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Molten-globule conformation of Arc repressor monomers determined by high-pressure ^{1}H NMR spectroscopy

(pressure dissociation/pressure denaturation/two-dimensional NMR/protein folding/β-sheet)

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ABSTRACT **The conformation of the pressure-dissociated monomer of Arc repressor was characterized by ^{1}H NMR spectroscopy. The NMR spectra of the monomer under pressure (up to 5.0 kbar; 1 bar = 100 kPa) are typical of a molten globule and they are considerably different from those of the native dimer and thermally denatured monomer. The two-dimensional nuclear Overhauser effect spectra suggest that the pressure-induced molten globule retains some secondary structure. The presence of nuclear Overhauser effects in the β-sheet region in the dissociated state suggests that the intermonomer β-sheet (residues 8–14) in the native dimer is replaced by an intramonomer β-sheet. Changes in one-dimensional and two-dimensional NMR spectra prior to pressure dissociation were found and suggest the existence of a "predissociated" state.**

To approach the question of how a protein acquires its biological three-dimensional structure or conformation, a perturbation is typically applied to the system. This perturbation can be chemical, such as pH extremes, urea, or guanidine hydrochloride, or physical, such as temperature or pressure. However, the equilibrium between the so-called native state (N) and the denatured state (D) might be affected in a different fashion depending on the nature of the perturbation. The subset of denatured states relevant to the equilibrium with the native state has been only transiently obtained, for example, by dilution of a denaturing agent. Therefore, to determine how a protein correctly folds from a random coil state into a native three-dimensional structure, one has to find a way to stabilize and characterize the folding intermediates (1–3). Several studies indicate that the folding intermediates have a compact structure, termed molten globule (4), with a secondary structure similar to the native state but with a disordered tertiary structure (5). The association of subunits creates an additional complexity in the case of oligomeric proteins (6) but it is expected that the interactions between the amino acid residues in the subunit interface are governed by the same forces as those determining the folding of a single-polypeptide protein.

In this report we describe a unique approach, the use of hydrostatic pressure, to stabilize and characterize the intermediate states for the dissociation and denaturation of Arc repressor. Hydrostatic pressure has been utilized to follow the denaturation and subunit dissociation of several proteins (7–9). Arc repressor is a small DNA-binding protein of 53 amino acid residues (M_r = 13,000) that is dimeric in solution (10, 11). It has been reported that Arc, Mnt, and Met repressors belong to a class of sequence-specific DNA-binding proteins that use a β-sheet as the DNA-binding motif in contrast to other structural motifs of DNA-binding proteins, including helix–turn–helix, zinc-finger, and leucine-zipper (12). Recent fluorescence experiments demonstrate that hydrostatic pressure induces reversible dissociation of Arc repressor (13). The dissociated monomer has properties characteristic of a molten globule. For example, it is compact with its partially exposed nonpolar core that binds bis(8-anilinonaphthalene-1-sulfonate) (13). NMR has been shown to be a powerful tool for the investigation of protein structure and dynamics of protein folding intermediates (14). The complete ^{1}H NMR assignments for Arc repressor have been reported (15, 16) and a tertiary and quaternary structure model for Arc repressor has been proposed (12).

In the present study, we have used high-pressure, high-resolution NMR techniques (17) to monitor the structural and dynamic changes of Arc repressor during the course of pressure dissociation and denaturation. The starting point for our analysis is the refined three-dimensional structure of the Arc repressor dimer with its intermonomer β-sheet as described by R. Kaptein (personal communication). Our results indicate a predissociation conformational change and suggest that the molten globule of Arc repressor monomer adopts the structure containing a β-strand, γ-turn, β-strand features (15, 16) in the 8- to 14-residue region that is believed to be the DNA-binding site.

MATERIALS AND METHODS

Arc repressor was expressed in *Escherichia coli* and purified as described (10, 11). All other reagents were of analytical grade. Experiments were performed in the standard buffer: 0.05 M Tris chloride/100 mM NaCl, pH 7.5 in ^2H_2O.

