Folding of a de Novo Designed Native-like Four-helix Bundle Protein*

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The folding of a model native-like dimeric four-helix bundle protein, (α2)2, was investigated using guanidine hydrochloride, hydrostatic pressure, and low temperature. Unfolding by guanidine hydrochloride followed by circular dichroism and intrinsic fluorescence spectroscopy revealed a highly cooperative transition between the native-like and unfolded states, with free energy of unfolding determined from CD data, \( \Delta G_{\text{unf}} = 14.3 \pm 0.8 \) kcal/mol. However, CD and intrinsic fluorescence data were not superimposable, indicating the presence of an intermediate state during the folding transition. To stabilize the folding intermediate, we used hydrostatic pressure and low temperature. In both cases, dissociation of the dimeric native-like (α2)2 into folded monomers (α2) was observed. van't Hoff analysis of the low temperature experiments, assuming a two-state dimer 171-monomer transition, yielded a free energy of dissociation of (α2)2 of \( \Delta G_{\text{diss}} = 11.4 \pm 0.4 \) kcal/mol, in good agreement with the free energy determined from pressure dissociation experiments (\( \Delta G_{\text{diss}} = 10.5 \pm 0.1 \) kcal/mol). Binding of the hydrophobic fluorescent probe 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS) to the pressure- and cold-dissociated states of (α2)2 indicated the existence of molten-globule monomers. In conclusion, we demonstrate that the folding pathway of (α2)2 can be described by a three-state transition including a monomeric molten globule-like state.

Understanding the transition of a polypeptide from a large ensemble of conformations to a well ordered three-dimensional structure is of fundamental importance to many areas of biochemistry and molecular biology. Of particular interest are the energetics governing the interplay of forces during the folding of proteins. One approach to address this issue is the design of proteins. Novel proteins made up of non-native amino acid sequences are intended to adopt predetermined folds and provide detailed information on the underlying thermodynamics. A common target for protein design has been the folding mechanism for the protein. Studies of binding of bis-ANS revealed that the pressure- or cold-dissociated state (α2, folded monomers) has characteristics of a molten globule. We propose that the folding process initially results in such species, which subsequently assemble to the native-like dimeric state.

EXPERIMENTAL PROCEDURES

Chemicals—All reagents were of the highest analytical grade available. Distilled water was filtered and deionized through a Millipore water purification system. Bis-ANS was from Molecular Probes (Eugene, OR).

Protein—The design and synthesis of (α2)2 has been described previously (8, 12, 13). Each monomer consists of a 62-amino acid residue peptide composed of two 27-residue α-helices and a glycine linker region of eight residues. The amino acid sequence of an individual chain of (α2)2 is given in Table I in single-letter codes; the heptad repeat assignments, a through g, used to design amphiphilic α-helices are shown above the first 8 amino acid residues.

The end charges are neutralized to stabilize helix formation; the amino terminus is acetylated (Ac) and the carboxyl terminus is amidated. The single tryptophan residue (Trp-15) is placed in a largely solvent-inaccessible pocket (8), which is consistent with its fluorescence properties (peak maximum at about 327 nm).

Fluorescence Measurements—Unless otherwise indicated, fluorescence emission spectra were measured at 25 °C on an ISS PC-1 spectrofluorimeter (ISS Inc., Champaign, IL). For intrinsic fluorescence cleavage in T4 lysozyme (5) and electron transfer in cytochrome b562 (6).

The design of four-helix bundle proteins is, in most attempts, based on amphiphilic secondary structure, leading to partitioning of nonpolar and polar amino acids into the interior and surface of the protein, respectively (7). Such a designed four-helix bundle protein, (α2)2, has recently been described and used as a template for anesthetic binding studies (8).

