The stability and equilibrium unfolding of a model three-helix bundle protein, a3-1, by guanidine hydrochloride (GdnHCl), hydrostatic pressure, and temperature have been investigated. The combined use of these denaturing agents allowed detection of two partially folded states of a3-1, as monitored by circular dichroism, intrinsic fluorescence emission, and fluorescence of the hydrophobic probe bis-ANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid). The overall free-energy change for complete unfolding of a3-1, determined from GdnHCl unfolding data, is +4.6 kcal/mol. The native state is stabilized by −1.4 kcal/mol relative to a partially folded pressure-denatured intermediate (I1). Cold denaturation at high pressure gives rise to a second partially (un)folded conformation (I2), suggesting a significant contribution of hydrophobic interactions to the stability of a3-1. The free energy of stabilization of the native-like state relative to I2 is evaluated to be −2.5 kcal/mol. Bis-ANS binding to the pressure- and cold-denatured states indicates the existence of significant residual hydrophobic structure in the partially (un)folded states of a3-1. The demonstration of folding intermediates of a3-1 lends experimental support to a number of recent protein folding simulation studies of other three-helix bundle proteins that predicted the existence of such intermediates. The results are discussed in terms of the significance of de novo designed proteins for protein folding studies.

Understanding the mechanisms by which a polypeptide adopts a stable and functional three-dimensional structure still represents a challenging problem (1). The folding of small proteins usually takes place on timescales close to a millisecond or less, and is believed to occur in a highly cooperative fashion without the presence of populated folding intermediates (2–8). However, recent simulation studies have suggested the existence of intermediate states during the folding of a small model three-helix bundle protein (9–12). Three-helix bundles represent a simple folding motif found in a variety of soluble and membrane proteins, including spectrin (13) and the extramembranous portion of Staphylococcus aureus protein A (14). Using sequence patterns discovered in coiled coils, the synthesis of amphipophilic α-helices that self-assemble into three- or four-helix bundles stabilized by a hydrophobic core has been successfully achieved (15–17). The de novo design of proteins represents a versatile tool to gain insight into the interplay of forces resulting in conformational stability. Artificial proteins are generally less complex than their native counterparts but at the same time retain the features responsible for the folding process.

Recently, Johansson and co-workers (18) reported the synthesis and initial characterization of a native-like three-helix bundle protein, designated a3-1. The three different helices of this 65-amino acid polypeptide are joined by (glycine) linkers. NMR solution studies revealed a well structured conformation with α-helical secondary structure. GdnHCl-induced unfolding of a3-1 followed by CD revealed a Gibbs free-energy of unfolding of +4.6 kcal/mol (18), comparable to that observed for small monomeric natural proteins of similar size, such as myoglobin (7.6 kcal/mol) (19) or the 43-amino acid residue peripheral subunit-binding domain of the pyruvate dehydrogenase complex (3.1 kcal/mol) (20).

In the last two decades, hydrostatic pressure has been extensively used as a reversible thermodynamic variable to characterize subunit association in oligomeric proteins (21, 22). In general, unfolding of monomeric proteins requires significantly higher pressures (i.e., 5–7 kilobars (kbar)) than those required for subunit dissociation of oligomers (typically up to 3.5 kbar) (21). Few examples to date demonstrate denaturation of monomeric proteins at pressures below 3 kbar. In the present study, we have used a combination of hydrostatic pressure (up to 3.5 kbar) and low temperatures to investigate the folding stability of a3-1. Interestingly, our results revealed the existence of partially (un)folded intermediate states of a3-1, giving support to the predictions from the above mentioned simulation studies. Bis-ANS binding studies revealed the existence of significant residual hydrophobic structure in the pressure-denatured and especially in the cold-denatured state of a3-1, suggesting molten globule-like conformations for these intermediates.

**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).

**Peptide**—The design, synthesis, and purification of a3-1 have been previously described (18). The amino acid sequence of a3-1 is given below in single-letter codes. The sequence is arranged in helixes, with the coiled-coil heptad positions labeled a through g (Table I). The N terminus of the peptide is acetylated, and the C terminus is amidated.