A home-built high-pressure, high-resolution NMR probe (17) was used for the pressure and temperature experiments. All NMR spectra were recorded on a GE-300 spectrometer system. Arc repressor concentrations of 0.3–1.5 mM were utilized. One-dimensional (1D) NMR experiments were performed with the following spectrometer parameters: 16,384 data points in the free induction decay and spectral width of 5 kHz, 60° excitation pulse with an acquisition delay of 2 s. The resolution (1.8 Hz) was the same in the range 1–5000 bar (1 bar = 100 kPa).

For two-dimensional (2D) NMR experiments, phase-sensitive correlated spectroscopy (COSY) and nuclear Overhauser effect spectroscopy (NOESY) or exchange-correlated spectra were recorded with spectral width of 4 kHz, 1–2 s of recycle delay, and two dummy scans before each free induction decay. A time domain data set of 512 × 2048 points was recorded and Fourier transformed in both dimensions after multiplication by a sine bell window function shifted by 0–45° and zero filling prior to the second Fourier transformation. The final data set consists of 1024 × 1024 real data points.

The structure of γ-turn monomers were obtained from molecular dynamics calculations and energy minimizations

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Abbreviations: NOESY, nuclear Overhauser effect spectroscopy; COSY, correlated spectroscopy; 1D, one-dimensional; 2D, two-dimensional.

by using dihedral angles (18) and experimental constraints. Calculations were carried out on a Silicon Graphics IRIS Indigo Elan workstation with INSIGHT II and DISCOVER.

RESULTS AND DISCUSSION

The pressure effects on the aromatic region of 1D ^{1}H NMR spectra of Arc are shown in Fig. 1*A*, and, for comparison, spectra at different temperatures are also shown in Fig. 1*B*. The spectra have been assigned according to Breg *et al.* (15); however, in this report we discuss only in a qualitative way the spectral changes due to pressure effects. It is evident from Fig. 1 that there are several major differences between the changes produced by pressure and those resulting from thermal denaturation. The spectra obtained on compression were equal to those recorded on decompression, suggesting complete reversibility over the course of the experiments.

