Packing and hydrophobicity effects on protein folding and stability: Effects of β -branched amino acids, valine and isoleucine, on the formation and stability of two-stranded α -helical coiled coils/leucine zippers

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Abstract

The aim of this study was to examine the differences between hydrophobicity and packing effects in specifying the three-dimensional structure and stability of proteins when mutating hydrophobes in the hydrophobic core. In DNA-binding proteins (leucine zippers), Leu residues are conserved at positions "d," and β -branched amino acids, Ile and Val, often occur at positions "a" in the hydrophobic core. In order to discern what effect this selective distribution of hydrophobes has on the formation and stability of two-stranded α -helical coiled coils/leucine zippers, three Val or three Ile residues were simultaneously substituted for Leu at either positions "a" (9, 16, and 23) or "d" (12, 19, and 26) in both chains of a model coiled coil. The stability of the resulting coiled coils was monitored by CD in the presence of Gdn·HCl. The results of the mutations of Ile to Val at either positions "a" or "d" in the reduced or oxidized coiled coils showed a significant hydrophobic effect with the additional methylene group in Ile stabilizing the coiled coil ($\Delta\Delta G$ values range from 0.45 to 0.88 kcal/mol/mutation). The results of mutations of Leu to Ile or Val at positions "a" in the reduced or oxidized coiled coils showed a significant packing effect in stabilizing the coiled coil ($\Delta\Delta G$ values range from 0.59 to 1.03 kcal/mol/mutation). Our results also indicate the subtle control hydrophobic packing can have not only on protein stability but on the conformation adopted by the amphipathic α -helices. These structural findings correlate with the observation that in DNA-binding proteins, the conserved Leu residues at positions "d" are generally less tolerant of amino acid substitutions than the hydrophobic residues at positions "a."

Keywords: coiled coils; interchain α -helical interactions; leucine zippers; packing and hydrophobic effects of β -branched amino acids; protein stability

It is generally accepted that hydrophobic interactions are the major forces involved in initializing protein folding and stabilizing the three-dimensional structures of proteins. Both hydrophobicity and the packing of hydrophobes in the hydrophobic core of a protein can affect protein stability (Kellis et al., 1988, 1989; Matsumura et al., 1988; Lim & Sauer, 1989; Sandberg & Terwilliger, 1989; Dill, 1990; Eriksson et al., 1992; Mendel et al., 1992). The role packing effects play in protein folding is still the subject of much debate (Behe et al., 1991). It is thus important to determine the relative contribution of

these effects in the hydrophobic interiors of proteins with different protein folds. In this article, we have addressed these issues by studying the two-stranded α -helical coiled coil motif.

Almost 40 years ago Crick proposed the existence of the two-stranded α -helical coiled coil (Crick, 1953). The first amino acid sequence of a two-stranded α -helical coiled coil (tropomyosin) was reported by Hodges and co-workers in 1972 when they identified the hydrophobic 3-4 (or 4-3) repeat responsible for the formation and stabilization of this unique structure (Hodges et al., 1972; Sodek et al., 1972). The two-stranded α -helical coiled coil is characterized by a heptad repeat denoted as abcdefg where positions "a" and "d" are normally occupied by large hydrophobic amino acids such as Leu, Ile, and Val

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(Cohen & Parry, 1986, 1990) and positions "e" and "g" by oppositely charged amino acids (McLachlan & Stewart, 1975; Stone et al., 1975). The hydrophobic side chains at positions "a" and "d" create an amphipathic α -helix where the nonpolar surfaces of two such subunits can dimerize in a parallel and in-register fashion to form a continuous hydrophobic core as a result of the "knobs-into-holes" packing as proposed by Crick in 1953. This structure was recently verified by the first high-resolution X-ray structure of a two-stranded α -helical coiled coil (O'Shea et al., 1991). It has been suggested that more than 200 proteins may contain this motif (Lupas et al., 1991).

Hodges and coworkers designed and synthesized the first model coiled coil protein and demonstrated its utility as an ideal model system for studying de novo design principles involving both intra- and intermolecular interactions related to protein folding and stability (Hodges et al., 1981; Talbot & Hodges, 1982; Hodges, 1992; Zhou et al., 1992a,b,c; Zhu et al., 1992). They demonstrated that the minimum length required for the formation and stabilization of a coiled coil was 28 residues based on the heptad repeat Lys-Leu-Glu-Ala-Leu-Glu-Gly (Lau et al., 1984a). Recently, leucine-zipper sequences, known to be important for mediating dimerization of many DNA-binding proteins (Landschulz et al., 1988; Vinson et al., 1989; McKnight, 1991) have been shown to fold as short two-stranded parallel α -helical coiled coils (O'Shea et al., 1989, 1991; Oas et al., 1990; Goodman & Kim, 1991). DeGrado and coworkers (O'Neil & DeGrado, 1990) have used the coiled coil model to determine the α -helical propensity of amino acids. Engel et al. (1991) have used the coiled coil motif as part of a much larger de novo designed protein.

