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Evidence for a Molten Globule-like Transition State in Protein Folding from Determination of Activation Volumes†

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ABSTRACT: One of the most important, yet elusive, aspects of the protein folding question lies in the nature of the transition state. Direct information about the structural properties of the transition state can be obtained from determination of the activation volumes for the folding and unfolding transitions. The present pressure-jump relaxation study on the folding/unfolding of staphylococcal nuclease reveals that the volume of the protein−solvent system is larger in the transition state than in either the folded or unfolded states. Moreover, the activation volume of folding is much larger than that of unfolding. These results support a molten globule-like model for the transition state of nuclease in which the polypeptide chain is in a collapsed, loosely packed, solvent-excluded structure. In this model, hydrophobic collapse with concomitant desolvation is the rate-limiting step in the folding of the polypeptide chain, and solvent-excluded expansion of the folded state is the rate-limiting step in protein unfolding.

For a chemical reaction, the volume difference ΔV between products and reactants is obtained from the derivative of the Gibbs free energy change ΔG with respect to pressure p:

$$d(\Delta G)/dp = \Delta V \quad (1)$$

Pressure-induced protein unfolding results from differential solvation (electrostriction and hydrophobic hydration) and excluded volume effects (Brandts *et al.*, 1970; Zipp & Kauzmann, 1973; Heremans, 1982; Weber & Drickamer, 1983; Prehoda & Markley, 1995). When hydrostatic pressure is applied, the protein−solvent system evolves toward the global configuration that occupies the least volume. Since in the unfolded state the excluded volume of packing defects is eliminated and charged and hydrophobic residues are exposed to solvation, it is this form of the protein that is favored by pressure.

This effect of pressure on the Gibbs free energy of unfolding necessarily arises from pressure effects on the rates of unfolding, refolding, or both. A reaction rate k_p at a given pressure p can be expressed in terms of the rate at atmospheric pressure k° and the activation volume $\Delta V^\ddagger$ for the formation of the transition state (Gladstone *et al.*, 1941):

$$k_p = k^\circ e^{(-p(\Delta V^\ddagger)/RT)} \quad (2)$$

where R is the gas constant and T is the kelvin temperature. Depending upon the relative volumes of the initial and transition states, increasing pressure can result in either an increase or a decrease in the reaction rate.

We have investigated the pressure dependence of the folding/unfolding relaxation kinetics of a recombinant nuclease produced from *Escherichia coli* (Alexandrescu *et al.*, 1989) whose sequence is that of nuclease A from the V8 strain of *Staphylococcus aureus*. Nuclease is a small,

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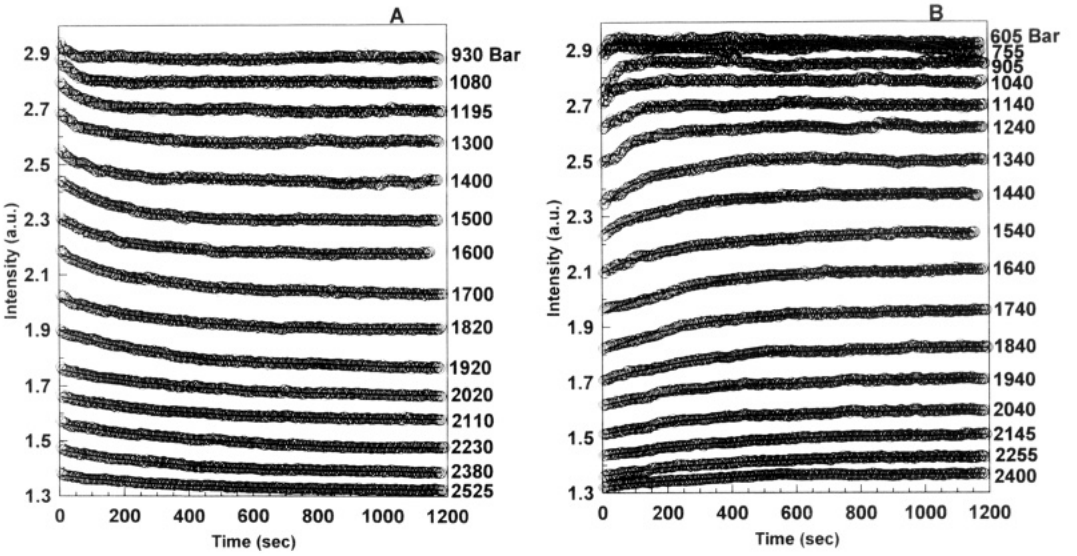

