Synthetic Model Proteins

POSITIONAL EFFECTS OF INTERCHAIN HYDROPHOBIC INTERACTIONS ON STABILITY OF TWO-STRANDED α-HELICAL COILED-COILS*

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We have designed a model protein that consists of two identical 35-residue polypeptide chains, parallel and in-register arranged in a two-stranded α-helical coiled-coil structure. This structure is stabilized by interchain hydrophobic interactions between leucine residues at positions “a” and “d” of a repeating heptad sequence. To determine the positional effects of interchain hydrophobic interactions on the stability of the coiled-coil, a single leucine residue in each chain at position “a” (9, 16, 23, 30) and “d” (5, 12, 19, 26, 33) was systematically replaced by an alanine. All these proteins formed two-stranded α-helical coiled-coils in benign conditions (0.05 M phosphate, 0.1 M KCl, pH 7). The stability of each mutant protein was determined by guanidine hydrochloride denaturation experiments, where the decrease in ellipticity at 220 nm was monitored by circular dichroism. The single alanine replacements of a leucine residue at hydrophobic positions a and/or d are all shown to destabilize the coiled-coil structure. The non-equivalent hydrophobic positions a and d make an equivalent contribution to protein stability along the majority of the coiled-coil structure (positions 9–30). The small decrease in coiled-coil stability caused by Leu → Ala substitution at either ends of the coiled-coil suggested that the Leu-Leu hydrophobic interactions are less important at the ends of the coiled-coil and the ends of the coiled-coil are more flexible. Analysis of the difference between the ellipticity in benign buffer and in 50% trifluoroethanol (Δε220) and the slope term from a plot of the free energy of unfolding versus guanidine hydrochloride concentration also supported the conclusion that the leucine residues at the ends of the coiled-coil are much less buried than in the middle section of the coiled-coil.

The de novo design of model proteins is an important endeavor that not only tests our understanding of protein folding and structure, but also lays the groundwork for the design of novel proteins with the desired biological/immunochemical activities. The purpose of our research is to design a small and unique protein molecule with defined secondary, tertiary, and quaternary structure, and then to modify systematically the structure to delineate the contributions that various amino acid side chains make to control folding, conformation, and stability (1–5). It is generally assumed that protein folding is not a random process and that elements of secondary structure such as β-turns or α-helices are the most likely candidates for nucleus formation in the unfolded protein (6–8). It seemed appropriate to design the model protein with only one type of secondary structure, i.e. the α-helix. An α-helical protein was chosen because it shows the least conformational variability among hydrogen-bonded secondary structural features found in globular proteins. Also, the α-helical structure is the easiest type of secondary structure to monitor in aqueous solution at neutral pH using such techniques as circular dichroism and NMR spectroscopy. To introduce tertiary and quaternary structure into a molecule, a minimum of two interacting α-helices is required. We concluded that the ideal model protein could be a two-stranded α-helical coiled-coil.

The coiled-coil consists of two right-handed α-helical polypeptide chains that are parallel, in-register, and coil about one another. The main advantage of studying coiled-coils in preference to single-stranded α-helices is that coiled-coils are stabilized by both intrachain and interchain interactions. Intercal interactions are the main feature responsible for the folding and stabilization of the three-dimensional structure of proteins and the stabilization of α-helices in aqueous solution by side chain interactions characterizes the globular proteins as well as α-helical coiled-coils (1, 5). Model coiled-coils can be used to investigate all the noncovalent interactions involved in maintaining the three-dimensional structure of proteins (i.e. hydrogen bonds, ionic interactions, van der Waals bonds, hydrophobic interactions, helix dipole, charge compensation effects, and side chain packing effects). The hydrophobic interactions between nonpolar residues are thought to be among the most important in determining the three-dimensional structure of a protein (9–11). In this study, we have focused on the hydrophobic interactions and their positional effects on the stability of the coiled-coil protein. The coiled-coils are biochemically significant in their own right and are involved in muscle regulation (12–14), DNA binding properties (15–18), and other functions (19). Thus, a detailed understanding of coiled-coil structure may delineate the reasons for a wide range of hydrophobic residues in the hydrophobic core and how a conformational change is transmitted along the coiled-coil.

