Protein Folding in the Absence of Chemical Denaturants

REVERSIBLE PRESSURE DENATURATION OF THE NONCOVALENT COMPLEX FORMED BY THE ASSOCIATION OF TWO PROTEIN FRAGMENTS*

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Small monomeric proteins are the best models for studying protein folding, but they are often too stable for denaturation using pressure as the sole perturbant. In the present work we subject [CI-2(1–40)·(41–64)], a noncovalent complex formed by the association of two complementary fragments of the chymotrypsin inhibitor-2, to high pressure to investigate the folding mechanism of a model protein. Pressures up to 3.5 kilobar do not affect the intact protein, but it can be unfolded reversibly by pressure in the presence of subdenaturing concentrations of guanidine chloride, with free energy and molar volume changes of 2.5 kcal mol⁻¹ and 42.5 ml mol⁻¹, respectively. In contrast, the complex can be reversibly denatured by high pressure without the addition of chemical denaturants. However, the pressure is clearly independent of the protein concentration, indicating lack of dissociation. We determined a change in the free energy of 1.4 kcal mol⁻¹ and a molar volume change of 35 ml mol⁻¹ for the pressure denaturation of the complex. A persistent quenching of the tryptophan adds further evidence for the presence of residual structure in the high pressure-denatured state. This state also appears to be compact as the small volume change indicates, compared with pressure denaturation of naturally occurring dimers. Based on observations of a number of pressure-denatured states and on characteristics of large CI-2 fragments with a solvent accessible core but maintaining tertiary interactions, the structure of the pressure-denatured state of the CI-2 complex could be explained by an ordered molten globule-like conformation.

The analysis of the fundamental processes that govern protein folding relies on the perturbation of the protein-solvent system to attain a denatured polypeptide. For this purpose, modifications in the solution are introduced such as temperature, pH, and particularly, chemical denaturants. The latter are generally more amenable for complete thermodynamic and kinetic analysis because changes in pH and temperature are not always fully reversible, making the interpretation more difficult. Other complications arise from changes either in volume or in the thermal energy of the system when temperature is the variable or from the fact that the action of chemical denaturants is based on the binding of these molecules to proteins (1). Often, temperature denaturation leads to aggregation, which further difficult a full thermodynamic characterization.

Proteins are assembled and evolved to work in biological systems, in defined ranges of pH and temperature, and in the absence of chemical denaturants. Under these conditions, however, proteins are normally in their folded conformation, and the population of unfolded molecules is extremely low. Pressure constitutes an ideal noninvasive technique to perturb the equilibrium between folded and unfolded states in protein systems, because it does not introduce changes in the composition of the solution, and its often fully reversible effect can be readily interpreted in thermodynamic and structural terms (2–4). A great advantage is that upon pressure release, it leaves no residue in the solution and the change produced can be analyzed in real time. It has been used extensively for the study of oligomeric systems and, to a lesser extent, in monomeric proteins in combination with other denaturing agents (5–9). This is because monomeric proteins are normally resistant to the mild effect of high pressure (10), compared with other denaturing agents. On the other hand, small monomeric proteins have been the object of intensive studies because their small size allows detailed structural and theoretical analysis in combination with experimental approaches to elucidate the molecular mechanisms of protein folding.

Chymotrypsin inhibitor-2 (CI-2)¹ is a small monomeric protein (64 residues) that constitutes a paradigmatic example of a simple two-state model for folding (11–13). CI-2 is a potent inhibitor of the proteases chymotrypsin and subtilisin and both x-ray crystal (14) and NMR solution structures (15) are available. It is, however, a highly stable protein in terms of the generally used denaturing agents, a property that, in principle, anticipates resistance to denaturation with pressure as the sole perturbant. CI-2 was cleaved into two peptide fragments of 40 and 24 residues, respectively, which were showed to reconstitute readily to yield a native-like noncovalent complex (16). Both the structure attained and its mechanism of folding are highly similar to the refolding of the uncleaved protein, except for the second order kinetic mechanism, which appears to differ only in the concentration (17–20). Because the complex formed

¹ The abbreviations used are: CI-2, chymotrypsin inhibitor-2; GdmCl, guanidine chloride; CM, center of spectral mass.