Analysis of known leucine zipper sequences have shown that generally β -branched amino acids are conserved at

positions "a" while Leu residues are conserved at positions "d" (McKnight, 1991). Hu et al. (1990) have determined the sequence requirements of the leucine zipper GCN4 for DNA binding and showed that the Leu residues conserved at positions "d" are less tolerant of amino acid substitutions than positions "a." Their studies raise a series of questions: Is this a structural or functional requirement? Are there any restrictions on which of the hydrophobic amino acids Leu, Ile, and Val can be placed at each of the "a" and "d" positions? Do β -branched amino acids at position "a" make similar contributions to the formation and stability of coiled coil compared to those at position "d"? The goal of this study was to explain, from both hydrophobicity and packing effects, the observed distribution of β -branched amino acids (Ile and Val) and their contribution relative to Leu residues to the formation and stability of coiled coils.

Results and discussions

Design of an α -helical coiled coil

To investigate the hydrophobic amino acid distribution in coiled coil proteins, a simple coiled coil model protein was designed. The amino acid sequence is shown in Figure 1. Position 2 contains a Cys residue to allow the formation of an interchain disulfide bond to ensure the two α -helices are in a parallel and in-register alignment. Moreover, a disulfide bond also stabilizes the coiled coil structure (Hodges et al., 1990). To study the preference of Ile, Val, and Leu in the hydrophobic interface of the coiled coil, three Leu residues at positions "a" or "d" were simultaneously mutated to Ile or Val in the central region of the coiled coil (Fig. 1). We chose triple mutations instead

Fig. 1. The amino acid sequences of the synthetic peptides used in this study to prepare homodimeric two-stranded α -helical coiled coils. The N-terminal amino group of each chain is acetylated and the C-terminal carboxyl group is amidated. The circled residues (Leu, Val, Ile, or Cys) denote the hydrophobic "a" and "d" positions responsible for the formation and stabilization of the coiled coil. The coiled coil analog in which three leucines at positions "a" are substituted by three isoleucines in both chains is denoted as LoIa (with the "o" denoting the oxidized form of the coiled coil, that is, a disulfide bond between the two cysteines at position 2 of each of the 35-residue polypeptide chains) and LrIa (with the "r" denoting the reduced form of the coiled coil) and similarly LoId, LrId, LoVa, LrVa, LoVd, or LrVd.

of a single mutation in order to amplify the effects in the analogues.

Structural characterization of the α -helical coiled coils

The criteria used to establish that the synthetic peptides have formed a two-stranded coiled coil structure are as follows: (1) The CD spectra of the designed peptides should show the double minima absorptions at 207 and 222 nm with high negative ellipticity values, characteristic of a fully formed α -helical structure. (2) The reduced peptides (without an interchain disulfide bond) and the oxidized peptides (with an interchain disulfide bond) should show the same apparent molecular weights in aqueous buffer, i.e., the reduced peptides should form two-stranded dimers and the oxidized peptides should form two-stranded monomers. This can be determined by size-exclusion chromatography and sedimentation equilibrium studies. (3) The ellipticities at 220 nm and the stability of reduced peptides as measured by denaturation studies in the CD should show a concentration dependence due to the monomer-dimer equilibrium, while the results of the oxidized peptides should show a concentration independence.

All the peptide analogues were studied according to the above requirements. Figure 2 is representative of the ultraviolet CD spectra of peptides LrVa and LrVd at pH 7

in aqueous buffer solution under the same conditions. Results for other peptide analogues are summarized in Table 1. At pH 7, peptides Lr, LrIa, LrId, and LrVa are indistinguishable by CD with $[\Theta]_{220}$ values of $-30,000 \pm 1,500$ ($^{\circ}\cdot\text{cm}^2\cdot\text{dmole}^{-1}$), in the range of 100–200 μM peptide concentration, corresponding to approximately 100% α -helical structure (Hodges et al., 1990). However, under the same conditions, LrVd only has ~30% α -helical content ($[\Theta]_{220}$ of $-11,250$) (Fig. 2). Size-exclusion chromatographic studies of these peptides compared to the 14-, 21-, 28-, 42-, and 56-residue peptide standards using methodology described previously (Lau et al., 1984b; Hodges et al., 1990; Zhou et al., 1990) demonstrated that the reduced peptides Lr, LrIa, LrId, and LrVa are dimeric (two-stranded) and the oxidized peptides LoIa, LoId, and LoVa are monomeric (two-stranded) in aqueous buffer solution, whereas the reduced peptide LrVd is monomeric (single-stranded) using the same conditions (Fig. 3). Analytical ultracentrifugation studies of peptides LrId and LoId gave essentially the same molecular weight of 8,100 (Fig. 4), which is in good agreement with that expected for the two-stranded structure (calculated molecular weight 7,508).

Recently, Alber¹ reported that synthetic peptides with the β -branched amino acid Ile in all the five "d" positions

¹ Reported by T. Alber at the Sixth Symposium of the Protein Society, San Diego, California, July 1992.