MATERIALS AND METHODS

All peptides were synthesized by the solid-phase technique starting with co-poly (styrene, 1% divinylbenzene) benzhydrylamine-hydrochloride resin using an Applied Biosystems peptide synthesizer model.
The amino acid sequence of the 70-residue synthetic two-stranded \(\alpha\)-helical coiled-coil (two identical 35-residue polypeptide chains) is shown in Fig. 1. In choosing the amino acid sequence for a particular protein, one must select a sequence that specifies one particular secondary, tertiary, and quaternary structure and disfavors alternative arrangements. In this regard, our choice of sequence was based upon the sequence of tropomyosin, the first two-stranded \(\alpha\)-helical coiled-coil to be dated. Each 35-residue polypeptide chain consists of five heptads. The coiled-coil analog in which an alanine substitution is made at each chain at the same position by an alanine residue to create a series of coiled-coil analogs with a single alanine replacing one of nine Leu-Leu pairs at positions 5, 9, 12, 16, 19, 23, 26, 30, and 33. The coiled-coil analog in which an alanine substitution is made at position 5 in chain 1 and chain 2 is denoted as A5 and similarly A9, A12, A16, A19, A23, A26, A30, or A33.

The amino acid sequence of the 70-residue synthetic two-stranded \(\alpha\)-helical coiled-coil is shown in the figure. The table below lists the residues at each position:

| Residue Number | Sequence |
|----------------|----------|
| 1              | Ac       |
| 2              | Lys-SH   |
| 3              | Ala      |
| 4              | Glu      |
| 5              | Leu      |
| 6              | Glu      |
| 7              | Gly      |
| 8              | Lys      |
| 9              | Leu      |
| 10             | Glu      |
| 11             | Ala      |
| 12             | Leu      |
| 13             | Glu      |
| 14             | Gly      |
| 15             | Lys      |
| 16             | Leu      |
| 17             | Glu      |
| 18             | Ala      |
| 19             | Leu      |
| 20             | Glu      |
| 21             | Gly      |
| 22             | Lys      |
| 23             | Leu      |
| 24             | Glu      |
| 25             | Ala      |
| 26             | Leu      |
| 27             | Glu      |
| 28             | Gly      |
| 29             | Lys      |
| 30             | Leu      |
| 31             | Glu      |
| 32             | Ala      |
| 33             | Leu      |
| 34             | Glu      |
| 35             | Gly      |
|                | NH2      |

The correct primary ion molecular weights were confirmed by time of flight mass spectroscopy on a BION ion-20 Nordic (Uppsala, Sweden).

Circular dichroism spectra were recorded at 20 °C on a Jasco J-500C spectropolarimeter (Jasco, Easton, MD) attached to a Jasco DP-500N data processor and an Lauda (model RMS) water bath (Brinkmann Instruments, Rexdale, Ontario, Canada) used to control the temperature of the cell. The instrument was routinely calibrated with an aqueous solution of recrystallized d-10-camphorsulfonic acid.

Ellipticity is reported as mean residue ellipticity with an aqueous solution of recrystallized D-10-camphorsulfonic acid.

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residues and 1 alanine residue in a and/or d positions. Thus, the A5 analog contains alanine at position 5 in chain 1 and position 5' in chain 2 or 1 alanine pair and 8 leucine pairs in the coiled-coil. The native coiled-coil model, in which all nine a and d positions are occupied by leucine residues results in 9 leucine pairs and is denoted as L. The questions to be answered in this study were as follows. First, do the hydrophobic (Leu-Leu) interactions along the length of coiled-coil contribute equally to the stability of the coiled-coil? Second, do the nonequivalent hydrophobic positions of 4–3 repeat (positions a and d) contribute equally to stability?