FIGURE 1: Relaxation profiles for (A) positive and (B) negative pressure jumps obtained by monitoring the intensity of the intrinsic tryptophan emission of nuclease as a function of time after the pressure jump. The scale of the *y*-axis is in arbitrary units (au). The lines represent the fits at each pressure.

monomeric, reversibly folding protein (Shortle & Meeker, 1986; Griko *et al.*, 1988; Alexandrescu *et al.*, 1989; James *et al.*, 1992) bearing one tryptophan residue at position 140 whose fluorescence becomes efficiently quenched upon unfolding (Shortle & Meeker, 1986). We and others have found by both fluorescence and NMR that at pH 5.5 nuclease becomes completely unfolded by 2500 bar (Eftink *et al.*, 1991; Royer *et al.*, 1993). This low stability makes it possible to study the pressure dependence of the folding/unfolding kinetics with existing pressure-jump apparatus.

MATERIALS AND METHODS

Protein Purification. Staphylococcal nuclease (nuclease) with the sequence of nuclease A from the V8 strain of *S. aureus* was produced in *E. coli* and purified as described by Alexandrescu *et al.* (1989) and Royer *et al.* (1993). The purified protein was lyophilized and stored in that form. Solutions of nuclease were prepared from the lyophylate at concentrations less than 10 μM (as determined by UV absorption at 280 nm and an extinction coefficient of 18050 cm^{-1} M^{-1}) in order to avoid any aggregation phenomena in the unfolding experiments which can occur at higher protein concentration. The buffer solution used was 10 mM bis-Tris at pH 5.5. The pK_a of bis-Tris is pressure insensitive due to the fact that the charge in the protonated and unprotonated forms is equivalent since, in the protonated form, the bis-Tris bears one positive charge and, in the unprotonated form, the proton bears a positive charge. There is thus no volume change due to electrostriction effects and no pressure dependence of the pK_a. All experiments were carried out at 21 °C.

Pressure-Jump Experiments. Pressure-jump relaxation kinetics profiles were carried out in a Vascomax high-pressure cell and using a high-pressure generating system similar to that described by Paladini and Weber (1981). A UV multifiber bundle (Oriel Corp., Stratford, CT) with focusing optics was used to bring the exciting light at 295 nm from the monochromator to the pressure cell. Tryptophan emission was monitored at 90° through a Corion 340-nm high-pass filter. Pressure jumps of 75−150 bar were made by closing the valve to the sample compartment, pumping to the desired new pressure, and rapidly reopening the valve. Fluorescence intensity data were acquired with 2−5-s integration times, which is fast relative to the relaxation time scales. The data were fitted to a single-exponential decay by using the time-domain fluorescence global analysis program described by Beechem *et al.* (1992). The pressure jumps were small enough that the adiabatic heating of the sample amounted to no more than 0.3 °C, which at the sample temperature of 21 °C would not perturb the folding equilibrium of nuclease significantly. Moreover, the extent of the perturbation of the equilibrium due to the pressure jump itself was less than 10%, such that the linearized rate expression holds (Eigen & de Maeyer, 1963). The values of the intensities at 25 min after the jump were taken as the equilibrium intensities, and these equilibrium profiles were fitted using the BIOEQS analysis program for the free energy and volume change of the unfolding (Royer *et al.*, 1990; Royer, 1993).

