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Thermodynamic characterization of an artificially designed amphiphilic α-helical peptide containing periodic prolines: Observations of high thermal stability and cold denaturation

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Abstract

To investigate the structural stability of proteins, we analyzed the thermodynamics of an artificially designed 30-residue peptide. The designed peptide, NH_2-EELLPLAEALAPLLEALLPLAEALAPLLKK-COOH (PERI COIL-1), with prolines at $i + 7$ positions, forms a pentameric α-helical structure in aqueous solution. The thermal denaturation curves of the CD at 222 nm (pH 7.5) show an unusual cold denaturation occurring well above 0 °C and no thermal denaturation is observable under 90 °C. This conformational change is reversible and depends on peptide concentration. A 2-state model between the monomeric denatured state (5D) and the pentameric helical state (H_5) was sufficient to analyze 5 thermal denaturation curves of PERI COIL-1 with concentrations between 23 and 286 μM. The analysis was carried out by a nonlinear least-squares method using 3 fitting parameters: the midpoint temperature, T_m, the enthalpy change, $\Delta H(T_m)$, and the heat capacity change, ΔC_p. The association number ($n = 5$) was determined by sedimentation equilibrium and was not used as a fitting parameter. The heat capacity change suggests that the hydrophobic residues are buried in the helical state and exposed in the denatured one, as it occurs normally for natural globular proteins. On the other hand, the enthalpy and the entropy changes have values close to those found for coiled-coils and are quite distinct from typical values reported for natural globular proteins. In particular, the enthalpy change extrapolated at 110 °C is about 3 kJ/mol per amino acid residue, i.e., half of the value found for globular proteins. Thus, the helices of PERI COIL-1, observed by CD, would be stabilized by entropic effect rather than enthalpic effect. This might be a general feature for de novo designed proteins that lack the rigid tertiary structure, and are mainly stabilized by nonspecific hydrophobic interactions, as well as for some molten globules of natural proteins.

Keywords: circular dichroism; native and molten globule states; protein design; sedimentation equilibrium; thermodynamic analysis

Several attempts of de novo designed proteins have been reported (for reviews, see, e.g., DeGrado et al., 1991; Sander, 1991), but the thermodynamic properties that stabilize the folded state of de novo designed proteins are rarely studied (Handel et al., 1993). Still, a careful thermodynamic analysis and a comparison with the properties of natural proteins may help our understanding of protein folding.

The leucine zipper motif provides hydrophobic interactions between helices in several transcription factors of the bZIP class (Landschulz et al., 1988; O'Shea et al., 1989a, 1989b; Vinson et al., 1989), as well as in tropomyosin (Holtzer et al., 1990; Lehrer & Stafford, 1991) and α-keratin. The X-ray crystal structure of the leucine zipper motif in GCN4 has been recently reported (O'Shea et al., 1991), and because of its apparent simplicity, the motif seems well suited for the study of interhelical interactions. Starting from this motif, the hydrophobic interactions between helices can be idealized and even modified to produce 3- or 4-stranded coiled-coils (e.g., Lau et al., 1984; Harbury et al., 1993). The PERI COIL-1 peptide was designed

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Abbreviations: Fmoc, 9-fluorenylmethoxy-carbonyl; HMP, 4-(hydroxymethyl) phenoxymethyl; PERI COIL-1, NH_2-EELLPLAEALAPLLEALLPLAEALAPLLKK-COOH; TFA, trifluoroacetic acid.

to fold into an amphiphilic helix with leucines on one side and glutamic acids on the opposite side and was expected to reproduce an interaction pattern similar to the leucine zipper. However, in addition to the leucine zipper motif, 4 prolines were placed periodically in the sequence to bend the helices. Proline is known as a helix-breaker, but it can produce a bend when located in the middle of an α-helix (Barlow & Thornton, 1988). Thus, the design of PERI COIL-1 is unique in combining the leucine zipper motif with prolines used as helical bends (Kitakuni et al., 1992).

In the present work, we investigated the thermal unfolding of PERI COIL-1, which shows 2 unexpected properties: (1) an uncommonly high thermal stability observed in aqueous solution, and (2) cold denaturation above 0 °C, that has been observed only in the presence of denaturant (Pace & Tanford, 1968; Chen & Schellman, 1989) or at low pH (Privalov et al., 1986; Griko et al., 1988; Tamura et al., 1991) in natural proteins. The thermodynamic parameters were analyzed, comparing with those of natural globular and coiled-coil proteins.

Results

Degree of association

We measured the degree of association of PERI COIL-1 at 20 °C for 5 samples with concentrations between 320 μM and 1.9 mM by sedimentation equilibrium (Fig. 1). The linear relationship between ln c and r^2 shows that the apparent molecular weight (M_{app}) calculated using Equation 1 (see Materials and methods) is independent of the concentration (Fig. 1; Table 1). This indicates that the weight-averaged molecular weight (M_w) is equal to the apparent molecular weight (M_{app}) over the concentration range examined. At 20 °C, the average M_w is 15,690, which is 5 times the value based on the amino acid composition of the monomer (M_w = 3,160), and indicates a pentameric state.