Table 1. CD results of the synthetic model peptides

Peptide	[Θ] ₂₂₀		Peptide concentration (μM) ^c	[Gdn·HCl] _{1/2} ^d (M)	ΔΔG ^e (kcal/mol)
	Benign ^a	TFE ^b			
pH 7				pH 7	
Lo	−29,800	−29,800	141	5.1	
LoIa	−29,900	−30,800	100	6.3	−1.8
LoId	−30,600	−30,800	142	4.8	0.9
LoVa	−30,400	−31,200	121	4.5	0.9
LoVd	−28,500	−31,100	103	1.5	5.3
Lr	−30,200	−25,400	368	2.5	
LrIa	−29,800	−27,300	226	3.4	−2.5
LrId	−29,300	−27,700	208	2.0	0.8
pH 2				pH 2	
Lr	−29,700	−27,900	176	5.3	
LrVa	−31,500	−29,000	189	4.0	2.8
LrVd	−30,400	−29,000	207	3.0	5.1

^a A 0.1 M KCl, 0.05 M PO_4 buffer, pH 7.0, was used for all benign measurements. Temperature was 20 $^{\circ}\text{C}$ for ellipticity measurements.

^b A 1:1 (v/v) mixture of benign buffer with trifluoroethanol (TFE). Temperature was 20 $^{\circ}\text{C}$ for ellipticity measurements.

^c Peptide concentrations used in Gdn·HCl denaturation experiments.

^d $[\text{Gdn}\cdot\text{HCl}]_{1/2}$ is the transition midpoint, the concentration of guanidine hydrochloride (M) required to give a 50% decrease in ellipticity.

^e $\Delta\Delta G$ is the free energy difference between the native protein and the mutant protein at the Gdn·HCl concentration required to give a 50% decrease in the ellipticity of the mutant protein at 220 nm. For example, $\Delta\Delta G(\text{LoVa}) = \Delta G(\text{Lo}) - \Delta G(\text{LoVa})$, where ΔG is the free energy of unfolding at 4.5 M Gdn·HCl.

Fig. 2. CD spectra of peptides LrVa and LrVd in 0.05 M PO_4 , 0.1 M KCl, pH 7, buffer. Temperature was 20 °C for ellipticity measurements.

and Leu in all the five "a" positions crystallized in a tetrameric coiled coil structure. However, in this study where three of five Leu residues were mutated to Ile, only the two-stranded α -helical coiled coil structure is observed in solution by size-exclusion chromatography and sedimentation equilibrium studies. This suggests that either the two hydrophobic residues at positions 5d and 33d and Ile occupies positions 12d, 19d, and 26d [Fig. 1] the structure is two-stranded, and when Ile occupies all five "d" positions, the structure is four-stranded) or the conditions used to crystallize the tetrameric coiled coil are favoring the formation of the tetramer or that other sequence differences between our model sequence and that used by Alber are influencing the final conformation. It will be of interest to see if the tetrameric structure observed by Alber is as stable as our dimeric structure or whether this tetrameric structure is less stable and converts to the dimeric structure in the unfolding pathway. Nevertheless, together these studies show the subtle control hydrophobic packing can have, not only on protein stability but on the conformation adopted by these amphipathic α -helices.

The oxidized peptides Lo and LoVd were dimeric (four-stranded form) as determined by size-exclusion chromatography (Fig. 3) and sedimentation equilibrium studies in benign medium (Fig. 4). The effect of Gdn·HCl concentration on the conformation of peptide Lo was determined by size-exclusion chromatography (Fig. 5). The sample is equilibrated in different Gdn·HCl concentra-

Fig. 3. Size-exclusion chromatography (SEC) of the synthetic reduced and oxidized peptides. Examples of the elution profiles (LoVd and LoVa) are shown in the upper panel, and the lower panel shows a plot of $\ln$ molecular weight vs. elution time. The elution time of each peptide was plotted against the multiple of its monomeric molecular weight, which most closely approaches the standard curve. The samples were dissolved in 1 M KCl, 50 mM PO_4 , pH 7, buffer. For the reduced peptides, 5 mM DTT was added in solution. The conditions for SEC are described in the Materials and methods. T, D, or M represents the four-stranded, two-stranded, or single-stranded form of the peptide, respectively. The nomenclature of the peptide is described in Figure 1. The numbers 14, 21, 28, 42, and 56 represent the number of residues in the size-exclusion chromatographic standards of α -helical peptides (Hodges et al., 1990; Zhou et al., 1990). The star (*) denotes that there are two 14-residue peptides, one is reduced peptide Ac-K-C-E-A-L-E-G-K-L-E-A-L-E-G-amide and the other is the two-stranded oxidized peptide of the sequence Ac-K-C-E-A-L-E-G-amide. The 21-residue peptide is Ac-K-C-E-A-L-E-G-[K-L-E-A-L-E-G]₂-amide in its reduced form. The two-stranded peptides of 28-, 42-, and 56-residues were formed by oxidation of the 14-, 21-, and 28-residue peptides.

tions and injected onto the size-exclusion column where the elution buffer is 1 M KCl, 50 mM PO_4 buffer, pH 7. In this experiment, the interconversion of the four-stranded and two-stranded coiled coil can be monitored. The four-stranded form of peptide Lo is not as stable as its two-stranded form. As the concentration of Gdn·HCl is increased, the four-stranded form converts to the two-stranded form. When the Gdn·HCl concentration is greater than 2 M, no significant amount of the four-stranded form was observed in size-exclusion chromatographic

Fig. 4. A $\ln Y$ vs. r^2 plot from typical sedimentation equilibrium experiments. See Materials and methods for experimental details.

studies (Fig. 5). However, at this Gdn·HCl concentration, peptide Lo still remains in its 100% α -helical structure (Fig. 6). These results suggest that the forces that stabilize the four-stranded structure are much weaker than the forces that maintain two-stranded α -helical coiled coil structure, and the peptide Lo has converted to the two-stranded coiled coil structure prior to its denaturation by Gdn·HCl as determined by CD studies. The main transition in Gdn·HCl denaturation profile (Fig. 6) of peptide Lo corresponds to the conversion of the two-stranded α -helical coiled coil to unfolded monomer.

