix stabilization is significantly greater for i, $i + 4$ Tyr–Leu, Tyr–Abu, Tyr–Nval, and Tyr–Nle pairs, and also for i, $i + 4$ Val–Leu, Ile–Leu, and Phe–Leu pairs. The results also indicate that for a given type of side-chain conformation (straight-chain or β-branched), an increase in the size of the nonpolar side chain by itself does not cause a significantly greater enhancement in helix content resulting from an i, $i + 4$ interaction. The i, $i + 4$ interactions between Tyr and the straight-chain Abu, Nval, Nle, or Leu do not differ significantly from each other even though side-chain hydrophobicity (as measured by nonpolar surface area) increases from Abu to Nval to Nle and Leu. Similarly i, $i + 4$ Leu–Val interactions are comparable to i, $i + 4$ Leu–Ile interactions even though the β-branched Val has a smaller nonpolar surface area than the β-branched Ile.

The explanation for the dependence of the strength of the interaction on side-chain conformation is straightforward. The side chains of 2 nonpolar residues must be in a fixed conformation when they make a helix-stabilizing interaction. The resulting loss of side-chain conformational entropy is the major obstacle to this stabilization. The side-chain rotamer conformations of the straight-chain Leu, Abu, Nval, or Nle are less restricted in an α-helix than the β-branched Val and Ile or the aromatic Tyr or Phe (MacGregor et al., 1987). The loss in side-chain conformational entropy when both interacting residues are straight-chain up to the Cγ carbon (Leu–Leu, Abu–Abu, Nval–Nval, or Nle–Nle) would be considerable if they were fixed in a single conformation, since their side-chain conformations are quite flexible. This explains why i, $i + 4$ Leu–Leu, Abu–Abu, Nval–Nval, or Nle–Nle pairs have little or no helix stabilizing effects. In contrast, there is a smaller loss in side-chain conformational entropy from interactions between a straight chain and a β-branched side chain (Leu–Val, Leu–Ile) or between a straight-chain and an aromatic side chain (Tyr–Abu, Tyr–Nval, Tyr–Nle, Tyr–Leu, or Leu–Phe) since Val, Ile, Phe, and Tyr side chains are intrinsically more constrained in a helix. The observation of a helix-stabilizing i, $i + 4$ Tyr–Val interaction, with Tyr N-terminal to Val suggests that these i, $i + 4$ interactions can also occur between a β-branched side chain and an aromatic side chain (Padmanabhan & Baldwin, 1994).

Restriction of side chain conformations helps to rationalize the lower helix propensities of Val, Ile, Phe, and Tyr relative to Leu, Abu, Nval, or Nle observed experimentally (Lyu et al., 1990; O'Neil & DeGrado, 1990; Padmanabhan et al., 1990; Park et al., 1993; Chakrabartty et al., 1994) and verified by Monte Carlo calculations (Creamer & Rose, 1992). Our present results indicate that it also plays an important role in determining the strength of interactions between nonpolar side chains in a helix.

Significance of nonpolar interactions in isolated α-helices for protein folding

A recent statistical survey of contact preferences for amino acid pairs carried out on 94 high-resolution X-ray structures of proteins showed that nonpolar i, $i + 4$ pairs, in particular i, $i + 4$ Phe–Met, Leu–Leu, Leu–Val, Leu–Ile, and Leu–Met pairs, constitute about 50% of the significant, nonrandom i, $i + 4$ pairs in protein α-helices (J. Singh, R. Fairman, & J.M. Thornton, pers. comm.). This observation suggests that these pairs contrib-

ute to protein stability, just as they do in isolated α-helices. Because protein stability is dependent primarily on tertiary packing interactions that are not present in an isolated helix (Kellis et al., 1988, 1989; Matsumara et al., 1988; Lim & Sauer, 1989; Sandberg & Terwilliger, 1989; Dill, 1990; Eriksson et al., 1992; Mendel et al., 1992), it is not straightforward to analyze the relation between the 2 phenomena. Nonpolar residues occur predominantly in the water-inaccessible interior of the protein, which is more analogous to helix formation in TFE solutions where helix-stabilizing interactions between nonpolar residues are unlikely. It is interesting that i, $i + 4$ Leu-Val and Leu-Ile pairs occur commonly in coiled-coil helices (Hu et al., 1990), but this observation can be explained by the structure of the nonpolar interface between the 2 helices. Hydrophobic interactions within single units of secondary structure, either α-helices or β-sheets, of the type that we have identified in isolated helices may be important in the initial stages of folding if, as has been suggested, there is coupling between secondary structure formation and a hydrophobic collapse (Baldwin, 1989; Dill et al., 1993).

Materials and methods

Peptide synthesis and purification

Peptides were synthesized and purified as described previously (Padmanabhan & Baldwin, 1991). Synthesis was performed on a Milligen 9050 automated synthesizer using the pentafluorophenyl esters of Fmoc (9-fluorenylmethoxycarbonyl) amino acids purchased from Milligen. Peptides containing Abu or Nval were synthesized using the free Fmoc amino acids, Fmoc-Abu (Peninsula Labs) or Fmoc-Nval (prepared as described by Stewart & Young, 1984), activated by HOBT (1-hydroxybenzotriazole; Sigma) and BOP (benzotriazolyltris (dimethylamino) phosphonium hexafluoro-phosphate; Biosearch). Peptides were acetylated at the N-terminus using acetic anhydride and then cleaved from the benzhydrylamine resin with 95:5 trifluoroacetic acid:anisole mixture for 2–4 h to yield peptides amidated at the C-terminus. Crude peptides were purified by reverse-phase fast protein liquid chromatography (FPLC; Pharmacia) using a C18 resin with gradients of 10–40% acetonitrile:water (0.1% trifluoroacetic acid). Peptide identity was confirmed using fast atom bombardment (FAB) mass spectrometry.

CD and NMR 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), 40–60 μM peptide, were obtained using a 1-mm-pathlength cuvette, 0.2-nm step size, and 1-s average time and were averaged over 4 scans. Mean molar residue ellipticity measurements at 222 nm ($[\theta]_{222}$ deg cm^2 dmol^{-1}) in 1 M NaCl, pH 7.0 (1 mM each of sodium citrate, sodium phosphate, and sodium borate/1 mM CD buffer) were made in 10-mm-pathlength cuvettes (10–30 μM peptide) or in 1-mm-pathlength cuvettes (>50 μM peptide). TFE titrations were carried out at 0 °C, 0.1 M NaCl, and pH 7.0 using the procedure described by Nelson and Kallenbach (1988). Percent helix contents were determined from $[\theta]_{222}$ measurements using +640 and $-40{,}000[1 - (2.5/n)]$ for 0% and 100% helix, respectively, n being the number of amino residues in the peptide (17 in this study) (Scholtz et al., 1991).

Acknowledgments

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