The last decade has witnessed an increasing number of studies using hydrostatic pressure (9), low temperature (10), or a combination of both to investigate the energetics and kinetics of folding and subunit interactions of various single or multidomain proteins. These studies have provided detailed characterization of thermodynamic parameters and provided a platform for the understanding of structure-energy relationships. Very recently, we have reported the use of hydrostatic pressure and low temperature in the characterization of partially folded intermediates of a monomeric de novo designed three-helix bundle protein (α2-1) (11). In the present work, we describe the effects of guanidine hydrochloride, pressure, and low temperature on the subunit association/folding of (α2)2 and determine the apparent thermodynamic parameters. The independent use of different perturbants (pressure and temperature) caused reversible dissociation of (α2)2 and allowed us to put forward a folding mechanism for the protein. Studies of binding of bis-ANS revealed that the pressure- or cold-dissociated state (α2, folded monomers) has characteristics of a molten globule. We propose that the folding process initially results in such species, which subsequently assemble to the native-like dimeric state.
measurements, excitation was at 280 nm, and emission spectra were recorded from 300 to 420 nm. Bio-ANS fluorescence was measured with excitation at 375 nm and emission from 420 to 600 nm. Paladini and Weber (14) equipped with sapphire optical windows performed fluorescence measurements under pressure using a pressure cell similar to that originally described. The temperature of the pressure cell was controlled by means of a jacket connected to a circulating bath and was monitored by a telethermometer. All experiments are representative of at least three experiments performed with different preparations of (α2)2, and were carried out in 20 mM Tris-HCl, pH 7.4, containing 130 mM NaCl. Protein concentration was determined using ε290 = 5,700 M⁻¹ cm⁻¹. Unless otherwise indicated, protein concentration was 1 μM (expressed in dimers), and bio-ANS concentration, when used, was 1 μM. Sample buffers were saturated with nitrogen prior to the experiments. In low temperature experiments, the sapphire windows of the pressure cell were flushed with nitrogen to prevent water condensation.

Fluorescence spectral centers of mass (intensity-weighted average emission wavelengths, λ_{cen}) were calculated with software provided by ISS Inc. as

$$\lambda_{cen} = \frac{\sum I(\lambda) \Delta\Sigma(\lambda)}{\sum I(\lambda) \Delta\Sigma(\lambda)} \quad (\text{Eq. 1})$$

where λ is the emission wavelength and I(λ) represents the fluorescence intensity at wavelength λ. Shifts in spectral center of mass were converted into extent of dissociation (α) at each pressure according to the relationship,

$$\alpha = \frac{1}{Q(1 - \lambda_1/\lambda_2 - \lambda_3)} \quad (\text{Eq. 2})$$

where λ2 and λ1 are the spectral centers of mass of native-like and monomeric protein, respectively, λ3 is the spectral center of mass at pressure p, and Q is the ratio of the spectral areas of dissociated and native-like (α2)2.

Circular Dichroism—CD measurements were carried out on a Jasco J-715 spectropolarimeter connected to a circulating bath, using an 0.2-cm path-length quartz cell.

Gel Filtration—Gel filtration experiments were carried out using a G2000SW TosoHaas column (7.5 mm inner diameter × 30 cm, 10 μm particle size). The flow rate was 0.5 ml/min with eluting buffer pressure of 100 p.s.i. Absorption detection (320 nm) was employed.

RESULTS

Pressure Dissociation of (α2)2—Fig. 1 shows the pressure-induced red shift of the intrinsic fluorescence spectral center of mass of (α2)2 at different concentrations (1 μM (●) and 8 μM (○)). As expected for the dissociation of a dimer, the curve at the higher protein concentration is displaced to higher pressure relative to the curve at the lower protein concentration. The pressure-induced transition exhibited a clearly defined plateau at about 346 nm for both protein concentrations. It is important to note that fully unfolded (α2)2 (i.e. in the presence of 6 M GdnHCl) exhibited much more red-shifted fluorescence emission with a spectral center of mass at 355 nm (Fig. 2). Upon stepwise release of pressure, the fluorescence spectra underwent a blue shift and reached complete recovery of the spectral center of mass at atmospheric pressure (data not shown). These results indicate that pressure induces reversible dissociation of (α2)2 to partially folded dimers.

The pressure dissociation data for (α2)2 were analyzed using a thermodynamic model for dimer dissociation. The equilibrium dissociation constant at atmospheric pressure (K0) and the molar volume change of dissociation (ΔV_{diss}) can be calculated from the following thermodynamic relationship,

$$K_p = K_0 \exp \left( -\Delta V_{diss} / R T \right) \quad (\text{Eq. 3})$$

where K_p is the dissociation constant at pressure p, and R and T have their usual meanings. The equation can be rewritten by introducing the degree of dissociation, α, at pressure p,

$$\ln(\alpha^2/(1-\alpha)) = \ln(K_0/4C_0) - p(\Delta V_{diss}/R T) \quad (\text{Eq. 4})$$

where ln(α^2/(1−α)) equals ln(K_0/4C_0) for the dissociation of a dimer. Thus, a plot of ln(α^2/(1−α)) versus pressure (Fig. 1, lower panel) yields the molar volume change of dissociation (ΔV_{diss}) from the slope and lnK_0 from the intercept on the ordinate. The parameters obtained for pressure dissociation of (α2)2 are summarized in Table II.