**Fluorescence Measurements**—Unless otherwise indicated, fluoro-
Quantum yields of unfolded and native-like a shows the degree of denaturation (inset) equilibrium unfolding of a in the presence of GdnHCl, the transition between native and folding profiles revealed by fluorescence and CD suggests that, to the unfolded state was observed in both fluorescence and CD been included. A single, cooperative transition from the native condensation.

The fluorescence spectral centers of mass (temperature-weighted average emission wavelengths, ) were calculated with software provided by ISS Inc. as follows,

where is the emission wavelength and represents the fluorescence intensity at wavelength Shifts in the spectral center of mass were converted into extent of denaturation at each pressure according to the following phenomenological relationship (24):

where and are the spectral centers of mass of native-like and fully unfolded protein obtained in the absence of denaturant and in the presence of a high concentration of GdnHCl, respectively, is the spectral center of mass at pressure and is the ratio of fluorescence quantum yields of unfolded and native-like a.

Results

Unfolding of a by GdnHCl and Hydrostatic Pressure—The equilibrium unfolding of a by GdnHCl was initially investigated. Control measurements showed that unfolding was very rapid and was complete within a few minutes after addition of GdnHCl. Samples were incubated at increasing GdnHCl concentrations for 2 h at room temperature, and intrinsic fluorescence emission spectra were recorded. Unfolding was accompanied by a significant red shift of the fluorescence emission of a-1 (Fig. 1), indicating increased exposure of the single tryptophan residue (Trp-32) to the aqueous medium. Fig. 1 (inset) shows the degree of denaturation (a) of a-1 as a function of GdnHCl concentration. For comparison, data on the unfolding of a-1 monitored by far-UV CD measurements (18) have also been included. A single, cooperative transition from the native to the unfolded state was observed in both fluorescence and CD measurements. The almost exact superimposition of the unfolding profiles revealed by fluorescence and CD suggests that, in the presence of GdnHCl, the transition between native and unfolded a-1, atmospheric pressure, and room temperature, is essentially a two-state transition with no evidence for the existence of populated folding intermediates. Fig. 1 also shows that unfolding of a-1 takes place between 1 and 3 M GdnHCl, with a transition mid-point at 2.4 M GdnHCl.

Fig. 2 shows the effect of pressure on the fluorescence spectral center of mass of a-1 in the absence or in the presence of GdnHCl. In the absence of GdnHCl (triangles) the center of mass did not reach a plateau even at the highest pressure used (3.5 kbar), indicating that complete unfolding was not achieved by the pressurization of a-1. From the equilibrium GdnHCl unfolding experiments (Fig. 1) it is apparent that GdnHCl concentrations up to 1 M are subdenaturing for a-1. Pressure unfolding experiments were then repeated in the presence of different subdenaturing concentrations of GdnHCl (0.4 M and 1 M, Fig. 2) to poise the system toward unfolding. Although addition of 0.4 M GdnHCl had little effect on the pressure sensitivity of a-1, pressure denaturation in the presence of 1 M GdnHCl exhibited a clearly defined plateau of the spectral centers of mass at about 342 nm (Fig. 2). It is important to note that fully unfolded a-1 (i.e., in the presence of 6 M GdnHCl) exhibited a very red-shifted fluorescence emission, with a spectral center of mass of 355 nm. Thus, the plateau observed for the spectral center of mass of the pressure-denatured state at 342 nm seems to correspond to a stable partially unfolded intermediate. 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), indicating reversible refolding of a-1 to a state qualitatively similar to the native-like protein. The fluorescence changes thus indicate that application of pressure in the presence of a subdenaturing concentration of GdnHCl (1 M) induced a transition to a stable conformation different from the fully denatured state induced by 6 M GdnHCl.

The pressure unfolding data for a-1 were analyzed using a two-state model for monomer unfolding. The dimensionless equilibrium denaturation constant at atmospheric pressure (Kp) and the molar volume change of folding (ΔV) can be calculated from the following thermodynamic relation,

where is the denaturation constant at pressure and and have their usual meanings. The equation can be rewritten by introducing the degree of unfolding, at pressure p:

where equals lnKp for the denaturation of a monomer. Thus, a plot of ln(1 - ) versus pressure (Fig. 2, lower panel) yields the molar volume change of folding (ΔV) from the slope and lnKp from the intercept on the ordinate. The parameters obtained for pressure unfolding of a-1 are shown in Table II.