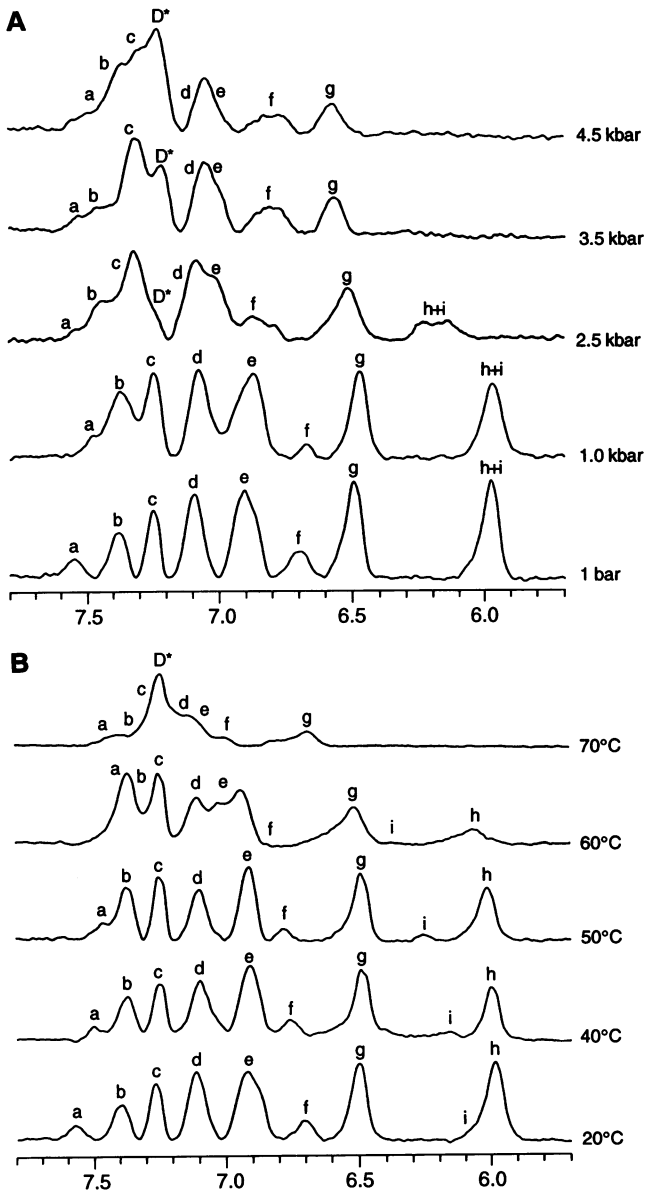

FIG. 1. Aromatic region of the 1D ^{1}H NMR spectra of Arc repressor at various pressures (20°C, pH 7.5) (*A*) and at various temperatures (1 bar, pH 7.5) (*B*). The resonance assignments are as follows (12): (a) W14 $C_{\delta 1}H$; (b) W14 $C_{\zeta 2}H$, $C_{\varepsilon 3}H$; (c) F45 $C_{\delta 2}H$; (d) F45 $C_{\varepsilon 2}H$, W14 $C_{\eta}H$; (e) W14 $C_{\zeta 3}H$, F45 $C_{\zeta}H$, F10 $C_{\delta 2}H$; (f) F10 $C_{\varepsilon 2}H$; (g) Y38 $C_{\varepsilon 2}H$; (h) Y38 $C_{\delta 2}H$; (i) F10 $C_{\zeta}H$; D*, resonance arising from partially denatured state.

Absence of hysteresis was also found in the temperature experiments. The results were reproducible for three different preparations.

According to previous studies (10), Arc repressor (pH 8.0) begins to denature at about 35°C and completely denatures at 70°C. Therefore, the spectrum at 70°C represents the completely denatured state of Arc repressor. According to Silva *et al.* (13), 1.0 mM Arc repressor at 20°C and pH 7.5 would begin to dissociate at about 1 kbar and completely dissociate at about 3.5 kbar. Our spectra in the pressure range of 1–5 kbar show substantial overlap and line broadening of many resonances. This kind of behavior has also been reported for the molten-globule state of guinea pig α-lactalbumin (19). Therefore our ^{1}H NMR spectra support the previously proposed molten-globule state of Arc repressor under high pressure (13). One also notices that the ^{1}H NMR spectra of the dissociated state in the pressure range of 3.5–5 kbar are substantially different from those of the native state (1 bar, 20°C) and the fully denatured state (1 bar, 70°C).

Fig. 2 illustrates the good agreement between the dissociation curve obtained from the NMR spectra and that reported in the fluorescence study (13). However, the different ΔVs and lack of overlap of the dissociation curves for different resonances will be presented elsewhere (unpublished work). This suggests that the transition from a native dimer to a molten-globule monomer is not very cooperative. Similar results have also been observed in the thermal denaturation of reduced and carboxymethylated basic pancreatic trypsin inhibitor (21) and the pressure unfolding of lysozyme (22).

The changes in the aliphatic region of ^{1}H NMR spectra of Arc repressor as a function of pressure further suggest the appearance of a molten-globule state. The changes in the linewidth and chemical shift in the pressure range from 1 bar to 1 kbar indicate conformational changes prior to the dissociation of Arc repressor dimer. The changes in the aliphatic protons promoted by increasing temperature were completely different. The spectra of molten-globule state in the pressure range from 3.5 to 5 kbar are substantially different from those of the native dimer at 1 bar and the thermally denatured monomer at 70°C.

To further characterize the structure of the molten-globule state of Arc repressor, we have carried out 2D NMR experiments at different pressures. Phase-sensitive COSY (23), NOESY, or exchange-correlated (24–26) spectra were recorded. The results of these experiments will be discussed elsewhere (unpublished work).

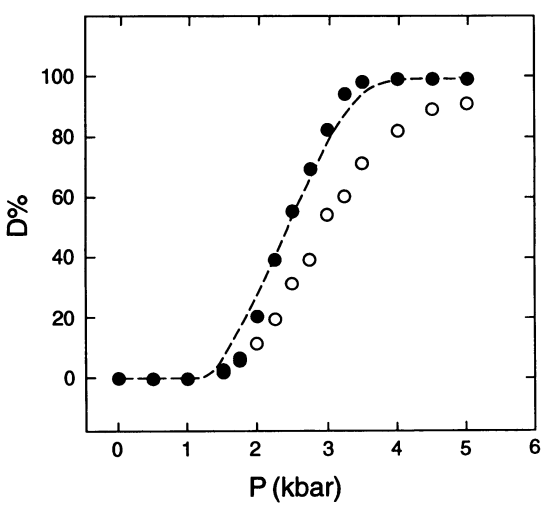

FIG. 2. Pressure dissociation curves obtained from NMR resonance for Tyr-38 $\varepsilon 2$ (○) and $\delta 2$ (●) protons compared with results from fluorescence study (dashed line) (10).