Table 1. *Determination of the sedimentation coefficients and the apparent molecular weights*

Conc (% and mM)[a]	$s_{20,w}$ (S)	M_{app} (20 °C)
1.0 (3.16)	1.13	
0.8 (2.53)	1.11	
0.6 (1.90)	1.13	15,510 ± 330
0.4 (1.27)	1.10	14,690 ± 80
0.3 (0.95)		16,200 ± 210
0.2 (0.63)		16,340 ± 180
0.1 (0.32)		15,720 ± 610
Average	1.12 ± 0.02	15,690 ± 270

[a] The values in parentheses indicate the concentration in mM. Sedimentation velocity and equilibrium analyses were performed at 20 °C in 50 mM citrate-phosphate buffer (pH 7.0).

Sedimentation velocity experiments showed a single peak. The apparent sedimentation constants (s_{app}) at 20 °C were estimated from the changes in peak position using Equation 2 (Materials and methods) and corrected for 20 °C in pure water ($s_{20,w}$). The averaged value ($s^0_{20,w}$) at infinite dilution is about 1.1 S, and values are identical for concentrations ranging from 1.27 to 3.16 mM (Table 1).

Thermal unfolding and analysis of the denaturation curves

The CD spectra at 40 °C and 70 °C, with a peptide concentration of 25.0 μM, show double minima at 206 and 222 nm, which is characteristic of a significant helical content (Fig. 2). At 0 °C, the CD spectrum is typical of a random-coil conformation, and

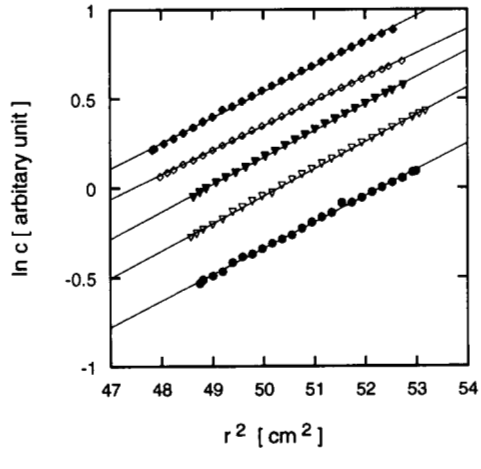

Fig. 1. Molecular weight determination of PERI COIL-1 from sedimentation analysis. Plot of the natural logarithm of the solute concentration (c) against the square of the distance (r) from the axis of rotation. The solution was sedimented in 50 mM citrate-phosphate buffer (pH 7.0) for 30 h at 20 °C. Initial concentrations were 0.1% (0.32 mM, ●), 0.2% (0.63 mM, ▽), 0.3% (0.95 mM, ▼), 0.4% (1.27 mM, ◇), and 0.6% (1.9 mM, ◆). Solid lines represent linear least-squares analyses of the values.

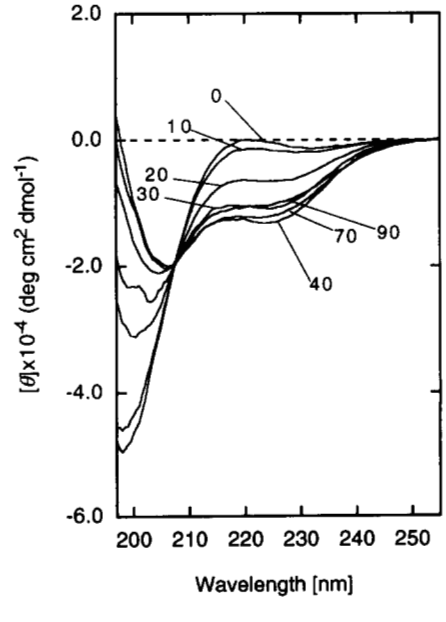

Fig. 2. CD spectra of PERI COIL-1 at different temperatures in 5 mM citrate-phosphate buffer, pH 7.5. The temperatures are indicated in °C. The peptide concentration is 25.0 μM.

an isodichroic point is observed at 207.5 nm (Fig. 2). The spectrum at 0.7 μM indicated a random coil conformation at any temperature.