As noted above, at higher protein concentration (8 μM) the midpoint of the transition was displaced toward higher pressure relative to the curve obtained with 1 μM protein. For the dissociation of a dimer, the molar volume change of dissociation (ΔV_{diss}) can be used to calculate the predicted displacement of the pressure dissociation curve (Δp_{50}) with protein concentration.

$$\Delta p = \left( RT / \Delta V_{diss} \right) \ln(C_0/C_1) \quad (\text{Eq. 5})$$

where C_2 and C_1 are the two concentrations of (α2)2 used (15). The Δp_{50} value of 760 bar thus calculated is in very good agreement with the experimentally determined Δp_{50} of 640 bar (Fig. 1, arrow), further indicating that the pressure transition reflects the dissociation of the dimeric (α2)2 helical bundle protein into monomers.

Denaturation of (α2)2 by GdnHCl—The equilibrium unfolding of (α2)2 induced by increasing concentrations of GdnHCl was monitored by intrinsic fluorescence and CD spectroscopy (Fig. 2). The unfolding was accompanied by a marked red shift of the fluorescence emission (from 337 to 355 nm in the spectral center of mass), indicating a transition of the single tryptophan residue (Trp-15) from a hydrophobic environment in the interior of the protein to the polar aqueous medium. For comparison, Fig. 2 also shows data on the unfolding of (α2)2 monitored by far-UV CD measurements (222 nm). Both curves indicate highly cooperative unfolding transitions. However, the midpoints of the curves are clearly different, i.e., −2.4 and 3.5 M.
when the transition was monitored by fluorescence and CD measurements, respectively. This type of behavior is characteristic of the existence of folding intermediates (i.e., a non-two state transition (16)) and indicates that changes in the tertiary structure of \((a_2)_2\) take place at lower concentrations of GdnHCl than changes in secondary structure. Of interest is the comparison with the pressure dissociation data shown in Fig. 1. The pressure dissociation curves (Fig. 1) level off at about 346 nm, which corresponds to 50% of the total fluorescence shift observed upon complete unfolding of \((a_2)_2\) at high concentrations of GdnHCl. Furthermore, GdnHCl-induced changes in the CD of \((a_2)_2\) only begin to take place at GdnHCl concentrations above 2.5 M when the unfolding of \((a_2)_2\) monitored by intrinsic fluorescence has already reached about 50% \((\alpha = 0.5)\) (Fig. 2). Taken together, these observations suggest that low concentrations of GdnHCl \((<2.5 \text{ M})\) promote dissociation of \((a_2)_2\), resulting in a shift in spectral center of mass to \(-346 \text{ nm},\) followed by unfolding of the dissociated subunits at \(>2.5 \text{ M}\) GdnHCl (with a further fluorescence red shift to \(-355 \text{ nm}\) and loss of the CD signal).

**Cold Dissociation of \((a_2)_2\)**—To further characterize the existence of a folding intermediate of \((a_2)_2\), we carried out low temperature unfolding experiments followed by fluorescence and CD spectroscopy. Fig. 3 shows the fluorescence spectral centers of mass (○) and ellipticity \([\theta]_{222}(\text{deg}^{-2}\text{cm}^2\text{dmol}^{-1})\) of \((a_2)_2\) as a function of decreasing temperature. Although the fluorescence curve reveals a red shift in the spectral center of mass, the CD data did not change with temperature. Therefore, it can be concluded that low temperature only disturbs the tertiary structure of \((a_2)_2\) while the helices remain intact. The fluorescence curve reaches a plateau at about \(1 \text{ °C}\), indicating a stable partially folded intermediate. After return of the sample to room temperature, the fluorescence spectral center of mass returned to the original value, reflecting the reversibility of the process (Δ). Interestingly, the fluorescence spectra of the state stabilized at low temperature and the pressure-dissociated protein (Fig. 1) level off at about the same spectral center of mass (\(-346 \text{ nm};\) Fig. 4), suggesting that a similar, dissociated state of \((a_2)_2\) is populated at low temperatures or at high pressure.

At constant pressure the temperature dependence of the equilibrium constant is described by the van’t Hoff equation,

\[
\Delta G/T = (1/T)\Delta H - \Delta S = R\ln K_T
\]

(Eq. 6)
where \( K_r \) is the equilibrium constant for dissociation at temperature \( T \) and \( \Delta G \) is the Gibbs free energy change of association. From a plot of \( \Delta G/T \) versus the inverse temperature, the changes in enthalpy (\( \Delta H \)) and entropy (\( \Delta S \)) of dissociation can be extracted (Fig. 3, lower panel). The thermodynamic parameters obtained from such analysis are summarized in Table II.