Cold Denaturation of a-1—To further characterize the existence of folding intermediates of a-1, we carried out low temperature unfolding experiments under pressure. The freezing point of water is significantly decreased under pressure (25), allowing aqueous samples to be analyzed at sub-zero temperatures without the need for addition of cryosolvent additives. Fig. 3 shows the fluorescence spectral centers of mass of a-1 as a function of decreasing temperature at 3.5 kbar in the absence and in the presence of 1.0 M GdnHCl. The starting points of the two curves (at 25 °C) are similar to the spectral centers of mass obtained in the pressure denaturation experiments at the corresponding GdnHCl concentrations. In the absence of GdnHCl circles the spectra became progressively red-shifted but did not reach a plateau at low temperatures (down to -12 °C), indicating that a stable partially unfolded intermediate had not been reached. By contrast, in the presence of 1.0 M GdnHCl, a further red shift of the fluorescence emission occurred, with a low temperature plateau observed at about -10 °C. Interestingly, the fluorescence spectra of the cold-denatured state and the completely unfolded protein (i.e.,
in 6 M GdnHCl) differ by about 8 nm in spectral center of mass (Fig. 4). 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 (Fig. 3, open symbols).

At constant pressure the temperature dependence of the equilibrium constant for a two-state unfolding transition is described by the van’t Hoff equation,

\[ \Delta G / T = (1 / T) \Delta H - \Delta S = -R \ln K_T \]  
(Eq. 5)

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

**DISCUSSION**

Structural transitions of a single-chain 65-amino acid three-helix bundle polypeptide, \( \alpha_3-1 \), induced by hydrostatic pressure and by a combination of low temperature and high pressure revealed the existence of partially folded intermediate states, which are not observable in GdnHCl unfolding experiments of this model protein. Bis-ANS binding studies support the idea that organized hydrophobic surfaces persist, or can form, at both high pressures and low temperatures. Taken into account the “new view” of protein (un)folding, which models the chain collapse of a polypeptide by a multiple pathways “funnel,” our results suggest that one possible unfolding transition of \( \alpha_3-1 \) can be summarized by the following scheme,

\[ N \rightarrow I_1 \rightarrow I_2 \rightarrow U \]

**Scheme 1**

where \( N \) is the native-like and \( U \) is the unfolded state, and \( I_1 \) and \( I_2 \) represent the two partially (un)folded intermediates revealed in high pressure and low temperature experiments, respectively (Fig. 6).

Pressure-induced changes in the intrinsic fluorescence emission spectrum of \( \alpha_3-1 \) took place between atmospheric pressure and 2.5 kbar in the presence of a subdenaturing concentration (1 M) of GdnHCl. The shift in spectral center of mass from 335 to 342 nm indicates partial exposure of the previously solvent-shielded tryptophan at the central heptad a position of helix II to the aqueous environment. The single-chain polypeptide nature of \( \alpha_3-1 \) renders this native-like three-helix bundle of particular interest for pressure unfolding studies. It is generally assumed that pressures below 5 kbar do not significantly disturb the secondary or tertiary structures of proteins (27). Hydrogen bonds, the stabilizing elements of helices and \( \beta \)-sheets, are permanent dipoles and relatively insensitive to pressure.
changes. Moreover, the volume change attendant on replacement of protein-protein hydrogen bonds by protein-water hydrogen bonds is rather small. Therefore, pressure-induced unfolding of small monomeric proteins is generally only observed at high temperature (28), at low pH (29, 30), or with mutant proteins (31, 32). The finding that $\alpha_{\text{R}}-1$ can be unfolded by pressure in the presence of a subdenaturing concentration of GdnHCl opens interesting possibilities for further studies of the stability of helical bundles and, in particular, of the structure of the pressure-stabilized partially folded state.