Characterization of the α-Helical Coiled-coils—The criteria used to establish that the synthetic peptides have formed a coiled-coil structure are as follows. 1) Size-exclusion chromatographic studies of these peptides compared to the 14-, 21-, 28-, and 35-residue α-helical peptide standards using methodology described previously (5, 30, 53) demonstrated once again that the peptides are dimeric in benign buffer (0.1 M KCl, 50 mM PO₄, pH 7) and monomeric in 50% TFE. 2) The ellipticity at 220 nm was dependent on peptide concentration in benign medium (inset of Fig. 3). 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. At peptide concentrations greater than 200 μM, the ellipticity of the peptide was independent of peptide concentration indicating the complete association of monomers to coiled-coil dimer. On the other hand the ellipticity at 220 nm was independent of peptide concentration in 50% TFE (6–615 μM, inset of Fig. 3) suggesting no dimer formation in 50% TFE. 3) Sedimentation equilibrium experiments carried out with the interfe-
ence optical system at a speed of 30,000 rpm and at an initial peptide concentration of 344 μM in the benign medium containing 2 mM dithiothreitol revealed the complete absence of monomer in the system. These results are in agreement with previous sedimentation equilibrium studies (3) which showed that the four or five heptad repeats of the sequence Ac-[Lys-Leu-Glu-Ala-Leu-Glu-Gly]-3-Lys-amide formed a homogeneous, two-stranded structure in benign media. 4) The CD spectra for all nine analogs (A5, A9, A12, A16, A19, A23, A26, A30, and A33) are very similar to each other and the native coiled-coil protein (L). All showed two minima, one near 207 nm and another at 207 nm in benign conditions (0.1 M KCl, 50 mM PO4, pH 7) with high ellipticity indicating high helical content. The peptides do not show any increase in helicity upon addition of the α-helix inducing solvent TFE, as measured by molar ellipticity at 220 nm (the ellipticities in 50% TFE were less than in benign buffer, Table I). The α→α* transition (220-nm CD band) is responsive to the α-helical content. The α→α* excitation band at 205 nm polarizes parallel to the helix axis and is sensitive to whether the a-helix is single-stranded or is an interacting helix as in the case of the two-stranded coiled-coils (27). The decrease in parallel band intensity, coupled with the red shift in the parallel band maximum, corresponds to the conversion of a rigid single-stranded α-helix to an α-helical coiled-coil structure (27). The maximum ellipticity at 207 nm in benign medium shifts to 205 nm in 50% TFE and the ratio of θ200/θ207 changes from 1.03 (+0.03) in benign medium to 0.86 (+0.03) in 50% TFE (Fig. 3). These results indicated that all analogs form the two-stranded α-helical-coil structure in benign medium and single-stranded α-helices in 50% TFE. These results are in good agreement with our previous studies on coiled-coil peptides (3–5).

**Table 1**

| PEP tide | θ200 (benign) | θ207 (50% TFE) | Δθ200 | [GdnHCl]1/2d | ΔG(50°)° | m° |
|----------|---------------|----------------|-------|---------------|---------|-----|
| L        | -30,000       | -22,850        | -7,150| 2.3           | 9.7     | 2.06|
| A5       | -20,980       | -20,090        | -990  | 1.8           | 8.5     | 2.10|
| A9       | -21,090       | -19,440        | -1,650| 0.7           | 6.7     | 2.20|
| A12      | -24,970       | -20,010        | -4,960| 0.7           | 6.8     | 2.44|
| A16      | -26,590       | -21,530        | -5,060| 0.7           | 6.8     | 2.32|
| A19      | -25,930       | -19,880        | -6,550| 0.7           | 6.7     | 2.32|
| A23      | -24,760       | -18,330        | -6,430| 0.7           | 6.8     | 2.33|
| A26      | -27,560       | -21,680        | -5,880| 0.8           | 7.3     | 2.37|
| A30      | -27,850       | -22,820        | -5,030| 1.1           | 7.7     | 2.22|
| A33      | -30,030       | -22,380        | -7,650| 1.6           | 8.8     | 2.13|

*A 0.1 M KCl, 0.05 M PO4 buffer, pH 7, was used for all benign measurements. Temperature was 20 °C for ellipticity measurements.