Figure 7 shows the dependence of the molar ellipticities of LrVa and LrVd on peptide concentration. As the concentration of peptide is increased, the monomer-dimer equilibrium is shifted toward the formation of coiled coil dimer, which increases the α -helical content of the peptide. The more stable the coiled coil, the lower the peptide concentration required to form the dimer. Peptides Lr, LrIa, LrId, and LrVa have ellipticity values at 220 nm of $-20,000$ at a concentration of $2 \mu\text{M}$, corresponding to approximately 60% helix. These results indicated that even at micromolar concentrations, the peptides Lr, LrIa, LrId, and LrVa are substantially in the coiled coil structure. Due to experimental limitations, it was not possible to accurately determine the ellipticity at concentrations below $2 \mu\text{M}$. A further decrease in ellipticity would be expected at lower dilutions due to the shift in the monomer-dimer equilibrium to monomer. However, peptide LrVd does not have α -helical structure in the 2 – $50 \mu\text{M}$ concentration range, but does form a coiled coil dimer when peptide concentration is higher than $1,000 \mu\text{M}$ at pH 7 (Fig. 7). From these results, we conclude that all peptide analogues including both oxidized and reduced peptides, with the exception of LrVd, form stable coiled coils in the micromolar concentration range at pH 7. On the other hand, at pH 2, peptide LrVd forms a stable coiled coil due

Fig. 5. Effects of concentration of Gdn·HCl on the conformation of peptide Lo monitored by size-exclusion chromatography. For the column description see Materials and methods. The column effluent was 1 M KCl, 50 mM PO_4 , pH 7, buffer. A: Peptide Lo in 1 M KCl, 50 mM PO_4 , pH 7, buffer. For the sample containing Gdn·HCl, the peptide was dissolved in 0.1 M KCl, 50 mM PO_4 , pH 7, buffer containing 6 M Gdn·HCl and then diluted to 0.6 M (B), 1 M (C), and 2 M (D) Gdn·HCl by buffer (1 M KCl, 50 mM PO_4 , pH 7) and incubated at room temperature for several hours prior to injection of sample. The sample volume was $100 \mu\text{L}$, and the sample concentrations were approximately 0.5 mg/mL . T and D represent the four-stranded and two-stranded forms of peptide, respectively.

to the increased stability at low pH. Previous studies on tropomyosin have shown that the coiled coil remains fully helical over a wide pH range (pH 2–9) and is exceptionally stable in acidic conditions (Noelken & Holtzer, 1964; Lowey, 1965). This behavior has also been observed more recently with synthetic model coiled coils (Lau et al., 1984a; Hodges et al., 1988; Zhou et al., 1992c). Clearly, some new contributions, which have yet to be determined, must be involved in the increased stability at low pH where the ionic interactions, thought to be important for coiled coil stability (Stone et al., 1975; McLachlan & Stewart, 1975), cannot contribute. The stability of the

Fig. 6. Gdn·HCl denaturation profiles of the synthetic coiled coils in 0.1 M KCl, 50 mM PO₄ buffer, pH 7 and pH 2. Θ/Θ_0 represents the ratio of molar ellipticity at 220 nm at the indicated molarity of Gdn·HCl concentration to the ellipticity without Gdn·HCl. The peptide concentrations are listed in Table 1. The coiled coil nomenclature is described in Figure 1.

coiled coil resulting from the hydrophobic residues at positions "a" and "d" should be independent of pH.

Protein stability determined by Gdn·HCl denaturation studies

The stability of the reduced and oxidized peptides were studied by measuring the ellipticity at 220 nm as a function of Gdn·HCl concentration. Figure 6 represents the Gdn·HCl denaturation curves at 20 °C. All studies were carried out at a similar concentration (Table 1) in order to avoid differences due to the concentration effect for the reduced dimers. The stabilities of proteins are expressed as either the Gdn·HCl concentration at which half the coiled coil is unfolded ($[\text{Gdn} \cdot \text{HCl}]_{1/2}$; Table 1) or in terms of the free energy of unfolding in the absence of denaturant (Fig. 8). The free energy difference ($\Delta\Delta G$) between the Leu coiled coil and the mutant protein at the Gdn·HCl concentration that is required to give a 50% decrease in the ellipticity of mutant protein is also listed in Table 1. The Gdn·HCl concentration at the midpoint of