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is much less stable than the intact protein (16) but conserves its folding mechanism, we decided to investigate possible effects of high hydrostatic pressure on this noncovalent complex. We hypothesize that the existing bimolecular equilibrium in the [CI-2(1–40)z(41–64)] complex will be the target of the mild action of pressure, and this will allow a reversible denaturation reaction that we aim to analyze in detail as a model for folding in the absence of chemical denaturants.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals used were of maximal purity available. Bis-Tris buffer and guanidine chloride (GdnCl) were purchased from Sigma. CI-2 was obtained recombinantly and its 1–40 and 41–64 fragments (abbreviated W and Y, respectively, for their contents of tryptophan and tyrosine) were cleaved and purified as described previously (16). Water was deionized and distilled twice prior to use.

**Fluorescence Spectroscopy and High Pressure Measurements**—Fluorescence spectroscopy was carried out either with Hitachi F-4500 (Tokyo, Japan) or ISS-K2 (Champaign, IL) equipment. Fluorescence measurements under pressure were made using a high pressure bomb described in (21), fitted with sapphire windows, and adapted to the ISS-K2 fluorimeter.

Tryptophan fluorescence was measured with excitation at either 278 or 295 nm, and the emission scanned from 300 to 400 nm or 315 to 415 nm, respectively. We focused on changes in spectral area, fluorescence

![Fig. 1](image-url)  
**Pressure denaturation of intact CI-2.**  
a, pressure titration experiment of 10 μM CI-2 in 50 mM Bis-Tris HCl buffer, pH 6.0 (closed circles), buffer plus 2.0 M GdnCl (open circles), and 2.25 M GdnCl (closed triangles). The inset shows a GdnCl denaturation curve of CI-2, followed by the change in the center of mass, in excellent agreement with the same process followed by fluorescence intensity (11). b, fluorescence spectra of intact CI-2 in 2.25 M GdnCl (and the same buffer as in a), pressure-denatured CI-2 in the same conditions and GdnCl-denatured protein at atmospheric pressure (6.0 M denaturant).
intensity and center of spectral mass (CM), defined as
\[ n = \frac{\sum \nu F_i}{\sum F_i} \]
(Eq. 1)
where \( \nu \) is the center of mass in wave numbers, \( F_i \) is the fluorescence emitted at wave number \( \nu_i \). Shifts in CM due to pressure were used to follow the denaturation process by converting them into fraction of denatured species at each pressure \( (\alpha_p) \) according to Weber (2).

\[ \alpha_p = \frac{[1 + Q(\nu_e - \nu_f)]}{[\nu_e - \nu_f]} \]
(Eq. 2)
where \( \nu_e \) is the CM at a given pressure, \( \nu_f \) and \( \nu_u \) the CMs corresponding to native and unfolded species, respectively. Thermodynamic parameters for the pressure unfolding for a unimolecular transition were determined according to Equation 3.

\[ \ln[\alpha_p/(1 - \alpha_p)] = \frac{\Delta V}{RT} + \ln K_U \]
(Eq. 3)
where \( \Delta V \) is the volume change and \( K_U \) is equilibrium constant for pressure unfolding extrapolated to atmospheric pressure (4).

Prior to pressure unfolding-refolding experiments, equimolar concentrations of fragments were incubated overnight at 25° C in 50 mM Bis-Tris buffer, pH 6.0, to form the complex. The mixture was placed in a capped quartz bottle, which was not sealed inside the pressure bomb, as previously described (21). Fluorescence spectra were recorded at atmospheric and different increasingly higher pressures. The temperature was kept at 25 ± 0.1° C using a circulating water bath connected to the high pressure bomb.

GdnCl denaturation experiments were carried out by incubating the preformed [CI-2(1–40)(41–64)] complex for 1 h with increasing concentrations of the denaturant in 50 mM Bis-Tris HCl, pH 6.0. Data for fluorescence intensity and CM were recorded for each concentration, and the resulting curve was analyzed using a two-state equation corresponding to a concentration-dependent equilibrium, fully described in Ref. 22.