It is interesting to compare the volume change measured for the unfolding of $\alpha_{\text{R}}-1$ with the volume changes reported for pressure denaturation of other proteins. The specific volume changes observed upon dissociation of dimeric proteins are dependent on the molecular weight. Arc repressor ($M_r 13,000$), for example, shows a specific volume change of $-7.7 \mu l/g$ (33), whereas Enolase ($M_r 80,000$) is reported to have a change in volume of $-0.7 \mu l/g$ (23). This can be explained by a larger proportion of buried amino acid residues, which becomes exposed to the solvent upon dissociation in smaller dimers, because in these cases the subunit interfaces involve a larger fraction of the entire structure. Alternatively, volume changes can also be interpreted in terms of the balance of forces responsible for protein stability. Disruption of electrostatic interactions leads to a large decrease in volume caused by electrostriction of water around the unpaired charged residues (34). By contrast, breaking of hydrophobic interactions is accompanied by much smaller volume changes. The denaturation of monomeric proteins is accompanied by similar effects, resulting in stronger hydration and the replacement of longer dispersion bonds by shorter dipolar interactions. Therefore, the relatively large specific volume change of $2.3 \mu l/g$ observed for the folding transition of the intermediate $I_3$ to the native-like state N of $\alpha_{\text{R}}-1$ (Table II) occurs most likely with the burial of polar side-chain groups.

Cold denaturation experiments at high pressures take advantage of the depression of the freezing point of water (25). Such an experimental setup and the presence of a subdenaturing concentration of GdnHCl allowed characterization of another folding intermediate, which showed strong bis-ANS binding. Destabilization of proteins at low temperatures indicates a

![Table II](image)

**Table II**

| GdnHCl denaturation | Pressure-denatured state ($I_3$) | Cold-denatured state ($I_1$) |
|---------------------|---------------------------------|-----------------------------|
| $\Delta G_{\text{unf}} = 4.6 \pm 0.3 \text{kcal/mol}$ | $\Delta V = 17 \text{ml/mol = 2.3 \mu l/g}$ | $\Delta H_{\text{unf}} = -7.1 \text{kcal/mol}$ |
| $\ln K = 2.3$ | $\Delta S_{\text{unf}} = -8.2 \text{kcal/mol}$ | $\Delta G_{\text{unf}} = 1.1 \pm 0.2 \text{kcal/mol, relative to } I_1$ |
| $\Delta G_{\text{unf}} = 1.4 \pm 0.1 \text{kcal/mol}$ | $\Delta G_{\text{unf}} = 2.5 \text{kcal/mol, relative to the native-like state}$ |

*a* Data from Ref. 18.

*b* Calculated assuming a two-state transition from native to the pressure-denatured state, using the relation $\Delta G = -RT \ln K$, $T = 298 \text{K}$.

*c* Calculated assuming a two-state transition from pressure to cold-denatured states, as described in the text.

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**FIG. 3.** Upper panel, cold denaturation of $\alpha_{\text{R}}-1$ under pressure (3.5 kbar) in the absence (●) or in the presence of 1 M GdnHCl (▲). During cooling, samples were allowed to equilibrate for 20 min at each temperature prior to the acquisition of the emission spectra. Open symbols correspond to data acquired with increasing temperature, and show the reversibility of the process. Lower panel, van’t Hoff plot for the cold denaturation of $\alpha_{\text{R}}-1$ at 3.5 kbar and 1 M GdnHCl. The degree of unfolding ($\alpha$) at each temperature was calculated using the values for spectral centers of mass of the native and the fully unfolded states of $\alpha_{\text{R}}-1$. Gibbs free-energy changes of unfolding were calculated from the relationship: $\Delta G_{\text{unf}} = -RT \ln (\alpha/(1-\alpha))$, where $R$ is the gas constant and $T$ is the absolute temperature. The errors are smaller than the symbols used.

**FIG. 4.** Fluorescence emission spectra of native (1), pressure-denatured (2), cold-denatured (3), and fully unfolded $\alpha_{\text{R}}-1$ in the presence 7 M GdnHCl (4). Pressure unfolding was obtained by incubation with 1 M GdnHCl and increasing the pressure to 3.5 kbar and 25°C (see Fig. 1). Cold denaturation was obtained by applying pressure up to 3.5 kbar in the presence of 1 M GdnHCl and decreasing the temperature to −10°C (see Fig. 2). Spectra are normalized for maximal emission intensity.