Fig. 3 shows the NOESY spectra in the αH-αH region where NOE cross peaks in the β-sheet region can be observed. The NOEs between α-Hs of Gln-9 and Arg-13 are observed throughout the pressure range studied, suggesting the proximity of theses two residues under those conditions. By contrast, no NOEs between α-Hs of Gln-9 and Arg-13 are observed in NOESY spectrum of Arc at 68°C, suggesting that Gln-9 and Arg-13 are no longer close to each other in the thermally denatured state.

It is well established (26, 27) that a relationship exists between the α-CH chemical shifts of individual residues and the protein secondary structure. Table 1 lists the chemical shift values of α-Hs for four residues located in the β-sheet region of the molten-globule state, thermally denatured state, and random-coil state. It can be seen from Table 1 that the chemical shift values for α-Hs of those four residues at different pressures are always much greater than the corresponding random coil values (28), supporting the view that these residues still maintain their β-sheet structure in the molten-globule state (28). However the chemical shift value of Asn-11 at 68°C is smaller than its corresponding random coil value, suggesting the disruption of the β-sheet in the thermally denatured state (28). The observed NOEs between α-Hs of Gln-9 and Arg-13 (Fig. 3) and the α-Hs chemical shifts (Table 1) at pressures above 3.0 kbar suggest that an antiparallel β-sheet is present in the Arc repressor monomer. The structure of intramonomer β-sheet in the molten-globule state could be similar to the one proposed by Breg *et al.* (15) and Zagorski *et al.* (16). Fig. 4 shows a structural model for the conversion from the intermonomer β-sheet in the native dimer to the intramonomer β-sheet with a γ-turn in the molten globule.

According to fluorescence experiments on Arc repressor (13), the native dimer at 1 mM concentration should start to dissociate at around 1 kbar. The relative changes in 1D spectra of aromatic and aliphatic regions at 1 kbar with respect to 1 bar indicate that there must be some changes in structure of Arc prior to dissociation. As pressure is increased from 1 bar to 1 kbar, the Arc dimer undergoes predissociation change in conformation, resulting in a predissociated dimer that might resemble the transition state in the protein folding theory described by Creighton (20). The NOE cross peak between Gln-9 and Trp-14 that appeared at 1.0 kbar (Fig. 3) might be caused by the predissociation changes in the intersubunit β-sheet, which would also explain the weakening of Gln-9/Arg-13 NOE peak. The intensities of NOEs at 1 kbar are relatively weak for α-Hs of Gln-9/Arg-13 and Gln-9/Trp-14 but strong for α-Hs of Gln-9/Met-7, suggesting that the conformation of the predissociated state could be an intermediate between the antiparallel β-sheet of native dimer and of the intramonomer β-sheet of molten-globule monomer (Fig. 4).

In the dissociated region (3.5–5 kbar), the substantial line broadening and overlap of many resonances are due to the interconversion of different conformations of the molten globule, suggesting a high degree of conformational hetero-

Table 1. Chemical shift values of α-protons of four residues in the β-sheet region in the random coil, thermally denatured, native, and molten globule (MG) states

				MG	
Residue	Random coil*	Denatured	Native	3.5 kbar	4.5 kbar
Gln-9	4.37	4.75	5.21	5.27	5.25
Asn-11	4.75	4.60	5.11	5.19	5.17
Arg-13	4.38	4.50	5.03	5.05	5.03
Trp-14	4.70	4.90	5.43	5.42	5.44

Chemical shifts in ppm are referenced to sodium 3-(trimethylsilyl)tetradeuteriopropionate.
*From ref. 27.