**Helicity of the Model Peptides**—The molar ellipticities of the peptide analogs (Table I) were measured in benign conditions and in the presence of TFE, a solvent that induces helicity in a single-stranded potentially α-helical polypeptide (28–29). Lau et al. (30) have shown previously that 50% TFE disrupts the quaternary structure of α-helices, i.e. TFE is a denaturant of tertiary and quaternary structure stabilized by hydrophobic interactions. While there are small differences in the ellipticities at 220 nm of some of the individual analogs relative to each other, all of the analogs (A5 → A33) in 50% TFE have values of about -20,600 ± 2,000°, implying that a replacement of leucine residue by alanine in the NH2 terminus, in the middle or in the COOH terminus of the peptide, has a similar effect on the single-stranded α-helical structure. These results are in agreement with the study of Merutka and Stellwagen (31). They substituted 1 alanine residue by serine or methionine at position 4, 9, or 14 in a 17-residue α-helical peptide and demonstrated that the effect of substitution on the stability of the α-helix is independent of the substitution position.

By comparing the difference in ellipticities of all the coiled-coil analogs in 50% TFE and in benign buffer (Δθ200), we can conclude that the hydrophobic interactions between the two α-helices in the coiled-coil are better than TFE in inducing α-helical structure. In addition, the observation that the Δθ220 value of peptide L is greater than its analogs (A5 → A33) suggests that the hydrophobicity of the hydrophobic face has a major impact on induction of α-helical structure through the interchain hydrophobic interactions which stabilize the coiled-coil and consequently stabilize the α-helices. Another interesting result is that the decrease in ellipticity (Δθ200) for the analogs with Ala substitution at the ends of the coiled-coil is less than for analogs with substitution in the central region (compare the Δθ220 value of A5 (−600), A9 (−1,650), and A33 (−2,650) with A12–A30 (−4,000 → −6,400)). These results indicated that the interchain hydrophobic interactions are less important at the ends of the coiled-coil, suggesting a more flexible structure at the ends compared to the center.

**Denaturation of Coiled-coil Analogs with Guanidine Hydrochloride**—The denaturation curves determined by monitoring the ellipticities of the peptides at 220 nm as a function of guanidine hydrochloride (GdnHCl) concentration at 20 °C are displayed in Figs. 4A and 5A. The transition midpoint of GdnHCl concentration, [GdnHCl]1/2, at which the helical content is 50%, depends on peptide concentration (Fig. 4A). The [GdnHCl]1/2 was 2.5 M for the higher peptide concentration (1347 μM) and 1.1 M for the lower peptide concentration (48 μM). The concentration dependence observed for the unfolding curves shows that chain dissociation is a normal feature in the denaturation of the coiled-coils. In contrast, the disulfide bridged coiled-coil (a disulfide bond formed between 2 cysteine residues at position 2 in each chain) did not show any concentration dependence in the GdnHCl denaturation experiments (data not shown).

If we assume that the single-stranded α-helix (folded monomer) is not present at significant concentrations in equilibrium, then only the transition between the two-stranded α-helical coiled-coil and the single-stranded random coil is monitored by the change in ellipticity at 220 nm. Therefore the unfolding reaction follows a two-state model of folded dimers in equilibrium with unfolded monomers.

\[ F_2 = K_d \]
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ride denaturation of coiled-coil peptide and 1345 A5 at 220 nm at the indicated molarity of guanidine hydrochloride to the KC1 buffer, pH

Prolation coefficient for the regression line was 0.94.

ellipticity without guanidine hydrochloride. Concentrations of peptide hydrochloride at different concentrations of peptide

proteins, represents analogs A9, A12, A16, A19, and A23.