**Bis-ANS Binding Studies**—The environment-sensitive fluorescent dye bis-ANS was used to characterize the partially folded, monomeric state of \((\alpha_2)_2\). Bis-ANS binds to hydrophobic domains of proteins (17). Upon non-covalent binding to such domains, the two naphthyl rings of the bis-ANS molecule become oriented in parallel, which brings about a significant increase in fluorescence quantum yield. Therefore, it is a sensitive probe to detect so called “molten globule” states of proteins, in which secondary structural elements are established, but the packing of the side chains in the hydrophobic core is not yet complete, giving rise to a fluctuating globular structure (18). Fig. 5 shows increased binding of bis-ANS to the dissociated state of \((\alpha_2)_2\) (trace c) compared with the native-like dimeric state (trace b), whereas the completely denatured protein (i.e. in the presence of 6 M GdnHCl, trace a) showed no binding. The increase in quantum yield of bis-ANS fluorescence upon binding to pressure-dissociated or cold-dissociated \((\alpha_2)_2\) is about the same (2.3-fold for pressure dissociation and 2.2-fold for cold dissociation; Fig. 5). In addition, the intrinsic fluorescence emission undergoes, in both cases, an identical red shift, for cold dissociation; Fig. 5). In addition, the intrinsic fluorescence emission undergoes, in both cases, an identical red shift, reaching a plateau at about 346 nm (Fig. 4, curves 2 and 3). Furthermore, the Gibbs free energy of dissociation determined from both experiments is in very good agreement (Table II). Taken together, these results indicate that hydrostatic pressure and low temperature stabilize a similar, but not necessarily the same dissociated state of \((\alpha_2)_2\).

**Gel Filtration Experiments**—To examine the monomeric state of \((\alpha_2)_2\) during unfolding induced by GdnHCl, we used size exclusion chromatography at different GdnHCl concentrations. In the absence of GdnHCl, \((\alpha_2)_2\) eluted with a retention time of 16.8 min, corresponding to a molecular mass of about 18,000 Da as compared with globular protein standards (Fig. 6A). The slightly higher determined molecular mass of \((\alpha_2)_2\) can be explained by its molecular shape, which is most likely cylinder-like rather than globular (8). In the presence of 2.5 M GdnHCl, the major peak is characterized by a retention time of 21.1 min, corresponding to a molecular mass of 6500 Da, in the expected mass range of the monomeric dissociated state of \((\alpha_2)_2\) (Fig. 6B). In addition, the chromatogram shows a second peak at 15.4 min, which most likely represents the fully unfolded hydrated monomers with an expanded Stokes radius. This result indicates that, at 2.5 M GdnHCl, the main peak corresponding to \(\alpha_2\) monomers is in equilibrium with a smaller fraction of fully unfolded monomers. In essence, gel filtration experiments at different GdnHCl concentrations strongly support the view that \((\alpha_2)_2\) unfolds via a monomeric intermediate state, which has also been stabilized by hydrostatic pressure and low temperature.

**DISCUSSION**

In this work, we describe the use of hydrostatic pressure and low temperature to stabilize the dissociated molten globule-like state of \((\alpha_2)_2\) (Fig. 6B). In addition, the chromatogram shows a second peak at 15.4 min, which most likely represents the fully unfolded hydrated monomers with an expanded Stokes radius. This result indicates that, at 2.5 M GdnHCl, the main peak corresponding to \(\alpha_2\) monomers is in equilibrium with a smaller fraction of fully unfolded monomers. In essence, gel filtration experiments at different GdnHCl concentrations strongly support the view that \((\alpha_2)_2\) unfolds via a monomeric intermediate state, which has also been stabilized by hydrostatic pressure and low temperature.
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state of a de novo designed four-helix bundle protein, \((a_2)_2\). These studies allowed us to suggest a folding mechanism for the protein. The foundation of our thermodynamic analysis lies in the observation that the dissociation curves are completely reversible, showing no hysteresis using either pressure or temperature as a perturbant of the equilibrium of subunit association of \((a_2)_2\).