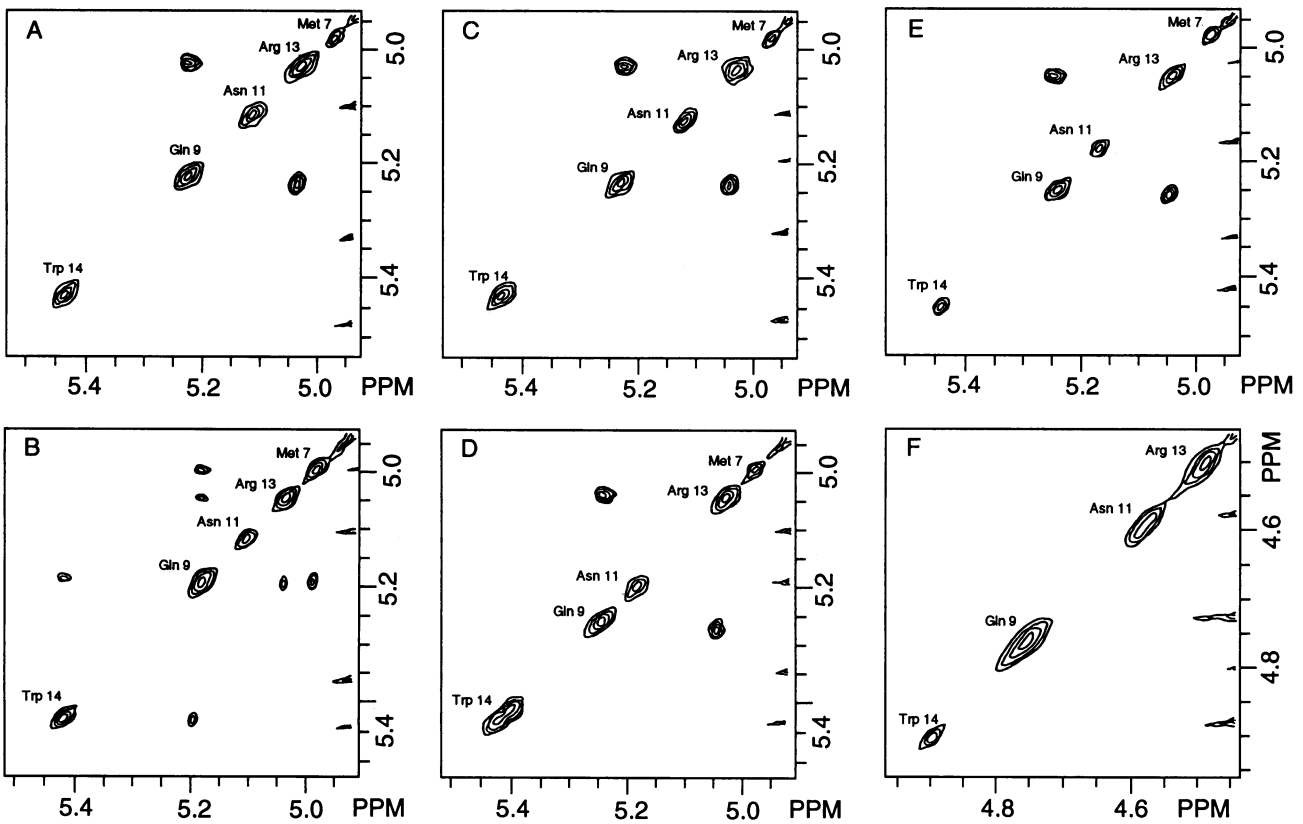

FIG. 3. Expanded region of NOESY spectra (pH 7.5, 100-ms mixing time) at 20°C and 1 bar (*A*), 1 kbar (*B*), 2.5 kbar (*C*), 3.5 kbar (*D*), and 4.5 kbar (*E*) and at 68°C and 1 bar (*F*).