220 nm at the indicated molarity of guanidine hydrochloride to the

Fig. 4. A, concentration dependence of the guanidine hydrochloride denaturation of coiled-coil peptide A5 in 0.06 M PO4 and 0.1 M KC1 buffer, pH 7 at 20 °C. R/θo represents the ratio of the ellipticity at 220 nm at the indicated molarity of guanidine hydrochloride to the ellipticity without guanidine hydrochloride. Concentrations of peptide A5 were 48 μM (●), 131 μM (△), 213 μM (○), 592 μM (▲), 888 μM (□), and 1345 μM (□). The denaturation studies of peptide A5 were also carried out at concentrations 266 and 403 μM (data not shown in A). B, plot of linear dependence of ΔGu on the concentration of guanidine hydrochloride at different concentrations of peptide A5. The correlation coefficient for the regression line was 0.94.

Fig. 5. A, guanidine hydrochloride denaturation profiles of all Ala substitution analogs and the native coiled-coil protein in 0.1 M KC1, 0.05 M PO4 buffer, pH 7. R/θo represents the ratio of the ellipticity at 220 nm at the indicated molarity of guanidine hydrochloride to the ellipticity without guanidine hydrochloride. ● denotes the native proteins, △ the A5 and A33 analogs, ○ the A30. + A26, and □ represents analogs A9, A12, A16, A19, and A23. B, plot of transition midpoint of guanidine hydrochloride concentration from denaturation profile (A) versus the position of Ala substitution (5, 9, 12, 16, 19, 23, 26, 30, or 33). Dashed line indicates the transition midpoint of guanidine hydrochloride concentration of the native coiled-coil protein (L).

mined from the ellipticity at 220 nm, f = (θo - θ)/θo, - θ, where θ is the observed ellipticity at any particular guanidine hydrochloride concentration; θo and θ are the ellipticities of the native and unfolded states, respectively (33). The value K, can be calculated at each different guanidine hydrochloride concentration in the transition zone and the free energy change (ΔG) can be determined using the equation ΔGu = -RT ln K, the free energy of unfolding in the absence of denaturant was estimated by linear extrapolation of the free energy of unfolding at each individual concentration of denaturant (ΔGu) to zero GdnHCl concentration (33, 34) according to the equation

\[ ΔGu = ΔGu^{H2O} - m[GdnHCl] \]

Fig. 4B shows the plot of the linear dependence of ΔGu on the concentration of guanidine hydrochloride at eight different concentrations (48-1347 μM) of peptide A5. Although the [GdnHCl]1/2 of coiled-coil dimer is dependent on peptide concentration (Fig. 4A), the ΔGuH2O is essentially the same for different peptide concentrations (the average ΔGuH2O = 8.30 ±0.2 ) (kcal/mol). The Gu values for all peptides are listed in Table I.