the denaturation curve is the most accurate value for determining protein stability and was therefore used to calculate the difference in free energy of unfolding (Fig. 8). The resulting error in $\Delta\Delta G$ is much smaller than the error in the measurement of $\Delta\Delta G$ in the absence of denaturant (Sali et al., 1991). It can be seen from Table 1 and Figure 6 (upper panels) that the $[\text{Gdn} \cdot \text{HCl}]_{1/2}$ is 6.3 for LoIa, 5.1 M for Lo, 4.8 M for LoId, 4.5 M for LoVa, and 1.5 M for LoVd. The difference in stability between LoVa and LoVd mutants in terms of $[\text{Gdn} \cdot \text{HCl}]_{1/2}$ values is 3 M, corresponding to 4.4 kcal/mol. Both mutants LoVa and LoVd are less stable than Lo. In the case of Ile substitutions, LoId (mutation at position "d") is substantially less stable than LoIa approximately by 2.7 kcal/mol. The Ile mutant at positions "a" (LoIa) is significantly more stable than Lo, and LoId is less stable than Lo. The preference of β -branched amino acids to positions "a" is further supported by studying the reduced coiled coils (lower panels in Fig. 6). The coiled coils with β -branched amino acid substitutions at positions "a" are much more stable than their counterparts at positions "d." LrIa is stabilized

Fig. 7. The concentration dependence of the mean residue molar ellipticity at 220 nm for peptides LrVa and LrVd. The symbols $\circ$ and $\bullet$ denote peptide LrVd in 0.1 M KCl, 50 mM PO_4 buffer at pH 7 and pH 2, respectively. $\square$ Denotes peptide LrVa in 0.1 M KCl, 50 mM PO_4 , pH 7. The peptide concentrations varied from 2 μM to 3 mM.

Fig. 8. Linear dependence of ΔG on the Gdn·HCl concentration ($\Delta G = \Delta G^{\text{H}_2\text{O}} - m[\text{Gdn} \cdot \text{HCl}]$). ΔG is the free energy of unfolding at each individual Gdn·HCl concentration and calculated according to the equation: $\Delta G = -RT \ln[(1 - fn)/fn]$, where fn is the fraction folded at a particular Gdn·HCl concentration. The arrows indicate the free energy difference ($\Delta\Delta G$) between the native coiled coil and the mutant protein at the Gdn·HCl concentration, which is required to give a 50% decrease in the ellipticity of the mutant protein at 220 nm.

by 3.3 kcal/mol relative to LrId at pH 7. As with the Ile mutations, LrVd is less stable than LrVa by 2.3 kcal/mol at pH 2. The Gdn·HCl denaturation studies for LrVa and LrVd were carried out at pH 2 because the peptide LrVd cannot form a stable coiled coil at pH 7. From these results, we conclude that there is a preference for β -branched amino acids at position "a" for the formation and stability of the coiled coil structure. The coiled coils with β -branched amino acids at positions "a" are more stable than those at positions "d."

Hydrophobic packing effects on protein stability

Hydrophobic interactions are the major factors in stabilizing the three-dimensional structures of proteins. Both hydrophobicity (Kellis et al., 1988, 1989; Matsumura et al., 1988; Lim & Sauer, 1989; Dill, 1990; Eriksson et al., 1992) and packing of interior hydrophobic residues affect protein stability (Sandberg & Terwilliger, 1989; Mendel et al., 1992). Normally, burying large hydrophobic residues will stabilize the folded structure. The hydrophobic effect is thought to be an attractive force that causes nonpolar residues to fold into the hydrophobic core in aqueous medium and can be related to the transfer free energy of amino acid side chains from water to organic solvent (Nozaki & Tanford, 1971; Fauchere & Pliska, 1983; Damodaran & Song, 1986; Shortle et al., 1990; Sharp et al., 1991). This hydrophobic effect is not related to the packing effect in the protein interior. It only depends upon how much hydrophobic surface area is buried in folded protein. It has been shown previously that protein stability is related to the hydrophobicity of the residues in the hydrophobic core of coiled coils (Hodges et al., 1990; Zhou et al., 1992a,b) as well as in globular proteins (Kellis et al., 1988, 1989; Matsumura et al., 1988; Lim & Sauer, 1989; Dill, 1990). The best comparison for the hydrophobic effect on protein stability is the comparison of the Ile and Val mutants where the side chains differ only by one methyl group on the extremity of the side chain (Hodges et al., 1990). When comparing the Ile to Val mutation, in both the reduced and oxidized coiled coils with the β -branched amino acids at positions "d," the Val mutants are less stable than the corresponding Ile mutants by a similar value, i.e., $\Delta\Delta G = 4.4$ kcal/mol for LoId $\rightarrow$ LoVd and 4.3 kcal/mol for LrId $\rightarrow$ LrVd, corresponding to 0.7 kcal/mol per Ile to Val mutation (Table 2). This result is in good agreement with the free energy of transfer of a methylene group from water to organic solvent (Shortle et al., 1990) and the result obtained by Rockwell et al. (1992) in their single Ile to Val mutation at position "a" in the coiled coil.