Pressure dissociation enabled the determination of the molar volume change and the free energy change of subunit dissociation of \((a_2)_2\). The volume changes upon pressure dissociation of a variety of dimeric proteins have been reported. Of note is that the specific volume change, obtained by normalizing the molar volume change to the molecular weight of the dimer, is significant increases in bis-ANS binding (Fig. 5). Very similar results have been reported for Arc repressor. Pressure dissociation studies of that small dimer monitored by tryptophan fluorescence and \(^1\)H-NMR revealed the stabilization of monomers. Based on bis-ANS binding and fluorescence anisotropy measurements, the authors concluded that the pressure-dissociated monomers are molten globules (23, 24). Interestingly, the native state of Arc repressor binds a certain amount of bis-ANS similar to the native-like state of \((a_2)_2\). The folding mechanism of the DNA-binding protein HU (19.5 kDa), structurally related to the Arc repressor, has been investigated using electrospray ionization mass spectrometry (25); these authors concluded that folding of the intertwined dimer proceeds via a monomeric intermediate.

Based on the pressure and low temperature studies presented here, the folding of \((a_2)_2\) can be summarized as depicted in Fig. 7. Interestingly, \((a_2)_2\) gains most of its conformational stability from the association of the two monomers \((a_2)\). Studies of the dissociation and unfolding of dimeric triose-phosphate isomerase also showed that the protein is stabilized mainly by the association of the monomers (26), pointing to the importance of subunit interactions for the folding stability of oligomeric proteins.

De novo design and mutagenesis studies have proved useful in furthering our understanding of the features responsible for a four-helix bundle fold. For example, Raleigh and DeGrado (27) reported a de novo designed protein \((a_2C)\) that featured most of the characteristics of a native protein, including temperature-induced transition between a native-like and a molten globule-like state as well as a well dispersed \(^1\)H-NMR up-field spectrum. Recent Monte Carlo simulations of three de novo designed helical bundle proteins, based exclusively on glycine, glutamic acid, lysine, and leucine residues, showed that the conformations obtained at low temperature have molten globule-like features (28).

Cold denaturation of proteins has been studied extensively over the last decade (see Refs. 10, 19, and 20 and references therein). The fact that proteins unfold at a low temperature has been attributed to the solvation of hydrophobic side chains, for which the Gibbs free energy is negative and increases in magnitude as the temperature is lowered. Thus, the nonpolar groups of the polypeptide chain, mainly responsible for the conformational stability, become exposed to the solvent at low temperatures and cause the protein to denature. Subunit dissociation at low temperatures has been reported for a variety of dimers such as yeast hexokinase (21) and phosphorylase A (22). In these studies, the enthalpy change upon association \((\Delta H_m)\) was found to be positive, which would thereby promote the dissociation into monomers. However, the entropy term \((T\Delta S_m)\) was also positive but larger than the enthalpic contribution, resulting in an overall negative free energy of association \((\Delta G_m)\). A similar situation was found for \((a_2)_2\) (Table II). The entropy term compensates the unfavorable enthalpy by about 10 kcal/mol at room temperature and is responsible for the stability of the helical bundle. Because changes in entropy during the folding process are attributed to changes in hydrophobic interactions, the dimerization of \((a_2)_2\) can thus be interpreted in terms of a hydrophobic collapse of nonpolar side chains.

The hydrophobic probe bis-ANS has been frequently used to detect molten globule folding intermediates. The conformational changes of \((a_2)_2\) upon pressure and temperature perturbation reveal the existence of a molten globule-like state, with significant increases in bis-ANS binding (Fig. 5). Very similar results have been reported for Arc repressor. Pressure dissociation studies of that small dimer monitored by tryptophan fluorescence and \(^1\)H-NMR revealed the stabilization of monomers. Based on bis-ANS binding and fluorescence anisotropy measurements, the authors concluded that the pressure-dissociated monomers are molten globules (23, 24). Interestingly, the native state of Arc repressor binds a certain amount of bis-ANS similar to the native-like state of \((a_2)_2\). The folding mechanism of the DNA-binding protein HU (19.5 kDa), structurally related to the Arc repressor, has been investigated using electrospray ionization mass spectrometry (25); these authors concluded that folding of the intertwined dimer proceeds via a monomeric intermediate.

In conclusion, we show that the de novo designed four-helix bundle protein \((a_2)_2\) has many characteristics of native proteins, including cooperative pressure and temperature transitions between a native-like and a molten globule-dissociated state. The design of novel proteins based on essentially non-native sequences thus appears as a versatile tool to improve our understanding of the forces and interactions that stabilize simple but biologically important folding motifs such as the four-helix bundle.
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