**Results**

Resistance of Uncleaved CI-2 to Pressure Denaturation—Monomeric proteins are often too stable for denaturation at the range of pressures normally attainable with standard pressure equipment used in fluorescence studies (21). CI-2 is not an exception. Being extremely stable to chemical denaturation, it cannot be fully unfolded by urea, and its thermal denaturation...
midpoint is above 75°C, even in the presence of denaturants (23, 25). The unfolding of CI-2 is accompanied by a maximum wavelength shift and a large increase in fluorescence caused by a yet unidentified residue or residues that quench the tryptophan side chain in the folded state (11). We applied increasingly high pressures to uncleaved CI-2 and followed changes in spectral area and in CM to assess solvent exposure of its unique tryptophan residue. The protein is resistant to denaturation at the maximum pressures tested (Fig. 1a). If we preincubate the inhibitor with subdenaturing concentrations of GdnCl (Fig. 1a, inset), its unique buried tryptophan residue is gradually exposed to the solvent as the change on its CM indicates. Subdenaturing GdnCl concentrations of 2.25 M are required to attain the maximum shift in the center of spectral mass of the tryptophan at the highest pressures, compatible with a large solvent accessibility. Despite this large shift (Fig. 1b), the area increases 2.5-fold in the high pressure state (3450 bar, 2.25 GdnCl), whereas the GdnCl-denatured state has its spectral area increased by 6-fold.

We can calculate the thermodynamic parameters associated with this transition using equation (3). In doing so, we obtain a ΔG of 2.5 kcal mol⁻¹ and a ΔV of 42.5 ml mol⁻¹.

**Pressure Denaturation of the [CI-2(1–40)z(41–64)] Complex—**
The spectrum of the tryptophan in the free fragment that contains the tryptophan residue, CI-2(1–40) displays a large quenching on association/folding to yield the noncovalent folded complex (16). A ribbon diagram for the structure of CI-2 is shown in Fig. 2a, indicating the position of the buried tryptophan. If we closely compare the normalized spectra of the complex with that of the intact folded CI-2, we find that the tryptophan fluorescence of the former is not completely shifted to the blue as in the case of the intact protein (Fig. 2b). Both crystal and solution structures are available for the complex (20), and the apparent exposure of the chromophore to the solvent is not an indication of major unfolding but probably of solvent accessibility and conformational dynamics. Nevertheless, the chemical shift of the tryptophan in the complex corresponds unequivocally to a folded conformation (20). The change exerted by pressure on the complex (Fig. 2b) is only partial in terms of the release of the fluorescence quenching of the Trp residue when we compare it with the change observed either on fragment association or GdnCl unfolding of the complex (Fig. 2b, inset).

The [CI-2(1–40)z(41–64)] complex, which shows similar folding behavior to the intact protein but with lower stability, was

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**Fig. 3.** Reversible pressure denaturation of the [CI-2(1–40)z(41–64)] complex. Top, pressure titration of 10 μM complex (see “Experimental Procedures”) followed by the change in the center of fluorescence spectral mass (closed circles) and the return to atmospheric pressure (open circles). Bottom, gel filtration chromatograms (Superdex 75) of the [CI-2(1–40)z(41–64)] complex before (solid line) and 45 min after (dashed line) pressure treatment. The peak eluting at 22 min corresponds to Bis-Tris buffer and marks the V₀.

**Fig. 4.** Concentration dependence of the GdnCl denaturation of the [CI-2(1–40)z(41–64)] complex. a, GdnCl denaturation of the complex (5 μM) followed by the change in CM. Inset, titration of CI-2(1–40) by the complementary fragment CI-2(41–64), indicating a similar overall change in CM. b, recovery of folded CM in 0.35 M GdnCl at increasing concentrations of the complex.
next analyzed for its ability to unfold under high hydrostatic pressure without the addition of chemical denaturants. The complex shows a large red shift of the intrinsic fluorescence upon the application of pressure due to the exposure of its unique tryptophan residue to the solvent (Fig. 2c), indicative of substantial denaturation (23, 24). The spectral area changed concomitantly by only 2-fold, which is considerably lower than what would be expected from the complete unfolding/dissociation of intact CI-2 or from the association of fragments in water (Fig. 2b, inset).

The effect of a gradual increase of pressure was followed by CM and shows a transition that is over at the highest pressures attainable by our equipment, with a value of 28500 cm$^{-1}$ at 3.5 kilobar (Fig. 3, top panel). The process is thus accompanied by a CM of 650 cm$^{-1}$ and a 2-fold increase in the spectral area or intensity. When the pressure was gradually released, both the CM and the area returned to their initial value, indicating a full reversibility of the effect (Fig. 2c).

