An isoleucine zipper peptide forms a native-like triple stranded coiled coil in solution

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Introduction

Studies of de novo designed proteins involve the construction of proteins forming predetermined three-dimensional structures and the creation of new functional proteins. Various structures, such as the β-barrel, TIM barrel, α-helical bundle and coiled coil were designed based on statistical and theoretical analyses (Hodges et al., 1981; Regan and DeGrado, 1988; Hecht et al., 1990; Quinn et al., 1994; Tanaka et al., 1994a,b). Most de novo designed proteins, however, usually exhibit the characteristics of a molten globule, a non-native state that is compact but possesses ill-defined tertiary structures. Recently, studies have focused on the construction of native-like, well-packed structures.

α-Helical bundles, especially coiled coils, have been the subject of extensive protein design because of their abundance in nature and their structural simplicity. A coiled coil is characterized by heptad repeats of seven amino acid residues, denoted a to g, with hydrophobic residues at the a and d positions. In early designs, Leu residues were often used in the hydrophobic interface (Lau et al., 1984; O’Neil and DeGrado, 1990; Wu et al., 1994). However, because of the presence of a wide range of rotameric states of Leu, designed coiled coils with Leu residues in the hydrophobic core have a tendency to form molten globules. For example, a 29 residue Coil-Ser peptide exists in a non-cooperative monomer–dimer–trimer equilibrium in solution, without efficient internal packing (Betz et al., 1995a), while the crystal structure was found to be an antiparallel triple-stranded coiled coil (Lovejoy et al., 1993). To facilitate native-like structural uniqueness, several strategies have been reported. They include the engineering of metal binding sites (Handel and DeGrado, 1990; Handel et al., 1993) and polar interactions in the buried positions (Lumb and Kim, 1995; Zeng et al., 1997), as well as the use of side-chain packing (Raleigh and DeGrado, 1992; Munson et al., 1994).

Natural proteins often use β-branched amino acids in α-helices, in spite of their low helical propensities. The importance of an Ile residue in the side-chain packing of the helix–turn–helix structure was demonstrated using the c-Myb DNA-binding protein (Furukawa et al., 1996). An Ile residue has only one favorable rotamer when in an α-helix; therefore, the Ile residue decreases the conformational entropy of the helical conformation. In the design of coiled coils, Ile or Val residues were incorporated at the hydrophobic positions to improve the side-chain packing in the hydrophobic core (Harbury et al., 1993; Betz and DeGrado, 1996; Boice et al., 1996; Gibney et al., 1997; Johansson et al., 1998). The GCN4-pII peptide, in which the amino acid sequence of the GCN4 leucine zipper was substituted with Ile residues at every a and d position, was shown to form a triple stranded parallel α-helical coiled coil, both in solution and in the crystal (Harbury et al., 1993, 1994). Each Ile side chain had adopted the preferred rotamer and was well-packed in the crystal structure. These results imply that the Ile residue might participate in the packing of the coiled coil structure in solution as well. As shown in the case of Coil-Ser, the coiled coil of a molten globule in solution can form a crystal with a high-resolution diffraction pattern. The crystal structures of α-helical peptides are sometimes different from those in solution (Hill et al., 1990; Lovejoy et al., 1993). Therefore, it is important to characterize the solution properties of designed peptides as well as the crystal structures (Betz et al., 1995b).

In this paper, we placed Ile residues at the a and d positions in our de novo design, and demonstrated that our Ile-zipper peptide (IZ) possesses native-like structural uniqueness in solution, without manipulation of metal binding or polar interactions.