The thermal unfolding of PERI COIL-1, monitored by the change in $[\theta]_{222}$, shows a reversible conformational transition between the coil and helical states. The minimum value for $[\theta]_{222}$ is about −15,000 (at ~40 °C for concentrations between 188.7 μM and 286.3 μM in Fig. 3A), which corresponds to a helical content of about 40%, assuming −40,000 and 0 deg cm^2 dmol^{-1} for 100 and 0% helicity, respectively. In the concentration range studied by CD, the helical conformation is retained even at 90 °C, whereas the peptide is unfolded below 10 °C. The midpoint of this cold denaturation phenomenon depends on the peptide concentration, as expected for a multimeric system (Fig. 3A). The denaturation curves were normalized before the fitting procedure, using the baselines represented in Figure 3A. The lower baseline, which represents the temperature dependence of $[\theta]$ of the helical state, was found by linearly fitting the denaturation curve of the solution containing 286.3 μM of peptide (Fig. 3A). The upper baseline, which represents the temperature dependence of the denatured state, was obtained by fitting the 0.7 μM data with a third power polynomial. The fitting of the 5 normalized curves for peptide concentrations between 23.7 and 286.3 μM was performed by a nonlinear least-squares method, using 3 fitting parameters (ΔH_m, T_m, and ΔC_p, Equation 7, Materials and methods). The association number was well determined by sedimentation equilibrium, so that we fixed n to 5. The 2-state model might be too simple, but it describes well the thermal unfolding curves of PERI COIL-1 at all temperatures (Fig. 3B). The isodichroic point at 207.5 nm (Fig. 2) further substantiates the assumption that a 2-state model should be essentially sufficient to analyze the data. The results are summarized in Table 2 and the fitting curves are shown in Figure 3B. Finally, the experimental data are slightly less well fitted at high temperatures (Fig. 3B); this might be a consequence of using a temperature independent heat capacity change.

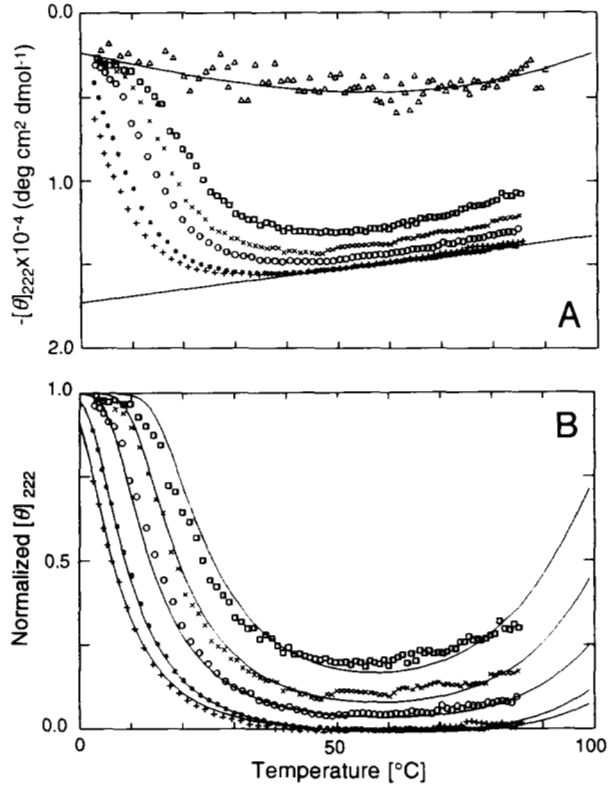

Fig. 3. A: Temperature dependence of the ellipticity at 222 nm and at pH 7.5. The peptide concentrations are 0.7 μM (△), 23.7 μM (□), 46.9 μM (×), 95.1 μM (○), 188.7 μM (*), and 286.3 μM (+). The temperature dependence of the peptide at 0.7 μM concentration does not show any transition. We assume, therefore, that at this concentration the peptide is in the denatured state over the entire temperature range. We also measured the temperature dependence in the presence of 3.0 M GdnHCl at the same pH, and it showed a good agreement with the temperature dependence curve at 0.7 μM. The baseline for the denatured state ($[\theta]_D = -2{,}375.1 - 68.8T + 0.197T^2 + 4.98 \times 10^{-3}T^3$, T in °C) was obtained by fitting the 0.7 μM data with a third-degree polynomial over the entire temperature range. When we fitted the 0.7 μM data between 0 °C and 55 °C with a linear baseline, we obtained a slope of −46, in good agreement with the value proposed for the random coil state of monomeric peptides ($[\theta]_D = -45T + 640$; Scholtz et al., 1991b). The baselines for the helical state ($[\theta]_N = 40.8T - 17{,}505.0$) was determined by fitting the 286.3 μM data between 50 °C and 90 °C with a linear function. The percentage change in $[\theta]_{222}$ per °C of the helical state at 25 °C is 0.24% and coincides well with the values reported for α-tropomyosin (about 0.3%; Holtzer & Holtzer, 1992). **B:** Temperature dependence of the normalized ellipticity at 222 nm using the baselines shown in A. The fitting curves are calculated with the parameters indicated in Table 2.