The contribution of hydrophobic packing to coiled coil stability can be separated from hydrophobicity and α -helix propensity effects. The difference between the Leu $\rightarrow$ Val mutation at positions "a" and positions "d" in terms of their contribution to the stability of the coiled

Table 2. The estimated stability difference for a single mutation in the reduced and oxidized coiled coils

Mutation	$\Delta\Delta G$ (kcal/mutation)					
	Observed ^a		Hydrophobicity ^b	Helix ^c propensity	Packing ^d	
	Reduced	Oxidized			Reduced	Oxidized
Ia → Va	0.88	0.45	0.8	0.09	-0.01	-0.44
Id → Vd	0.72	0.73	0.8	0.09	-0.17	-0.16
La → Ia	-0.42	-0.30	-0.1	0.39	-0.71	-0.59
Ld → Id	0.13	0.15	-0.1	0.39	-0.16	-0.14
La → Va	0.46	0.15	0.7	0.48	-0.72	-1.03
Ld → Vd	0.85	0.88	0.7	0.48	-0.33	-0.30

^a The stability difference for the single mutation determined from Gdn·HCl denaturation was calculated based on the data shown in Table 1. For example: $\Delta\Delta G_{\text{observed}}(\text{Id} \rightarrow \text{Vd}) = [\Delta\Delta G(\text{LoVd}) - \Delta\Delta G(\text{LoId})]/6 = (5.3 - 0.9)/6 = 0.73$

^b $\Delta\Delta G_{\text{hydrophobicity}}$ is the free energy difference of transfer of amino acids from water to *n*-octanol (Fauchere & Pliska, 1983; Shortle et al., 1990).

^c $\Delta\Delta G_{\text{helix propensity}}$ is the free energy difference for a single mutation in a solvent-exposed position of the coiled coil as determined from CD studies (O'Neil & DeGrado, 1990).

^d The stability difference between the mutant proteins in terms of hydrophobic packing effects was calculated based on equation: $\Delta\Delta G_{\text{packing}} = \Delta\Delta G_{\text{observed}} - (\Delta\Delta G_{\text{hydrophobicity}} + \Delta\Delta G_{\text{helix propensity}})$

coils can be explained by the difference in packing effects of the hydrophobic side chains. If one only considers the hydrophobic effect, LoVa should be destabilized by 4.2 kcal/mol (6 Val instead of 6 Leu per dimer, 6×0.7 kcal/mol) relative to the native coiled coil Lo (Fauchere & Pliska, 1983; Shortle et al., 1990). This mutant is indeed less stable than the native coiled coil, but is only destabilized by 0.9 kcal/mol (Table 1), which is much less than 4.2 kcal/mol. O'Neil and DeGrado (1990) reported that the substitution of a Leu by Val in a solvent-exposed position of the coiled coil destabilized the coiled coil by 0.48 kcal/mol (Table 2, Helix propensity column). Taking account of both hydrophobicity and helix propensity effects, a packing effect can be calculated. Therefore, there is a packing effect that stabilizes the coiled coil by 1.03 kcal per Val residue (Table 2). These results imply that the interhelical Val–Val packing effect at position “a” provides more stabilization than the Leu–Leu packing effect at “a.” Comparing LoIa and Lo, LoIa is much more stable than Lo. Leu → Ile mutations should stabilize the coiled coil structure by 0.6 kcal/mol (6 Ile instead of 6 Leu residues per dimer, 6×0.1 kcal/mol) in terms of hydrophobic effect (Fauchere & Pliska, 1983; Shortle et al., 1990). But the stability difference between LoIa and Lo is around 1.8 kcal/mol (Table 1), implying that the Ile–Ile hydrophobic packing effect at positions “a” stabilizes the coiled coil relative to Leu–Leu packing, by 0.59 kcal per Ile residue (Table 2). This same mutation on the outside of the coiled coil (referred to as helix propensity in this study) destabilizes the coiled coil by 0.39 kcal/mol/residue (Table 2). Both the hydrophobic effect and packing effects contribute to the coiled coil stability to overcome the decrease in stability due to the helix propensity

effect, resulting in LoIa being much more stable than Lo. From mutation studies at positions “a,” it can be concluded that β -branched amino acids actually stabilize the coiled coil structure at positions “a” relative to Leu residue in terms of the packing effect. In contrast, when mutations were carried out at positions “d,” both Ile and Val destabilize the coiled coil structure. The hydrophobic effect of Ile mutations at positions “d” should stabilize the coiled coil by 0.6 kcal/mol, but in fact LoId is destabilized by 0.9 kcal/mol (Table 1), which is mainly due to the helix propensity effect (Table 2). In the reduced coiled coil LrId, the similar value is obtained. Similarly, peptide LoVd is destabilized by 5.3 kcal/mol relative to Lo (Table 1). This is mainly the result of a hydrophobic effect (Table 2). From these results, we conclude that β -branched amino acids stabilize the coiled coil at positions “a” relative to Leu residues in terms of packing effects in the hydrophobic core.