The Positional Effects of Hydrophobic Interactions on the Coiled-coil Stability—In order to compare the stability of coiled-coil analogs, the GdnHCl denaturation for all these coiled-coils was carried out at similar peptide concentrations (~200 μM) to rule out the possible concentration effects on stability. If the stability of a two-stranded coiled-coil is due to hydrophobic interactions in the a and d positions (Fig. 1), one would expect to find a decrease in coiled-coil stability with a substitution of any leucine residue with a less hydrophobic alanine residue at these positions. All alanine-substituted coiled-coils are less stable than the native coiled-coil as shown by the transition midpoints and ΔGuH2O values (Fig. 5 and Table I). These results are in agreement with previous studies on coiled-coils (4, 5). However, the relative contribution of these hydrophobic interactions at different positions in the coiled-coil are not identical. A5 and A33 coiled-coils are the most stable of the analogs as indicated by the same denaturation curve (Fig. 5A). A30 shows a further decrease in stability compared to A5 and A33. All remaining analogs have essentially identical stability and are the least stable of the analogs. Fig. 5B plots the transition midpoint of GdnHCl concentration versus the position of the alanine substitution. From Fig. 5B, it is clearly seen that hydrophobic interactions at positions 9, 12, 16, 19, 23, and 26 are very important and contribute equally to the coiled-coil stability. Substitution of a leucine residue with an alanine at each of positions 9-26 greatly reduces protein stability, while substitution of a leucine residue with an alanine at position 5 or 33 only slightly decreases the coiled-coil stability. The small decrease in stability found for Leu → Ala substitution at either end of the coiled-coil suggests that Leu-Leu interactions at the ends of the coiled-coil do not contribute significantly to the coiled-coil stability, and leucine residues at the ends of the coiled-coil are much less buried than in the middle section of the coiled-coil. An explanation based on the theoretical model of the coiled-coil structure is that, in a parallel coiled-coil, a leucine sidechain in one helix makes inter-chain contact with the equivalent leucine in the opposing α-helix (such as 16-18') and also interacts extensively with 2 leucine residues from the opposing helix, one above and one below (such as 16-12' and 16-19'). All of these hydrophobic interactions between leucine residues in chain 1 and chain 2 are shown in Fig. 2 (bottom panel, left side). In the native coiled-coil, a leucine residue at the middle part of the coiled-coil interacts...
with 3 leucine residues in the opposing helix (one green side chain contacts with three brown side chains or one brown with three greens), while a leucine residue at either end of the coiled-coil (positions 5 and 33) only interacts with 2 leucines in the opposing helix. If one considers a Leu-Leu pair in the central region of the coiled-coil such as 16-16', the total number of interchain Leu-Leu interactions are five (16-16', 16-12', 16-19', 16'-12, and 16'-19). On the other hand, a Leu-Leu pair at the end of the coiled-coil (33-33') has only 3 Leu-Leu interchain interactions (33-33', 33-30', and 33'-30). This analysis would predict that the ends of the coiled-coil would be less stable and more flexible. The molecular dynamics simulation of the model coiled-coil (Fig. 6) is in good agreement with our experimental results. When an alanine substitution is made at the end of the coiled-coil (such as position 33), 3 leucine-leucine hydrophobic interactions are lost (33-30', 33-33', and 33'-30). By comparison, 5 leucine-leucine hydrophobic interactions would be lost, when Ala was substituted in the middle part of the coiled-coil (for example, 19-16', 19-19', 19-23', 19'-16, and 19'-23) in the A19 coiled-coil analog (bottom panel in Fig. 2). One of the mechanisms by which hydrophobic side chains stabilize protein structure is through van der Waals interactions. The presence of an empty cavity in the mutant protein where the wild-type side chain normally resides would destabilize the native state relative to the denatured state because of reduced interactions (35).