Discussion

Although PERI COIL-1 has been designed with the leucine zipper motif, it shows an unusual high temperature stability and also a cold denaturation above 0 °C. Thus, the thermodynamic parameters associated with PERI COIL-1 are interesting to understand the stability of natural proteins. For this purpose, we examined the contribution of the enthalpy change and the entropy change to the Gibbs free energy (Fig. 4A), which is largely positive at high temperature.

At 110 °C, the specific enthalpy and entropy changes for a variety of globular proteins extrapolate to respective common values, which are considered to reflect the chain enthalpy and entropy (Privalov, 1979). For PERI COIL-1, the specific enthalpy change at 110 °C, assuming a constant heat capacity difference, is 2.9 kJ/mol per residue (Table 2; Fig. 4B). This value is small compared to the average value of 6.25 kJ/mol per residue found for globular proteins (Privalov & Gill, 1988). It is also at the lower limit of the enthalpy changes observed for coiled-coils, which are between 3 and 4.5 kJ/mol per residue (Privalov, 1982; Thompson et al., 1993). A possible interpretation is that PERI COIL-1 has less hydrogen bonds and van der Walls contacts than globular proteins (Privalov & Gill, 1988). The contribution of the intra- and intermolecular interactions to the

Table 2. *Thermodynamic parameters*[a]

T_m (K)	ΔH_m (kJ/mol)	ΔC_p (kJ/mol/K)	Δ
246.3	−719.9	8.66	4.8×10^{-4}

[a] Fitting results of the thermal denaturation curves of PERI COIL-1 with peptide concentrations ranging from 23.7 μM to 286.3 μM. The degree of association, n, was determined by sedimentation equilibrium, and fixed to 5 for the fitting calculations. The RMS deviation, Δ, is calculated as $\Delta^2 = \sum(\theta_{calc}[T_i] - \theta_{exp}[T_i])^2/N$, where $\theta_{calc}[T_i]$ and $\theta_{exp}[T_i]$ are, respectively, the normalized calculated and experimental data points at temperature T_i. N is the number of experimental points. Δ has no unit because the thermal denaturation curves are first normalized to 0–1.