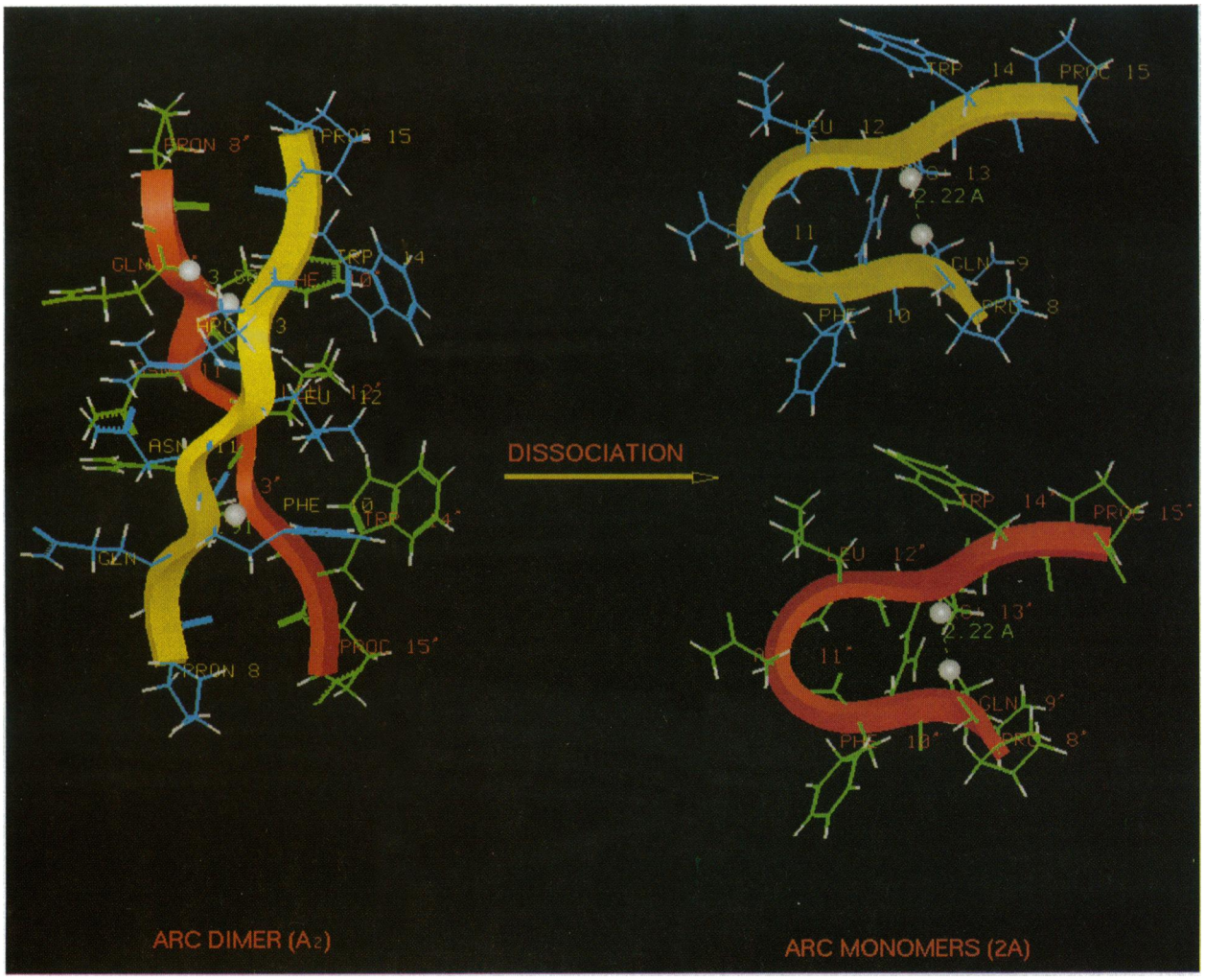

FIG. 4. Schematic representation of the β-sheet structure in the pressure-induced dissociation from native dimer to molten-globule monomer. The ribbons of the two subunits are in red and yellow, respectively. The dimer structure was kindly provided by R. Kaptein (personal communication).

geneity in comparison to the native state. The NMR results corroborate previous fluorescence lifetime data (13).

In this study, high-pressure NMR techniques have been used to explore the conformational changes during the pressure dissociation of Arc repressor dimer into a molten-globule monomer. The observed changes prior to dissociation suggest the existence of a predissociated state. We also illustrate the great potential of high-pressure NMR spectroscopy in characterizing the conformation of intermediate states of proteins.

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