To confirm that the stability differences arose from interhelical hydrophobic packing effects, unfolding studies of single-stranded α -helices of peptides LrVa and LrVd in 50% TFE (Lau et al., 1984b; Hodges et al., 1990; Zhou et al., 1990) were monitored as a function of temperature. As shown in Figure 9, both peptides LrVa and LrVd show the similar unfolding curves at pH 2 and pH 7. The amino acid compositions of these α -helices are identical in the nonpolar face of the α -helix and one might expect these single-stranded α -helices to have similar hydrophobicities and stabilities, as observed. However, positions “a” and “d” respond differently to substitution when these peptides pack together to form coiled coils. This suggests that the large difference in stabilities of these coiled coils is a result of differences between the interhelical packing of

Fig. 9. The thermal melting profiles of LrVa (squares) and LrVd (circles) in 0.1 M KCl, 50 mM PO_4 buffer with TFE (1:1, v/v), pH 7 (closed symbols) and pH 2 (open symbols). Θ/Θ_5 represents the ratio of the ellipticity at 220 nm at the indicated temperature to the ellipticity at 5 °C. The star denotes the $\Theta/\Theta_5 = 1.0$ for the four profiles.

the hydrophobes. The first high-resolution X-ray structure (O'Shea et al., 1991) of the coiled coil protein GCN4 at 1.8 Å resolution indicated that the local stereochemical environment for position "a" and "d" was different. Typically, the $\text{C}\alpha$ - $\text{C}\beta$ vectors for position "a" and "d" point in different directions relative to the dimer axis: the hydrophobic side chains at position "d" point into the dimer interface, while those at position "a" point away from the hydrophobic interface. The β -carbons of symmetry-related residues are 2 Å closer at positions "d" than the β -carbons at positions "a." Similar observations were also made by Phillips et al. (1986) in analyzing the low-resolution X-ray structure of α - and β -tropomyosin where the regions of the coiled coil-containing β -branched amino acids at positions "d" were distorted. At positions "a," all side chains make side-to-side contacts and have ideal van der Waals interaction, and the hydrophobic core is well packed. Amino acid sequence analysis of tropomyosin, keratin, and other coiled coil proteins by Parry also showed that there is a preference for Ile and Val at position "a" to "d" (Parry, 1982; Conway & Parry, 1990). Similarly, Ile and Val are found almost three times more frequently at position "a" than at position "d" in the coiled coil domain of myosin (McLachlan & Karn, 1983). Analysis of other known coiled coil proteins such as muir lipoprotein also shows a similar trend (McLachlan, 1978). These analysis results are in good agreement with our mutation studies.

Implications for the DNA-binding proteins and protein folding

Hu et al. (1990) have determined that the Leu residues in the heptad repeat do have a special function in regulating the dimerization and the DNA-binding activity. Most

single mutants at "a" and "d" are functional but the amino acids must be hydrophobic. Multiple mutations showed that Leu residues conserved at "d" are important in function. From our results, the mutants with Ile or Val at positions "d" instead of Leu still form the coiled coil structure but with lower stability. Though it is the basic 3-4 hydrophobic repeat that specifies the basic folding of a coiled coil, hydrophobicity, helix propensity, and packing effects will regulate conformation, stability, and biological activity. Lim and Sauer (1991) have determined how internal packing interactions contribute to the stability, structure, and activities by mutagenesis of interacting hydrophobic residues in the core of λ repressor. Of the 78 mutants, only 2 fold into structures with stabilities and activities comparable to the wild type even though 70% of the mutants show some level of activity. Positions 36 and 47 in λ repressor must be occupied by a β -branched Ile or Val residue to maintain high thermal stability and activity. Again, the core hydrophobic residues contribute to determining the conformation and stability of a protein at two different levels. At the first and more general level, it is the pattern of the hydrophobic core residues and their general hydrophobicity that dictates the overall protein fold with moderate stability. If this is true, then many different sequences will yield a particular type of fold, as long as they maintain the appropriate overall pattern and hydrophobicity. At the second and detailed level, the specific steric features of these core residues and their packing interactions determine the protein's precise conformation, stability, and biological activity. Thus it may be straightforward to design a sequence that will adopt a particular fold with reasonable stability. However, it will be far more difficult to design a protein that has the exact properties with regard to stability and packing necessary to perform a desired activity in an efficient manner.

Materials and methods

All peptides were synthesized by solid-phase peptide synthesis methodology using a benzhydrylamine-hydrochloride resin on an Applied Biosystems peptide synthesizer Model 430A (Foster City, California) with conventional *N*-*t*-butyloxycarbonyl (*t*-Boc) chemistry as described previously (Hodges et al., 1988). The peptides were cleaved from the resin by reaction with HF (20 mL/g resin) containing 10% anisole and 2% 1,2-ethanedithiol for 1 h at -5 to 0 °C. The crude reduced peptides were purified by reversed-phase high performance liquid chromatography (HPLC) on a SynChropak RP-P semipreparative C_{18} column (250 × 10 mm inner diameter, 6.5 μm particle size, 300 Å pore size) (SynChrom, Lafayette, Indiana), with a linear AB gradient of 0.5% B/min and 2 mL/min, where solvent A is 0.05% trifluoroacetic acid (TFA) in water and solvent B is 0.05% TFA in acetonitrile. Disulfide-bridged peptides were obtained by air oxidation of reduced pep-

tides in 0.1 M NH_4HCO_3 (1 mg/mL), pH 8.0, at room temperature. The reaction was monitored by analytical reversed-phase chromatography (RPC). The disulfide-bridged peptides were purified by RPC on an analytical C_{18} column (SynChropak RP- PC_{18} , 250×4.6 mm inner diameter, 300 Å pore size; SynChrom, Inc., Lafayette, Indiana) with a linear AB gradient of 0.5% B/min and 1 mL/min, where solvent A is 0.05% TFA in water and solvent B is 0.05% TFA in acetonitrile.