Materials and methods

Peptide synthesis

Ile-zipper peptide was synthesized by the solid-phase peptide synthesis method on Rink amide resin, using N-Fmoc (9-fluorenylmethoxycarbonyl) protected amino acids, HBTU [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate], and hydroxybenzotriazole, on an Applied
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Biosystems peptide synthesizer, model 430A. Deprotection and cleavage were performed by treatment with trifluoroacetic acid containing 5% ethanedithiol and anisole (1/3, v/v) for 1.5 h. Peptide purification was carried out at room temperature by reversed-phase HPLC using a YMC-pack ODS-A column (10 mm i.d.×250 mm, 120 Å, 5 μm, YMC Inc., Japan) eluted at 3 ml/min. A linear elution gradient was used with 35–50% CH3CN/H2O containing 0.1% trifluoroacetic acid for 30 min. The final product was characterized by analytical HPLC and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

For the fluorescence quenching assay, a Cys residue was coupled to the N-terminus of IZ. After deprotection and purification as above, the peptide was dissolved in 500 μl of 0.2 M acetate buffer (pH 4.0) to a peptide concentration of about 300 μM. N-(9-Acridinyl) maleimide (DOJINDO, Japan) was dissolved in 350 μl acetone (3 mM), and then was mixed with the peptide solution. After 1 h at room temperature, the acridinyl IZ was purified by reversed phase HPLC.

Sedimentation equilibrium

Sedimentation equilibrium analysis was performed with a Beckman XL-1 Optima Analytical Ultracentrifuge equipped with absorbance optics. The initial peptide concentration was 50 μM in 10 mM sodium phosphate, pH 7.0, and 100 mM NaCl. The sample was centrifuged at 25 000, 30 000 and 35 000 r.p.m. at 25°C, and the absorbance was monitored at 280 nm. The oligomerization state was determined by fitting the data to a single species, using Origin Sedimentation Equilibrium Single Data Set Analysis (Beckman). The partial specific volume used for the data analysis was 0.776 ml/g, calculated from the weighted average of the amino acid content using the method of Cohn and Edsall (1943).

Fluorescence studies

Fluorescence studies were performed with a HITACHI F-4500 fluorescence spectrophotometer at room temperature with a 1-cm path-length cuvette. For the fluorescence quenching assay, an acridinyl random coiled peptide was used for a reference sample to exhibit full-fluorescence (non-quenching). The emission spectrum between 380–600 nm was measured with excitation at 362 nm. The spectra and the thermal transition curve were obtained with 2 mm path-length cuvette. The temperature was increased at a rate of 1°C/min. The spectra and the thermal transition curve were obtained by measuring the tyrosine absorbance in 6 M guanidine hydrochloride (GdnHCl) solutions, using ε222 = 1450 M⁻¹cm⁻¹ (Brandts and Kaplan, 1973). The thermal transition curve was obtained by monitoring the ellipticity at 222 nm ([θ]222) as a function of temperature with a 2-mm path-length cuvette. The temperature was increased at a rate of 1°C/min. The spectra and the thermal transition curve were obtained with 2 μM and 20 μM peptide solutions, respectively, in 10 mM phosphate buffer containing 0.1 M NaCl (pH 7). The GdnHCl denaturation curve, which was monitored [θ]222 by averaging ten 1.0-s readings, was obtained with a 20 μM peptide solution in 10 mM Tris–HCl buffer containing 0.1 M NaCl (pH 7) at 25°C. For a two state equilibrium between monomer and trimer, the equilibrium constant for unfolding, K, was obtained as K = (3c²f²)/(1 – f), where c is the total peptide concentration, f is the fraction of unfolded (monomeric) peptide, ΔG is the free energy of unfolding at a given concentration of GdnHCl, R is the gas constant, and T is the absolute temperature. The free energy of unfolding in water (ΔG°) was estimated from a linear extrapolation, according to ΔG = ΔG° – m[GdnHCl] (Pace, 1986).

Nuclear magnetic resonance (NMR)

NMR spectroscopy was performed on a Bruker DMX600 spectrometer operated at 600.13 MHz for 1H. Samples were prepared at the approximate concentration of 1 mM in D2O (pH 7.0). Chemical shifts were referenced internally to 0 ppm with trimethylsilylpropionic acid. One-dimensional data sets were measured at 25°C with suppression of residual water signal by presaturation. The data sets were defined by 8 k complex points and 64 scans were accumulated using a spectral width of 8289.3 Hz. Hydrogen exchange studies were initiated by dissolving a lyophilized sample in D2O. An observed exchange rate constant (kobs) was calculated from a two-parameter nonlinear least-squares fit of the exponential decay curve of the peak intensities between 5 min and 10 days (by KaleidaGraph, Synergy Software).