Although the CM attained by the complex at the highest pressures is compatible with the tryptophan residue being fully exposed to the solvent (28600 cm$^{-1}$), we confirmed the end point of the transition by unfolding [CI-2(1–40)] by pressure titration in the presence of 0.7 M GdnCl, corresponding to the midpoint at atmospheric pressure and gives an identical result (not shown). As a control experiment, we also carried out a pressure titration of the isolated CI-2(1–40) fragment, with no significant changes observed.

To check for complete reversibility, we carried out gel filtration chromatography of the complex before and after pressurization. Fig. 3 (bottom panel) shows that the chromatograms in both conditions are superimposable; there is no evidence of dissociation into fragments when the complex is renatured immediately after pressure release.

Comparative Unfolding of CI-2 and [CI-2(1–40)] by GdnCl and Pressure—Pressure appears to exert only partial changes in the fluorescence quantum yield of the chromophore, and the changes in spectral properties of the complex and intact CI-2 appear to be much more extensive in response to GdnCl than to high pressure. In similar conditions to the pressure experiments, we analyzed the denaturation/dissociation of [CI-2(1–40)] by GdnCl by monitoring the change in CM (Fig. 4a). The latter changes 750 cm$^{-1}$, coincident with the total change observed by pressure, indicative of complete dissociation of the tryptophan probe. On the other hand, the change in spectral area is 6-fold, much larger than the change observed at high pressures (Fig. 2c).

To further investigate these differences, we monitored the
formation of the complex upon mixing CI-2(1–40) with increasing concentrations of CI-2(41–64) in the same manner as described previously (16) but followed the change in CM. The change in this parameter is of similar magnitude to those observed for pressure and GdnCl denaturation (Fig. 4a, inset). However, the spectral area changes 7-fold as in the case of GdnCl denaturation and larger than the change exerted by high pressures (16).

**Monitoring Dissociation during GdnCl Denaturation of [CI-2(1–40)z(41–64)]**—The question arises whether the GdnCl denaturation equilibrium is linked to protein concentration. As Fig. 4a shows, the transition of the CM is over at 1.2 M GdnCl, with a total change of 750 cm$^{-1}$. A protein concentration dependence would translate into a displacement of the curve and may be followed by the change in the CM at a concentration near the [GdnCl]$_{50}$ of the transition, with increasing concentrations of the complex. For this purpose, we incubated [CI-2(1–40)z(41–64)] in 0.35 M GdnCl at concentrations ranging from 2 to 50 μM complex and measured the CM. Fig. 4b shows the recovery of the CM as the protein concentration is increased, shifting to values corresponding to a fully folded conformation. The change in CM for the complex either in the absence of denaturant or at 3.5 M GdnCl with increasing complex concentrations is minimal (not shown). A CM of $-390$ cm$^{-1}$ is expected from near midpoint values to folded complex in the absence of denaturant (Fig. 4a), and this value is approached by the protein concentration-dependent CM recovery (Fig. 4b). This is equivalent to a shift in the GdnCl curve, which indicates an increase in the stability of the complex to denaturation that depends on protein concentration.

Next, we followed changes in molecularity directly for the GdnCl denaturation process, making use of gel filtration chromatography. The [CI-2(1–40)z(41–64)] complex at 5 μM was incubated with different concentrations of the denaturant and then injected into a gel filtration column equilibrated in the same denaturant concentration. At zero denaturant, only complex can be observed, but as the GdnCl concentration increases a clear dissociation is observed that is completed at 2.0 M denaturant, coincident with the end of the denaturation transition observed by fluorescence intensity data (Figs. 5 and 6).

The GdnCl denaturation process is linked to the dissociation of the fragments and can thus be analyzed using a concentration-dependent two-state approach as in the case of a dimer (22). Fluorescence intensity changes give the most accurate data,
which were used to calculate a free energy of unfolding of 8.4 kcal mol$^{-1}$ and an $m$ value (the cooperativity unfolding parameter) of 1.8 kcal mol$^{-1}$ M$^{-1}$ (Fig. 6). The overall fluorescence change observed in previous spectra is measured as accurately as 6-fold, as the arrow in the plot indicates. The elution times from the gel filtration experiment (Fig. 5) change in parallel with fluorescence intensity (Fig. 6), supporting a concerted unfolding/dissociation process.