The purified peptides were homogeneous as determined by reversed-phase HPLC and confirmed by amino acid analysis and mass spectroscopy. For amino acid analysis, purified peptides were hydrolyzed in 6 N HCl containing 0.1% phenol at 110 °C for 24 h or 1.5 h at 160 °C in evacuated sealed tubes. Amino acid analysis was performed on a Beckman model 6300 amino acid analyzer (Beckman, San Ramon, California). The correct primary ion molecular weights of the reduced peptides were confirmed by time of flight mass spectroscopy on a BIOION-20 Nordic (Uppsala, Sweden).

CD spectra were recorded at 20 °C on a Jasco J-500C spectropolarimeter (Jasco, Easton, Maryland) equipped with a Jasco DP-500N data processor and a Lauda (model RMS) water bath (Brinkmann Instruments, Rexdale, Ontario, Canada), used to control the temperature of the cell. Constant N_2 flushing was employed. The instrument was routinely calibrated with an aqueous solution of recrystallized *d*-10-(+)-camphorsulfonic acid at 290 nm. Molar ellipticity at 220 nm is reported as mean residue molar ellipticity ($[\Theta]_{220}$, °·cm²·dmol⁻¹) and calculated from the equation:

$$[\Theta] = [\Theta]_{\text{obs}} \text{mrw}/10lc.$$

$[\Theta]_{\text{obs}}$ is the ellipticity measured in degrees, *mrw* is the mean residue molar weight of peptide (molecular weight divided by the number of amino acid residues), *c* is the peptide concentration in grams per milliliter, and *l* is the optical path length of the cell in centimeters. CD spectra were the average of three scans obtained by collecting data at 0.25-nm intervals from 254 to 190 nm. Gdn·HCl denaturation studies were carried out by preparing mixtures of a stock solution of peptide in buffer (0.1 M KCl, 50 mM PO_4 , pH 7), buffer alone, and a solution of 6 M Gdn·HCl (or 8 M Gdn·HCl for LoIa) in buffer where the ratios of buffer and 6 M Gdn·HCl (or 8 M Gdn·HCl) solutions varied to give the appropriate final Gdn·HCl concentrations. Peptide concentrations were determined by amino acid analysis.

The molecular weights of the peptides in aqueous buffer solution were determined by size-exclusion chromatography and sedimentation equilibrium experiments. Size-exclusion chromatography was carried out on a Spherogel TSK 2000SW column (300×7.5 mm inner diameter, 10 µm particle size, 125 Å pore size) (Beckman Instruments, Toronto) at a flow rate of 0.2 mL/min at

room temperature. The eluent solvent was a 0.5 M KCl, 50 mM PO_4 , pH 7, buffer. The 14-, 21-, 28-, 42-, and 56-residue α -helical peptides were used as molecular weight standards (Hodges et al., 1990; Zhou et al., 1990). Sedimentation equilibrium experiments were carried out with a Beckman Model E Analytical Ultracentrifuge equipped with electronic speed control, RTIC temperature control, Rayleigh interference optics, ANH titanium rotor, and double sector charcoal-filled Epon cells was used for all runs. Measurements of photographic plates were performed on a Nikon Model 6 Microcomparator at a magnification of 50×. Determinations of molecular weights were made using the conventional sedimentation equilibrium technique described by Chervenka (1970). Prior to running in the ultracentrifuge, the samples were dialyzed for a minimum of 48 h at a temperature of 4 °C. Fringe counts were then performed on the samples to determine initial concentrations using a conversion factor of 4.1 fringes: 1 mg/mL (Babul & Stellwagen, 1969). Sedimentation equilibrium runs were performed for a minimum of 36–48 h prior to taking equilibrium photographs. Molecular weight calculations were carried out using a computer program written in the APL language. An assumed value of 0.728 mL/g was used for the partial specific volume. Apparent weight average molecular weights were determined from the slopes of the $\ln \gamma$ vs. r^2 plots. Peptide Lo was dissolved in 0.1 M KCl, 50 mM PO_4 , pH 7, buffer containing 6 M Gdn·HCl, dialyzed against 1 M KCl, 50 mM PO_4 , pH 7, buffer and run at an initial concentration of 2.2 mg/mL, at a speed of 20,000 rpm. Peptides LoId and LrId were dissolved in 0.5 M KCl, 50 mM PO_4 , pH 7, buffer, dialyzed against 0.5 M KCl, 50 mM PO_4 , pH 7, buffer and run at 28,000 rpm, and at concentrations of 1.31 mg/mL and 1.85 mg/mL, respectively. For reduced peptide LrId, 10 mM dithiothreitol (DTT) was added in the buffer to prevent the formation of an interchain disulfide bond. The lower slope corresponding to a higher molecular weight for the Lo peptide in comparison with the other peptides is a result of the lower operating speed and higher solution density for this sample.

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