When exchange occurs only from the globally unfolded protein, and the folded protein is stable (f<sub>u</sub> << 1), the predicted protection factor (k<sub>unf</sub>/k<sub>f</sub>) is, in principle, equal to exp(ΔG<sub>unf</sub>/RT), where k<sub>unf</sub> is the average intrinsic exchange rate constant, and ΔG<sub>unf</sub> is the free energy of unfolding per peptide chain (ΔG<sub>unf</sub> = ΔG°/3) (Bai et al., 1994; Dürr and Bossard, 1997). Naturally occurring globular proteins typically contain a subset of amide protons with a hydrogen exchange rate that is approximately an order of magnitude slower than that expected if the exchange occurred only from globally unfolded molecules (Roder, 1989; Mayo and Baldwin, 1993; Loh et al., 1993; Bai et al., 1994). An average intrinsic amide exchange rate of 31 s⁻¹ was used for k<sub>f</sub>, corresponding to three times the rate of amide exchange for poly-DL-alanine at pH 7 and 25°C (Englander et al., 1972; Lumb and Kim, 1995).

Results and discussion

Design of the Ile-zipper peptide (IZ)

The amino acid sequence of IZ for a triple stranded coiled coil was determined based on DeGrado’s heptad pattern (O’Neil et al., 1994). An average intrinsic amide exchange rate of 31 s⁻¹ was used for k<sub>f</sub>, corresponding to three times the rate of amide exchange for poly-DL-alanine at pH 7 and 25°C (Englander et al., 1972; Lumb and Kim, 1995).

Circular dichroism (CD)

Circular dichroism spectroscopy was carried out on a JASCO 720 spectrometer at 20°C with a 1-mm path-length cuvette. The peptide concentration was determined by measuring the tyrosine absorbance in 6 M guanidine hydrochloride (GdnHCl) solutions, using ε<sub>222</sub> = 1450 M⁻¹cm⁻¹ (Brandts and Kaplan, 1973). The thermal transition curve was obtained by monitoring the ellipticity at 222 nm ([θ]222) as a function of temperature with a 2-mm path-length cuvette. The temperature was increased at a rate of 1°C/min. The spectra and the thermal transition curve were obtained with 2 μM and 20 μM peptide solutions, respectively, in 10 mM phosphate buffer containing 0.1 M NaCl (pH 7). The GdnHCl denaturation curve, which
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Fig. 1. Helical wheel representation of the heptad pattern of IZ in the parallel orientation, viewed from the N- to the C-terminus. The side chains at the a and d positions are segregated into non-equivalent layers, forming the hydrophobic core. The Glu and Lys residues at the e and g positions undergo electrostatic interactions between adjacent helices. The amino acid sequence of IZ is YGG(IEKKIEA)₄.

Fig. 2. Circular dichroism spectrum of the IZ peptide. The measurement was performed in 10 mM sodium phosphate containing 0.1 M NaCl (pH 7.0) at 20°C. The peptide concentration was 2 µM. which is proceeded by a Tyr-Gly-Gly sequence for the peptide quantitation. The C-terminal was amidated to stabilize the helix formation.

Structural characterization of IZ

The CD spectrum of IZ was characteristic of an α-helical structure, with minima at 222 and 208 nm at pH 7.0 (Figure 2). The ratio of [θ]₂₂₂ and [θ]₂₀₈ was 1.06, indicating that the peptide forms an α-helical coiled coil structure (Graddis et al., 1993; Zhou et al., 1994; Kohn et al., 1995). Using a [θ]₂₂₂ of about 40 000 deg.cm².dmol⁻¹ for 100% helicity, the peptide was found to have an almost completely α-helical structure.