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**Folding of a Model Three-Helix Bundle Protein**
significant contribution of hydrophobic interactions to the folding process. Studies of the small dimeric protein Arc repressor showed that folding and association are accompanied by the displacement of solvent molecules, suggesting the burial of previously solvent-exposed nonpolar side chains (35). According to Privalov (36), hydration of polar residues decreases the entropy of the folding process. On the other hand, Weber (37) described the entropy-driven condensation of proteins as a consequence of the conversion of stronger solvent-protein interactions into weaker (entropy-rich) protein-protein interactions (London dispersion forces). In several cases, protein-protein interactions involved in folding and subunit association have indeed been found to be predominantly entropy-driven. For example, the subunit association of hexokinase is characterized by a strong entropic contribution (\(T\Delta S = +38 \text{ kcal/mol}\)), which outweighs the unfavorable enthalpy of +17 kcal/mol (38). The folding of \(\alpha_3\)-1 reveals an entropy-driven transition from \(I_2\) to \(I_1\), with \(T\Delta S = +8.2 \text{ kcal/mol}\) at 25 °C and a van’t Hoff enthalpy of \(\Delta H = +7.1 \text{ kcal/mol}\), resulting in \(-1.1 \text{ kcal/mol}\) of conformational stability (\(\Delta G\)). The entropy-driven nature of the \(I_2 \rightarrow I_1\) transition suggests that a hydrophobic collapse may be involved at this stage of folding of \(\alpha_3\)-1.

The design of \(\alpha_3\)-1 contains six distinct hydrophobic core layers, each consisting of three amino acids of either two \(a\) and one \(d\) or one \(a\) and two \(d\) heptad positions of the corresponding helices \(I\), II, and III. Very likely, these areas are involved in formation of the organized hydrophobic domains revealed by bis-ANS binding at low temperatures. Of note is that the changes in intrinsic fluorescence emission of \(\alpha_3\)-1 induced by pressure and low temperature were fully reversible, indicating that the protein refolds to a state that is qualitatively similar to the native-like state upon return to atmospheric conditions.

The folding of small (<100 amino acid residues), single-domain proteins is assumed to occur in a concerted fashion well accounted for by a two-state transition without well populated intermediates (39, 40). On the other hand, there is strong evidence, especially from hydrogen exchange experiments, that partially folded conformations can be present (41, 42). For a number of proteins, residual structure has been detected and linked to partially folded states, which are believed to be important as nucleation sites for condensation (43, 44). For example, small patterns of stable residual structure were found in barnase during acid denaturation and were assumed to be formed during the early stage of folding (45). In addition, urea denaturation of the chaperonin GroEL also revealed persistent hydrophobic surfaces at high urea concentrations as probed by bis-ANS binding (46). Low temperature unfolding studies of \(\beta\)-lactamase provided evidence for the existence of two equilibrium intermediates between native and unfolded states (47).

Small synthetic helical proteins that undergo metal-directed transitions from molten globule-like to native-like states via folding intermediates have also been reported (48, 49). Thermodynamic calculations of a model three-helix bundle using either a simple off-lattice or an all-atom approach revealed the existence of a metastable minimum (9–11). In addition, Zhou and Karplus (12) have very recently used the same model protein to calculate different folding trajectories (12). The phase diagram could be varied by changing a single parameter related to the relative stability of native and non-native contacts. The simulation revealed that the folding mechanism for helical proteins changes from a cooperative (diffusion-collision) transition to one that involves on-pathway intermediates depending on the difference between the strength of native and non-native interactions (12). Our results on \(\alpha_3\)-1 give direct support to the idea that even small helical bundle proteins may indeed present metastable folding intermediates, which can be detected under appropriate experimental conditions designed to stabilize them.

In conclusion, the present results, together with the small size of this three-helix bundle protein, make \(\alpha_3\)-1 an ideally suited system for detailed protein folding studies. In this regard, an interesting possibility could be the use of high pressure NMR studies to characterize the structure of the folding intermediates.

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