**Concentration Dependence of [CI-2(1–40)(41–64)] Denaturation by Pressure**—Although with structure and folding mechanisms similar to those of the intact CI-2 protein, [CI-2(1–40)(41–64)] clearly differs in that its folded equilibrium is in principle linked to an association-dissociation process and its kinetic mechanism of association/folding in solution is indeed second order. To test for concentration dependence, we carried out pressure titrations at different concentrations of the complex, ranging from 5 to 100 µM (Fig. 7a). Surprisingly, the process does not depend on protein concentration, although in all the concentrations tested there is a similar displacement of the spectral parameters corresponding to the exposure of its unique tryptophan residue to the solvent. We cannot rule out a protein concentration dependence at lower concentrations, but the 7-fold change in the fluorescence of the single tryptophan residue precludes a sensitive measurement below 5 µM because the folded conformation at atmospheric pressure is largely quenched, posing limits to the sensitivity.

It is very important for us to confirm that the high pressure state remains associated but the availability of techniques to measure directly the molecularity at high pressure is limited to gel electrophoresis (26), which is not able to operate at the 3.5 kilobar required to denature the complex. Taking advantage of a slow renaturation process after pressure release, we decided to use gel filtration chromatography. Data from our laboratory indicate that the overall refolding ($t_{1/2}$) at 25°C is approximately 150 s (not shown). To slow down the renaturation process and allow a characterization of the state attained at high pressure, we decreased the temperature to 10°C and the $t_{1/2}$ for the renaturation after pressure release increases 5-fold. This leaves enough time to release the pressure and inject the sample immediately in a gel filtration column, equilibrated at the same temperature. Fig. 7b shows a control experiment indicating the eluting times of the complex and the free fragments under these conditions, monitored by absorbance at 220 nm. A very similar chromatographic behavior is observed for a complex that has been pressurized for 2.5 h and for 10 h; both show only an incipient shoulder that could be interpreted as a small degree of dissociation (Fig. 7c). If we instead monitor tryptophan fluorescence, two peaks of identical height are observed after release from 2.5 h of pressurization (Fig. 7d). This cannot be interpreted as 50% dissociation, because the tryptophan is 6–7-fold quenched in the folded complex. This means that the actual dissociated fragment, at the lowest concentrations we use in our studies ($5 \mu$M), is less than 10%. Nevertheless, it is something significant, and the fraction that dissociates will probably become higher as the concentration of the complex is lowered. After 10 h of pressurization, the peak corresponding to the free fragments increases concomitantly with a decrease of that corresponding to the complex, suggesting that an extremely slow dissociation may take place.

**Thermodynamic Parameters Associated to the Pressure Denaturation Process**—From the pressure titration data at different concentrations we can calculate the parameters associated with the pressure denaturation process (5). The lack of protein concentration dependence and the chromatographic experiments led us to conclude that the greater population of the pressure-denatured state is not dissociated in the range of concentrations at which the sensitivity of our equipment allows us to work in equilibrium conditions (5–100 µM). If we add the limited change in the quantum yield at high pressure, which does not recover the values of the free CI-2(1–40) fragment, we assume a process in which no changes in molecularity take place in our present experimental conditions. These, besides protein concentration, include pH, temperature, and the experimental time frame.

Thus, for the analysis of the thermodynamic parameters, we do not need to account for protein concentration, and we use the equation described in the experimental section for a unimolecular unfolding transition. By analyzing the linear transformation of the data of the pressure titration curves at 5, 50, and 100 µM (Fig. 7a and see “Experimental Procedures”), we obtain an average free energy of 1.36 ± 0.03 kcal mol$^{-1}$. The corresponding value for the volume change upon pressure denaturation is 35 ± 0.7 ml mol$^{-1}$.

**DISCUSSION**

Intact CI-2 proved to be resistant to denaturation by high pressure, something not at all unexpected for small monomeric proteins. Subdenaturing concentrations of GdnCl allowed pressure denaturation, indicated by a shift in the CM of its tryptophan. This reversible qualitative spectral change was, however, not accompanied by a quantitative change of the fluorescence quantum yield as in the case of GdnCl unfolding. Thus, at high pressure, there must be a structure different from a fully extended denatured polypeptide. Nevertheless, the structural basis for the large fluorescence quenching in the folded state of both intact CI-2 and fragment complex remains unknown.