We used sedimentation equilibrium centrifugation to determine the oligomerization state of IZ in aqueous solution (pH 7, 10 mM phosphate, 100 mM NaCl, 25°C). The apparent molecular size of the peptide at 25°C and pH 7 was 10 736 ± 595 daltons (Figure 3), indicating that the peptide was trimerized (the calculated molecular mass for the trimer is 10 627 daltons).

There are two possible helical orientations of the trimerized peptide, parallel and antiparallel (up-up-down). To analyze the orientation and the bundle structure of the coiled coil peptide, a fluorescent probe, acridine, was coupled to the N-terminus of IZ. When the acridine fluorescence was measured, the fluorescence of the acridinyl IZ was quenched to below one-eighth of that of an acridinyl random coiled peptide in aqueous buffer (Figure 4). This result indicates that the three peptide chains of IZ have the parallel orientation, since the fluorescence intensity should be decreased to at least one third if the peptide has an up-up-down orientation.

In the NMR studies of IZ, a Tyr residue of IZ was constrained on the core of the coiled coil, since the NOEs between the aromatic protons and the methyl group at 0.81 p.p.m. (probably the Ile residue at position 4) were observed (data not shown). Only a single set of aromatic protons of the Tyr residue appeared in the NMR spectra (see Figure 6A), which also supports the parallel orientation of the coiled coil structure. Taken together with the results from CD, sedimentation equili-
Fig. 5. (A) GdnHCl denaturation profile of the IZ (20 µM) at 25°C in 10 mM Tris–HCl buffer containing 0.1 M NaCl (pH 7.0). The fraction of unfolded peptide (f_u) was calculated as f_u = (Θ_f − Θ_i)/([Θ_f − Θ_o]), where [Θ] is the observed mean residue ellipticity at 222 nm at any particular denaturant concentration, and [Θ_i] and [Θ_o] are the mean residue ellipticities at 222 nm of the folded and unfolded states, respectively. (B) Linear dependence of ΔG on the GdnHCl concentration (ΔG = ΔG° + m[GdnHCl]). ΔG is the free energy of unfolding at a given concentration of GdnHCl, and was calculated as described in the Materials and methods. The data yields a ΔG° value (25°C; pH 7.0) of 24 kcal/mol (m = 3.6 kcal mol⁻¹ M⁻¹).

Fig. 6. (A) 1H NMR amide hydrogen exchange studies of IZ (25°C, pH 7.0, 1 mM peptide concentration). The spectrum at 5 min shows well dispersed peaks in the amide region. The resonances indicated by asterisk arises from a Tyr residue. (B) Time-course of 1H occupancy of the slowest exchanging amide proton of IZ. The data were fitted for the exponential decay to yield a k_ex of about 3 × 10⁻⁸ (s⁻¹), leading to the observed protection factor (k_prot/k_ex) of about 10⁷. The protection factor predicted from the ΔG° of IZ is 10⁸ (see text), suggesting that the most protected amide proton exchanges through a global unfolding mechanism.

Native-like behaviour of IZ

We analyzed the GdnHCl denaturation of IZ by CD spectroscopy. The [Θ] value at 222 nm was measured as a function of the concentration of GdnHCl at 25°C and pH 7. As shown in Figure 5, the GdnHCl transition displays a cooperative unfolding curve, with a 3.0 M GdnHCl midpoint concentration (m = 3.6 kcal mol⁻¹ M⁻¹). The high m value indicates strong cooperativity, which is suggestive, but not conclusive, evidence for the uniqueness of the structure. For the monomer–trimer equilibrium, the apparent ΔG° determined by GdnHCl denaturation was 24 kcal/mol. The structure was quite stable to thermal denaturation, with a midpoint of more than 80°C.