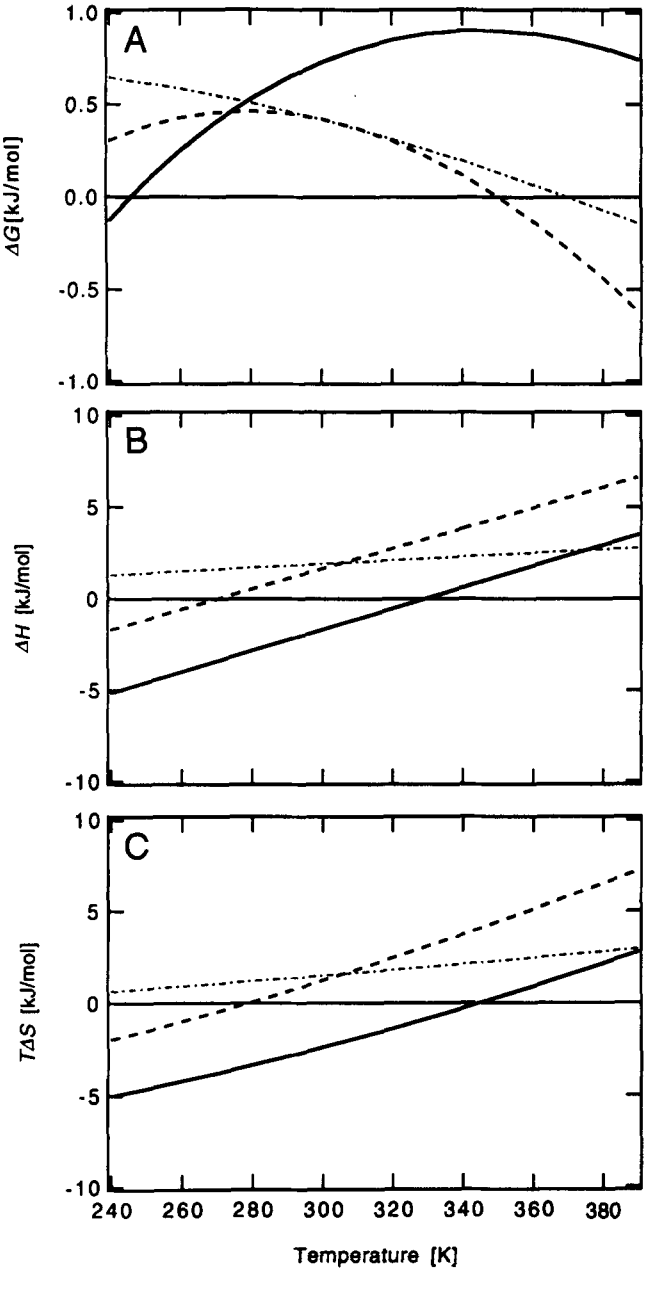

Fig. 4. Plot of the Gibbs energy change, ΔG (**A**), the enthalpy change, ΔH (**B**), and the entropy change, $T\Delta S$ (**C**), of PERI COIL-1 (——) versus the temperature. The values are calculated per mole of amino acid residues, using the parameters listed in Table 2 (with $C_0 = (1/n)^{1/(n-1)}$). For comparison, the Gibbs energy, the enthalpy, and the entropy changes of cytochrome *c* (– – –) are computed with ΔH (0 K) = −1,565.88 kJ/mol, T_m = 77 °C (350 K), and ΔC_p = 5.76 kJ/mol/K (Kuroda et al., 1992). -·-·- Represent the corresponding curves for the coiled-coil region of GCN4 ($\Delta H(T_m)$ = 145 kJ/mol, T_m = 96.9 °C [370 K], and ΔC_p = 0.56 kJ/mol/K; Thompson et al., 1993).

enthalpy change can be estimated if we assume 4.2 kJ/mol per residue (and $\Delta C_p \approx 0$) for the enthalpy change at 110 °C accompanying the formation of a single helix (Ooi & Oobatake, 1991; Scholtz et al., 1991a). Because the helical content of PERI COIL-1 is 40%, the enthalpy change for the formation of 1 helix will be 4.2 × 0.40 = 1.7 kJ/mol per residue. The enthalpy change due to the intermolecular interaction among the helices will then be 2.9 − 1.7 = 1.2 kJ/mol per residue. Thus, the intramolecular interactions accompanying the formation of helices contribute as much as the intermolecular interaction among the helices to the enthalpy change. A similar situation occurs in coiled-coils, where the intermolecular contribution to the enthalpy change is small, if not a negative (Privalov, 1982; Thompson et al., 1993).

On the other hand, the heat capacity difference of PERI COIL-1 (Table 2) is significantly larger than values found for coiled-coils (Privalov, 1982; Thompson et al., 1993) and it is close to that of globular proteins. For a globular protein with 150 residues, the heat capacity difference is between 8 and 9 kJ/mol (Pace et al., 1989; Equation 32 of Oobatake & Ooi, 1993). The heat capacity difference is attributed to the burial of hydrophobic and aromatic residues in the folded state. Thus, the large heat capacity of PERI COIL-1 is compatible with a model where the leucine residues are essentially buried in the folded pentameric state and exposed in the denatured one, because for a linear α-helix no residue can be buried.

The stability of PERI COIL-1 at high temperature was unexpected. Because the enthalpy change is smaller than in globular proteins and also at the lower limit of coiled-coil proteins, the stability of PERI COIL-1 should arise from the entropic effect, that is, from a decrease in entropy change. Assuming that the ionization effect is small at pH 7, one can see that the entropy change is smaller than for globular proteins (Fig. 4C). Two possibilities may be accounted for the small entropy change at 110 °C. First, PERI COIL-1 contains many more prolines than a natural protein (4 over 30 residues). This restrains the conformational space of the denatured state and decreases the entropy of the denatured state. Secondly, the NMR spectrum is unusually broad, even for a protein with a molecular weight of 15,000 Da, and the chemical shifts are poorly dispersed (data in H_2O at 40 °C; not shown). This suggests that the helices are in a molten globule-like state with a large number of conformations, and this could increase the entropy of the helical state. The first explanation relates to the entropy term within each helix; the second refers to the entropy among and within helices. Still, the decrease in the entropy change at 110 °C is not fully clear because coiled-coils also have a small entropy change, even though they have few prolines and should adopt a well-defined native structure. For natural proteins, cold denaturation occurs usually at subzero temperatures. Two possible reasons for the observations of cold denaturation above 0 °C for PERI COIL-1 are as follows. First, the present transition is a monomer-pentamer transition, and the observed transition temperature ($T_{1/2}$) increases when the association number (n) increases, as mentioned in the Materials and methods section (Fig. 5). A second reason is the result of the small enthalpy change combined with a large heat capacity difference. This would increase the cold denaturation temperature ($T_{cold} = T_m - \Delta H/\Delta C_p$; Privalov et al., 1986). In other words, the destabilizing hydration effect overrides the chain contribution, which is lower than for natural proteins, at a temperature higher than that for natural proteins. For GCN4 (Thompson et al., 1993) and other coiled-coils, cold denaturation does not occur above 0 °C even though the enthalpy change at 110 °C is small, because the hydration effect (ΔC_p) is also small.