Without the addition of chemical denaturants, the complex [CI-2(1–40)(41–64)] undergoes a reversible denaturation transition. The final value for the center of spectral mass is coincident with the tryptophan residue in CI-2(1–40), which is disordered and solvent exposed already at atmospheric pressure. The fact that the fluorescence intensity changes only 2-fold compared with 7-fold in fragment association or complex unfolding by GdnCl strongly suggests the presence of residual structure. The lack of fluorescence quenching in the free CI-2(1–40) fragment at atmospheric pressure indicates that the basis for the quenching in the pressure-denatured state cannot be caused by interaction with neighboring residues and must have a long range, i.e. tertiary nature.

The lack of protein concentration dependence in the pressure denaturation equilibrium must be interpreted as the absence of dissociation. A deterministic behavior, found in multi-protein subunit assemblies such as virus or large protein oligomers or aggregates, can be ruled out for the small size and structural simplicity of the complex (5). Gel filtration chromatographic experiments after pressure release support the idea of a major population of undissociated species at high pressures in our experimental conditions.

GdnCl denaturation of [CI-2(1–40)(41–64)] is accompanied

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2. R. Mohanna-Borges and G. de Prat-Gay, unpublished results.

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**TABLE I**

Free energies of unfolding of [CI-2(1–40)(41–64)] obtained from different reactions

| Reaction | ΔG | kcal · mol$^{-1}$ |
|----------|----|-----------------|
| Pressure denaturation | +1.4 | |
| GdnCl denaturation | +8.5 | |
| Fragment association (equilibrium)* | -10.0 | |
| Fragment association (kinetics)* | -9.5 | |

*a From $K_d = e^{-\Delta G/RT}$ (16).

*b From $K_{off}/K_{on} = e^{-\Delta G/RT}$ (17).
by a concerted dissociation into fragments. A major difference is that the state at high GdnCl (dissociated fragments), although with a similar CM to the high pressure state, shows no quenching of the tryptophan. We conclude that there must be a link between the dissociation and the complete release of the quenching.

It is clear that the pressure-denatured state is different from that of GdnCl. The latter is expected to be a better denaturant, for its ability to solvate hydrophobic side chains and for its strong ionic effect that would be more effective in releasing quenching of chromophores by charged residues. It is difficult to imagine how the [CI-2(1–40)(41–64)] complex can be denatured and remain associated. This puzzling observation can be explained by the existence of a persistent residual structure in the high pressure state, associated and still quenched. Because the tryptophan is located at the center of the main hydrophobic core, which nucleates all the major secondary structure elements into the tertiary structure, it is tempting to suggest that any structure present at the high pressure state should be non-native, compatible with a full exposure of the chromophore to the solvent. However, fluorescence is extremely sensitive to the environment of the probe, and the CM shift could be due to a large solvent accessibility of the residue but not to full global unfolding. A molten globule-like conformation appears more compatible with these results. This hypothesis is supported by previous work with large CI-2 molten globule-like fragments, in which the fluorescence indicated solvent accessibility to the single tryptophan residue, but both its chemical shift and the overall backbone conformation as judged by its far UV circular dichroism spectrum corresponded unequivocally to a folded conformation (27–30).

Pressure does not affect all interactions as GdnCl does. Although pressure weakens electrostatic interactions and hydrophobic contacts, it is known to stabilize hydrogen bonds in macromolecules (31). An explanation consistent with our results would be that intrafragment interactions in the folded state are more affected by pressure, and a more drastic treatment, i.e., GdnCl, is needed to perturb interfragment interactions leading to dissociation. The natural protease inhibitory function of CI-2 requires that it remains stable for long enough after cleavage by the target enzyme, resulting in a very slow dissociation. The nature of the interactions that hold together the cleaved protein must be different from those of nature designed dimers that evolved for eventually undergoing dissociation.

Table I shows the free energy values calculated for the different reactions analyzed for this system in this and previous work (16, 17). GdnCl denaturation and fragment association/folding are in very good agreement, considering the different approaches involved. Partly structured and undissociated intermediate species were neither detected in the fragment association/folding nor in the GdnCl denaturation reactions. Thus, the complete transition that takes place upon either fragment association/folding or GdnCl denaturation/dissociation of the complex is accompanied by an absolute free energy change of 8.5 to 10 kcal mol$^{-1}$, whereas the pressure transition involves only 1.4 kcal mol$^{-1}$ change.