One important criteria of the native state of a protein is the presence of amide protons that are protected from hydrogen exchange (Betz et al., 1993). The most protected amide protons of the ACID-p1/BASE-p1 heterodimer exchange 10-fold more slowly than expected, if the exchange occurs only from globally unfolded molecules (O’Shea et al., 1993; Lumb and Kim, 1995). This feature is a hallmark of native-like levels of structural uniqueness (Roder, 1989; Mayo and Baldwin, 1993; Loh et al., 1993; Bai et al., 1994). In contrast, designed proteins often exhibit faster amide proton exchange rates, presumably due to poor packing of the side chains or structural fluctuations (DeGrado et al., 1991; Betz et al., 1993; Lumb and Kim, 1995).

We measured the hydrogen exchange rate of IZ, at a 1 mM peptide concentration at pH 7 and 25°C (Figure 6). Under these conditions, the ΔG°m was calculated to be 8 kcal/mol peptide chain, from the ΔG° value obtained by GdnHCl denaturation, and the predicted protection factor is estimated to be 10⁶ (see Materials and methods). For the slowest exchanging amide protons of the peptide, which were detectable even after 10 days, the observed protection factor (k_prot/k_ex) was calculated to be 10⁷. These results indicate that the amide protons exchange through a global unfolding mechanism, and provide strong evidence that the peptide has a well-packed structural uniqueness.

Native proteins have interior side chains with unique conformations, which result in a large degree of signal dispersion in their NMR spectra (Betz et al., 1993). Although the number of peaks in the amide region of IZ is significantly less than the number of residues of IZ (the top of Figure 6A), this observation is well explained by the repeated sequence and the parallel orientation of the peptide. Thus the NMR spectrum of IZ showed well dispersed peaks in the amide region. This result also implies that the interior-facing amino acids of IZ are considered to be well-ordered and native-like.

Molten globules and many designed proteins bind the hydrophobic dye ANS, presumably because these proteins are loosely packed (Goto and Fink, 1989; Semisotnov et al., 1991; Handel et al., 1993). The ANS has a very low fluorescence intensity in water, but fluoresces strongly in the 440–480 nm range when in an apolar environment. When we measured the ANS fluorescence in the mixture of IZ, the ANS did not show any fluorescence, indicating that it did not bind to the IZ trimer. From the NMR and ANS binding studies, it can be concluded that the peptide has native-like properties.

Conclusion

Designed coiled coils with Leu residues at both the a and d positions often exhibit molten globule-like behaviour (Lumb and Kim, 1995; Betz et al., 1995a). One of the major reasons is the wide range of rotameric states available for Leu, which induce poor packing of the side chain in the hydrophobic interface.

In the hydrophobic core, GCN4 has a unique Asn residue, which has been suggested to determine the uniqueness of the coiled coil structure (Lumb and Kim, 1995). Other natural coiled coils have several hydrophilic amino acids in the hydrophobic core (Woolfson and Alber, 1995). One approach toward obtaining a native-like structure is, therefore, the introduction of a hydrophilic residue at the a or d position. Another approach is the incorporation of a metal binding site (Handel and DeGrado, 1990; Handel et al., 1993). For example, the H3zε4 peptide, which was incorporated with two Zn²⁺ binding sites into a molten globule-like parent peptide, showed well dispersed signals in the NMR spectrum (Handel et al., 1993). Both methods can improve the structural uniqueness of designed proteins to some extent.
In the present study, we substituted Leu with Ile to improve the side chain packing. This Ile-zipper peptide, IZ, formed a stable triple stranded parallel coiled coil, as shown by CD, sedimentation equilibrium and quenching of the fluorescent probe. The cooperative GdnHCl-induced denaturation, the well dispersed NMR spectrum, the slow amide hydrogen exchange rate and the lack of ANS binding provide strong evidence that the peptide folds into a well-packed structure that is very similar to the native state of natural proteins. These results suggest that by simply improving the side chain packing of the hydrophobic interface, a high level of native-like structural uniqueness can be imparted to the designed coiled coils.

The triple stranded coiled coil is now being recognized as a structurally and functionally important motif (Schneider et al., 1998). Due to its native-like properties, our triple stranded coiled coil, IZ, will be useful as a scaffold for the functional and structural investigations of natural proteins, as well as for the further de novo design of new functional proteins.

References