In summary, the peptides form pentamers rather than dimers as designed (Kitakuni et al., 1992). The large heat capacity difference suggests that the attempt to stabilize the helices through

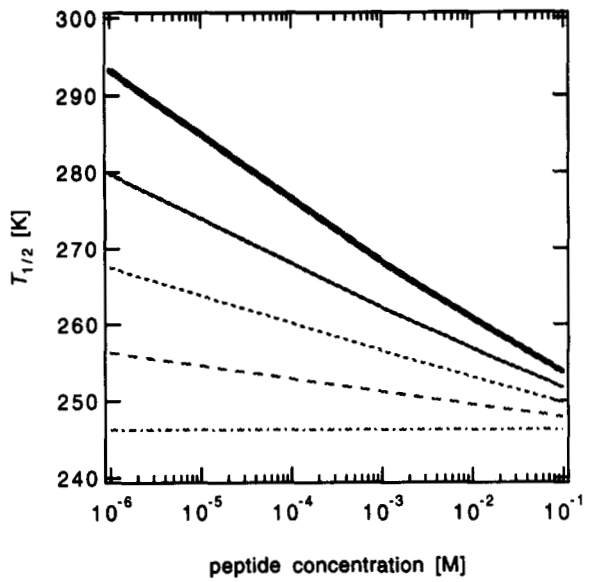

Fig. 5. Plot of the observed transition temperature $T_{1/2}$ versus peptide concentration for different association numbers n. $T_{1/2}$ was computed using Equation 9 with the parameters listed in Table 2 (——). The influence of n is examined by fixing it to 1 (-·-·-), 2 (— — —), 3 (-----), and 4 (——) and keeping the same values for the other parameters. A slight discrepancy between the calculated $T_{1/2}$ and the values observed in Figure 3 arise from neglecting ΔC_p in Equation 9. The error arising from setting ΔC_p to 0 is significant only at peptide concentrations lower than about 30 μM.

hydrophobic interactions with other helices (Kitakuni et al., 1992) was successful. However, it is reasonable to assume that the folded pentameric state is not one with a well-defined overall conformation, but an ensemble of helical structures interacting with each other in a nonspecific manner. Thus, the folded state of PERI COIL-1 should be considered as a molten globule state rather than a native one, with the folded state being stabilized by entropic effect instead of enthalpic effect. This situation may be similar to that of most de novo designed proteins and to some molten globule states of natural proteins that have a low stabilization enthalpy. Small enthalpy and entropy changes are also observed in coiled-coils, but in opposition to coiled-coils, PERI COIL-1 has a large heat capacity difference, comparable to those observed in globular proteins. Both the thermal stability and the cold denaturation at high temperature result from the combination of the small enthalpy and entropy changes with the large heat capacity. The comparison of the thermodynamic parameters of PERI COIL-1 with those of globular proteins and coiled-coils may suggest that the tight packing observed in natural proteins produces not only a large enthalpy change, but also a possible correlation between the heat capacity and the enthalpy change values.

Materials and methods

Peptide synthesis

PERI COIL-1 was synthesized by the stepwise solid-phase method with an Applied Biosystems peptide synthesizer, model 430A. The solid support was HMP-resin (0.94 mmol of amino groups/g). The N^{α}-protecting group was Fmoc, and the side-chain protecting groups were *t*-butyl for Glu and *t*-butyloxycarbonyl for Lys. The peptidyl resin was cleaved with TFA and H_2O at room temperature. After 1 h, cold ether was added and the precipitate was collected by suction. It was then dissolved in AcOH-H_2O (1:4) and the crude peptide was purified by preparative reverse-phase HPLC on an Aquapore Prep-10 column (C-8, 300-Å pore size; Applied Biosystems) with 0.1% TFA in a gradient of acetonitrile in water, at a flow rate of 3.5 mL/min. The homogeneity of the purified peptide was determined by analytical reverse-phase HPLC using a micropore octyl-silica column 9C-8 (300-Å pore size; Applied Biosystems). The column was eluted with a linear gradient of acetonitrile in water, containing 0.1% TFA, at a flow of 1.0 mL/min. The amino acid sequence was confirmed by automated Edman degradation on an Applied Biosystems model 477A sequencer.

Molecular weights

Sedimentation velocity and sedimentation equilibrium experiments were performed in an analytical ultracentrifuge (Beckman Spinco model E) equipped with Schlieren and interference optical systems. Double-sector cells with a pathlength of 12 mm were used at 21,740 rpm in an AnG rotor for the sedimentation equilibrium analysis, and at 48,000 rpm in an AnD rotor for the sedimentation velocity analysis. The effective running time for the sedimentation velocity experiment was 5 h and 20 min and the bar angle was 60°. For the sedimentation equilibrium experiments, it took 30 h to attain equilibration. The initial concentrations were determined from the synthetic interfacial pattern using a capillary-type double-sector cell.

For a regular solution, the apparent molecular weight (M_{app}) is a function of the solute concentration (c) and of the radial distance from the rotation center (r) and is expressed as (Williams, 1972)

$$M_{app} = \frac{2RT\, d \ln c}{(1 - \bar{\nu}\rho)\omega^2\, dr^2}, \qquad (1)$$

where $\bar{\nu}$ is the partial specific volume of the solute at infinite dilution and is estimated to be 0.79 from the amino acid composition using the additivity rule and specific values for each amino acid residue (Zamyatnin, 1972). ρ is the solvent density, ω is the angular velocity (21,740 rpm), R is the gas constant, and T is the absolute temperature. Five samples of 320 μM to 1.9 mM (0.1–0.6%, w/v) were used for the low-speed sedimentation equilibrium experiments at 20 °C in 50 mM citrate-phosphate buffer (pH 7.0).