**DISCUSSION**

Previous studies by our laboratory (3-5) have demonstrated that a large part of the stability of the coiled-coils is due to the presence of hydrophobes in the 3-4 repeat positions exactly as predicted. It seems reasonable that the two helices are stabilized primarily by the burial of closely packed, hydrophobic side chains (36), although hydrogen bonds and salt bridges between interacting helices, usually involving nonburied atoms, also occur quite often (37). Mutations or modifications which disturb either the close packing or the hydrophobicity of a helix pairing site would then destabilize that interaction and consequently the protein (38). Several experiments indicated that helix pairing sites are indeed sensitive to these two types of disruptions (39-42). Such disruptions have also been observed in this model coiled-coil protein (5). When the leucine residues at position 16 and 19 of each chain (Fig. 1) are replaced with Ile, Val, Ala, Phe, or Tyr, the stability of the protein with regard to the hydrophobic aliphatic amino acid residue substitution correlates with the hydrophobicity of the side chain (order of protein stability is Leu > Ile > Val > Ala). The aromatic amino acids did not stabilize the proteins to the same extent as aliphatic side chains with similar hydrophobicities (5), suggesting that the aliphatic side chains provide much better interchain packing than the aromatic side chains. Similar results have been reported in studies of the globular proteins tryptophan synthase a subunit (43) and bacteriophage T4 lysozyme (44). In this regard, it is important to note that the relative contributions of nonpolar residues to protein stability using our two-stranded a-helical coiled-coil model and the study by Matsumura et al. (44) on T4 lysozyme showed an excellent correlation. In both proteins, the mutations were made in the hydrophobic residues involved in the hydrophobic core responsible for overall protein stability. These results verify the general utility of our model to investigate the contribution of side chains to protein stability. In the present study, the single alanine replacements of a leucine residue at hydrophobic positions a and/or d are all shown to destabilize the coiled-coil structure, implying that all of the hydrophobic interactions between these positions contribute to the coiled-coil stability. However, the positions at the ends of the coiled-coil make a smaller contribution to stability. These variations must be a consequence of the variations in the environment surrounding the different residue positions. For instance, a leucine residue would be expected to make a greater contribution to protein stability if it was fully buried than if it was fully exposed to solvent. Indeed, the reverse hydrophobic effects on protein stability have been observed on λCro protein, whereupon amino acid substitution on the surface of the protein resulted in a mutant protein which showed a good correlation between stability and decreasing side chain hydrophobicity (45).

Analysis of the difference between the ellipticity in benign buffer and in 50% TFE (Δθ_TFE) (see “Results”) and the slope term (m value in ΔG_m = ΔG_m,420 - m[GdnHCl]) also supported the conclusion that the end of the coiled-coil structure is more flexible and Leu-Leu hydrophobic interactions are less important at the end of the coiled-coil. The chemical interactions by which GdnHCl and other denaturants destabilize protein structure have not been established, with the result that the detailed structural basis of the variations in m value cannot be elucidated clearly. However, as demonstrated by Shorttle et al. (35), the values of m for mutant forms of staphylococcal nuclease (in each mutation, single alanine and glycine substitutions were constructed for each of the 11 Leu, 9 Val, 5 Ile, 4 Met, and 3 Phe) are dependent upon the sites of mutations. Mutations that increased the m value only involved residues that contribute side chains to the major hydrophobic core of the protein, whereas mutations that caused the m value to decrease are located outside of this major hydrophobic core. Upon comparison of the m values of all coiled-coils in this study, it is noted that there is an increase in the m value for alanine substitution in the central region of the coiled-coil. For example, comparing the m values of A12 → A26 (2.32 → 2.44) with L (2.06) suggests that the hydrophobic residues at positions 12 → 26 form the major hydrophobic core of this.

**FIG. 6.** Molecular dynamics structure of the model coiled-coil protein in which only backbone atoms are shown. Sixteen trajectory structures from four different molecular dynamics were selected and superimposed to minimize the root mean square deviation of backbone atoms of residues 15–21 in two chains.
coiled-coil structure. The similar m values of A5 (2.10) and A33 (2.13) compared to the native coiled-coil L (2.06) indicate that the hydrophobic positions 5 and 33 are outside the major hydrophobic core.

Integrating the results from this study with other studies on mutants of globular proteins, such as bacterial ribonuclease barnase (46), staphylococcal nuclease (35), and glycerol-3-phosphate dehydrogenase (47), it would appear that the importance of hydrophobic interactions varies considerably with position within a protein. The mutations which use less hydrophobic residues in place of more hydrophobic ones are generally more deleterious for protein stability at buried than at less buried positions (35, 38, 46-49).

Although hydrophobic residues at positions a and d contribute to the coiled-coil stability, one may ask the question, does a Leu residue at position a make the same contribution to the coiled-coil stability as a Leu at position d? According to the survey by Cohen and Parry (14) on naturally occurring coiled-coils (tropomyosin, myosin, par-...