The free energy change for several other systems studied using high pressure ranged from 9 to 14 kcal mol$^{-1}$ (5, 7). In cases of dimers with intertwined topologies such as the arc repressor or the E2 DNA binding domain of human papillomavirus (32–34), dissociation was accompanied by substantial unfolding, which nevertheless led to partially structured monomers with proposed molten globule characteristics. As previously discussed, the difference with larger dimers lies in that the latter dissociate to monomers with defined topologies, whereas in the small intertwined dimers, the intersubunit interactions at the interface are crucial for stabilizing the overall tertiary structure. In the case of pressure denaturation of an unstable variant of the monomeric staphylococcal nuclease (nuclease concanavalin A), ΔG values of 1.4 kcal mol$^{-1}$ were observed in the absence of any added chemical denaturant (35), similar to the value reported for the CI-2 complex. Table II summarizes and compares data for the above mentioned unimolecular and bimolecular transitions.

The value of the ΔV for the CI-2 noncovalent complex is lower than in the other model reactions, which, together with the change in the normalized specific volume and small ΔG, supports the idea of a compact, structured state at high pressure. The larger change in molar volume per mass of protein can be explained by a larger fraction of buried residues exposed to the solvent upon dissociation (5). Smaller volume changes have been observed for other monomeric proteins (8, 36, 37), and it is noticeable that the larger changes in free energy take place only on species that dissociate (Table II and Ref. 5).

Scheme 1 illustrates the main aspects discussed in this work. The equilibrium I, i.e., the transition to the high pressure state, shows the major change in solvent accessibility of the tryptophan probe, whereas the dissociation equilibrium (II) must be accompanied by the release of the quenching. The energy change of this step estimated by subtraction would be −7.5 kcal.

**Table I**

Comparison of pressure denaturation in various monomeric and dimeric model proteins

| Protein                        | ΔG (kcal mol$^{-1}$) | ΔV (mol$^{-1}$) | Δµ (µl g$^{-1}$) | Pressure denatured state |
|-------------------------------|----------------------|-----------------|-----------------|--------------------------|
| [CI-2(1–40)(41–64)]$^a$       | 1.4                  | 25              | 4.8             | Undissociated            |
| CI-2$^b$                      | 2.5                  | 43              | 5.9             | Monomeric                |
| Staphylococcal nuclease$^c$   | 2.3                  | 57              | 3.8             | Monomeric                |
| E2 DNA binding domain (HPV)$^d$ | 9.9                  | 78              | 4.2             | Dissociated              |
| Arc repressor$^e$             | 9.8                  | 100             | 7.7             | Dissociated              |

$^a$ For both complex and CI-2, errors in ΔG are less than 5%, and errors in ΔV are less than 10% (this work).

$^b$ In the presence of 2.25 M of GdnCl (this work).

$^c$ Wild type protein at pH 5.1 (40).

$^d$ Ref. 34.

$^e$ Ref. 32.

![Diagram](Image 308x473 to 556x582)

**Scheme 1. Proposed model for the pressure denaturation of [CI-2(1–40)(41–64)].** In [WY], the subscript F denotes folded, and HP denotes high pressure.
The lack of availability of techniques such as circular dichroism under high pressure complicates the assessment of the overall backbone conformation of the proteins in these conditions. A recent report describes the possibility of exploring the secondary structure of the pressure-denatured state of staphylococcal nuclease using Fourier-transformed infrared spectroscopy (38). The question of whether the pressure-denatured state of the [CI-2(1–40)(41–64)] complex is a compact non-native denatured state or a native-like molten globule could be unveiled by the application of NMR techniques under pressure (33, 39). Clearly, the [CI-2(1–40)(41–64)] complex is not a naturally designed dimer as in the case of the above mentioned examples such as arc, E2, or larger proteins.

Because pressure is a subtle denaturant that does not alter the chemical composition of the system, we can argue that pressure-denatured states could model a denatured state in conditions compatible with biological systems. Because molten globule is a broad definition that does not necessarily mean a loose native-like conformation, precise atomic definition of structures observed at high pressures constitutes a major challenge for structural biologists, with strong implications for protein folding both in vitro and in vivo.

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mol $^{-1}$, assuming similar overall changes taking place in GdnCl denaturation/dissociation, fragment association/folding and that a complete pressure transition would in theory include dissociation. This is much higher than the 1.4 kcal mol $^{-1}$ from the transition to an undissociated pressure-denatured state and similar to the dissociation of above mentioned examples like arc or E2. As a general conclusion, the main energetic change would therefore be driven by the dissociation process.