Sedimentation coefficients (s_{app}) are calculated from log r vs. t (time) plots of the experimental Schlieren patterns, using the following equation

$$s_{app} = \frac{2.303\, d \log r}{60\omega^2\, dt}, \qquad (2)$$

where ω is the angular velocity. s_{app} was corrected according to Svedberg and Pedersen (1940) to yield the value at 20 °C in water. Four samples of 1.27–3.16 mM (0.4–1.0%, w/v) were used for the sedimentation velocity analysis at 20 °C in 50 mM citrate-phosphate buffer (pH 7.0).

CD measurements

CD spectra were recorded on a J-600 spectropolarimeter (Jasco). Thermal unfolding processes were followed by measuring $[\theta]_{222}$ as a function of the sample solution temperature, with a 2- or 5-mm-pathlength cuvette. The temperature was measured with a thermistor inserted directly in the solution. The heating rate was about 0.6 °C/min. The peptide concentration ranged from 0.7 to 286.3 μM in 5 mM citrate-phosphate buffer (pH 7.5). The peptide concentration used to calculate the mean residue ellipticity ($[\theta]$, deg cm^2/dmol) was determined by quantitative amino acid analysis of the stock solution. The data were stored on a floppy disk and analyzed on a VAX-8810 computer.

Thermodynamic analysis

The thermal denaturation curves of PERI COIL-1 were analyzed assuming the following standard model:

$$N_n \leftrightarrow nD, \tag{3}$$

where N_n represents the n-mer helical (native) state, D is the monomer coil (denatured) state, and n (=5) is the association number determined by sedimentation equilibrium experiments.

The equilibrium constant K is written as:

$$K = [D]^n/[N_n], \tag{4}$$

where the brackets refer to the concentration. Using the normalized CD value θ and the total monomer concentration C_0, the concentrations of the 2 states are given by the following equations:

$$[D] = (1 - \theta)C_0 \tag{5}$$

$$[N_n] = \theta C_0/n. \tag{6}$$

With these values, K is written as:

$$K = \frac{(1-\theta)^n}{\theta} nC_0^{n-1} = \exp\left(-\frac{\Delta H_m}{R}(1/T - 1/T_m) - \frac{\Delta C_p}{RT} \times \left((T - T_m) + T\ln\frac{T_m}{T}\right)\right) \tag{7}$$

where ΔC_p is the heat capacity change, ΔH_m and ΔS_m are the enthalpy and entropy differences at T_m, respectively, and T_m ($= \Delta H_m/\Delta S_m$) is the temperature at which $K = 1$. The sign of ΔH_m is defined as the transition from helix to coil. $T_{1/2}$, the temperature at which half of the (monomer) peptides are in the denatured state ($\theta = 1/2$) is related to T_m by

$$\ln\frac{(1-\theta)^n}{\theta} = -\frac{\Delta H_m}{R}(1/T - 1/T_m) - \ln(nC_0^{n-1}) \tag{8}$$

$$\frac{1}{T_{1/2}} = \frac{1}{T_m} - \frac{R}{\Delta H_m}\ln\left(\frac{n}{2^{n-1}}C_0^{n-1}\right). \tag{9}$$

Equations 8 and 9 are approximations with ΔC_p set to zero. Equation 9 shows that $T_{1/2}$ depends on both the concentration and the association number. For a peptide concentration of about 1 mM, Equation 9 implies that the observed transition temperature ($T_{1/2}$) of cold denaturation is shifted to a temperature higher than T_m, because of the negative enthalpy change. Similarly, as the enthalpy change of the (normal) thermal denaturation is positive, the observed denaturation temperature will be shifted to a temperature lower than T_m.

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References

Barlow DJ, Thornton JM. 1988. Helix geometry in proteins. *J Mol Biol 201*:601-619.

Chen B, Schellman JA. 1989. Low-temperature unfolding of phage T4 lysozyme: Equilibrium studies. *Biochemistry 28*:685-691.

DeGrado WF, Raleigh DF, Handel T. 1991. De novo protein design: What are we learning? *Curr Opin Struct Biol 1*:984-993.

Griko YV, Privalov PL, Sturtevant JM, Venyaminov SY. 1988. Cold denaturation of staphylococcal nuclease. *Proc Natl Acad Sci USA 85*:3343-3347.

Handel TM, Williams SA, DeGrado WF. 1993. Metal ion-dependent modulation of the dynamics of a designed protein. *Science 261*:879-885.

Harbury PB, Zhang T, Kim PS, Alber T. 1993. A switch between two-, three- and four-stranded coiled-coils in GCN4 leucine zipper mutants. *Science 262*:1401-1406.

Holtzer A, Holtzer ME, Skolnick J. 1990. Does the unfolding transition of two-chain, coiled-coil proteins involve a continuum of intermediates? In: Gierasch LE, King J, eds. *Protein folding. Deciphering the second half of the genetic code*. Washington, D.C.: AAAS. pp 177-190.

Holtzer ME, Holtzer A. 1992. α-Helix to random coil transitions: Interpretation of the CD in the region of linear temperature dependence. *Biopolymers 32*:1589-1591.

Kitakuni E, Horiuchi T, Oda Y, Oobatake M, Nakamura H, Tanaka T. 1992. Design and synthesis of an α-helical peptide containing periodic proline residues. *FEBS Lett 298*:233-236.

Kuroda Y, Kidokoro S, Wada A. 1992. Thermodynamic characterization of cytochrome *c* at low pH. Observation of the molten globule state and of the cold denaturation process. *J Mol Biol 223*:1139-1153.

Landschulz WH, Johnson PF, McKnight SL. 1988. The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. *Science 240*:1759-1764.

Lau SYM, Taneja AK, Hodges RS. 1984. Synthesis of a model protein of defined secondary and quaternary structure. *J Biol Chem 259*:13253-13261.

Lehrer SS, Stafford WF III. 1991. Preferential assembly of the tropomyosin heterodimer: Equilibrium studies. *Biochemistry 30*:5682-5688.

Oobatake M, Ooi T. 1993. Hydration and heat stability effects on protein unfolding. *Progr Biophys Mol Biol 59*:237-284.

Ooi T, Oobatake M. 1991. Prediction of the thermodynamics of protein unfolding: The helix-coil transition of poly(L-alanine). *Proc Natl Acad Sci USA 88*:2859-2863.

O'Shea EK, Klemm JD, Kim PS, Alber T. 1991. X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled-coil. *Science 254*:539-544.

O'Shea EK, Rutkowski R, Kim PS. 1989a. Evidence that the leucine zipper is a coiled-coil. *Science 243*:538-542.

O'Shea EK, Rutkowski R, Stafford WF III, Kim PS. 1989b. Preferential heterodimer formation by isolated leucine zippers from Fos and Jun. *Science 245*:646-648.

Pace NC, Shirley BA, Thomson JA. 1989. Measuring the conformational stability of a protein. In: Creighton TE, ed. *Protein structure, a practical approach*. Oxford, UK: IRL Press. pp 311-330.

Pace NC, Tanford C. 1968. Thermodynamics of the unfolding of β-lactoglobulin A in aqueous urea solutions between 5 and 55°. *Biochemistry 7*:198-208.

Privalov PL. 1979. Stability of proteins: Small globular proteins. *Adv Protein Chem 33*:1-104.

Privalov PL. 1982. Stability of proteins which do not present a single cooperative system. *Adv Protein Chem 35*:168–241.

Privalov PL, Gill SJ. 1988. Stability of protein structure and hydrophobic interaction. *Adv Protein Chem 39*:191–234.

Privalov PL, Griko YV, Venyaminov SY. 1986. Cold denaturation of myoglobin. *J Mol Biol 190*:487–498.

Sander C. 1991. De novo design of proteins. *Curr Opin Struct Biol 1*:630–637.

Scholtz JM, Marqusee S, Baldwin RL, York EJ, Stewart JM, Santoro M, Bolen DW. 1991a. Calorimetric determination of the enthalpy change for the α-helix to coil transition of an alanine peptide in water. *Proc Natl Acad Sci USA 88*:2854–2858.

Scholtz JM, Qian H, York EJ, Stewart JM, Baldwin RL. 1991b. Parameters of helix–coil transition theory for alanine-based peptides of varying chain lengths in water. *Biopolymers 31*:1463–1470.

Svedberg T, Pedersen KO. 1940. *The ultracentrifuge*. London/New York: Oxford University Press.

Tamura A, Kimura K, Takahara H, Akasaka K. 1991. Cold denaturation and heat denaturation of streptomyces subtilisin inhibitor: 1. CD and DSC studies. *Biochemistry 30*:11307–11313.

Thompson KS, Vinson CR, Freire E. 1993. Thermodynamic characterization of the structural stability of the coiled-coil region of the bZIP transcription factor GCN4. *Biochemistry 32*:5491–5496.

Vinson CR, Sigler PB, McKnight SL. 1989. Scissors-grip model for DNA recognition by a family of leucine zipper proteins. *Science 240*:911–916.

Williams JW. 1972. *Ultracentrifugation of macromolecules*. New York: Academic Press.

Zamyatnin AA. 1972. Protein volume in solution. *Progr Biophys Mol Biol 24*:107–123.