RESULTS AND DISCUSSION

Changes in the intrinsic fluorescence emission intensity of nuclease at pH 5.5 and 21 °C were monitored as a function of time after small, rapid pressure jumps. In Figure 1 are plotted a series of relaxation profiles for successive positive (Figure 1A) or negative (Figure 1B) pressure jumps of 75−150 bar in magnitude at final pressures ranging from 600 to 2500 bar. Positive pressure jumps result in perturbation of the equilibrium toward the unfolded state, as evidenced by relaxation profiles exhibiting a decrease in fluorescence intensity; negative pressure jumps result in perturbation of the equilibrium toward the folded state and an increase in fluorescence intensity. Unlike the unfolding or refolding of nuclease at atmospheric pressure by pH or denaturant jumps, which occur in less than 1 s (Chen *et al.*, 1992 a,b; Nikano *et al.*, 1993), the times cales for the relaxations under pressure are on the order of minutes. The equilibrium profiles for increasing and decreasing pressure obtained from the final intensity values at each pressure demonstrate that the reaction is reversible (Figure 2). These profiles yielded a free energy for unfolding of 3.2 ± 0.3 kcal/mol and a volume change for unfolding of −77 ± 8 mL/mol.

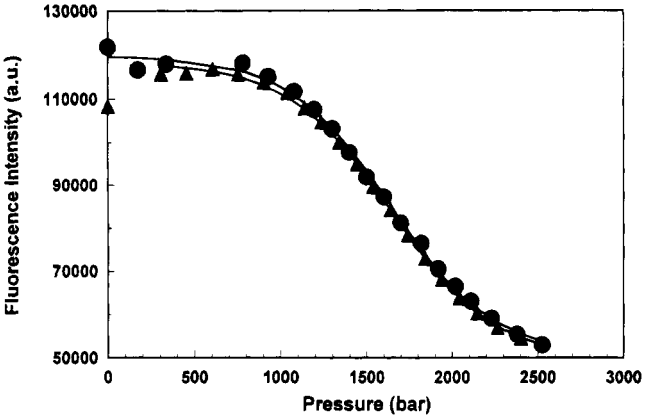

FIGURE 2: Equilibrium fluorescence intensity values as a function of pressure. These values indicate the equilibrium extent of reaction at each pressure from the (●) positive and (▲) negative pressure jumps. The line through the data represents the fit to eq 1 in terms of ΔG_f and ΔV_f. The scale of the y-axis is in arbitrary units (au).

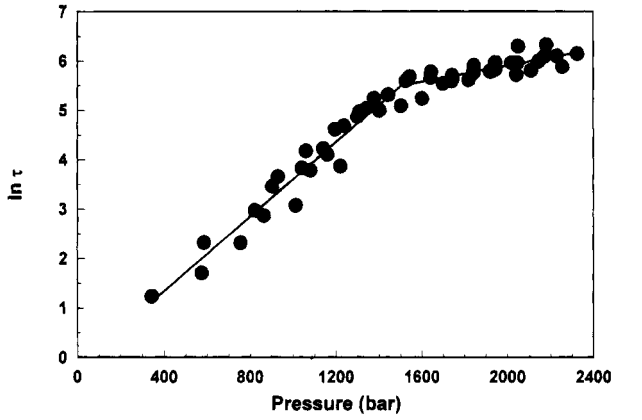

FIGURE 3: Natural logarithm of the relaxation time, ln τ, as a function of pressure for two independent positive and negative pressure-jump experiments, one of which corresponds to the data in Figure 1. The relaxation times are those obtained from the nonlinear analysis of the relaxation data according to a single exponential decay model as described in Materials and Methods. Lines through the points represent the results of linear regression analysis of the points above and below the midpoint of the transition as described in the text.

All pressure-jump relaxation profiles above 600 bar could be described reasonably by a single relaxation time, as expected for a two-state reaction with a single rate-limiting step, and the value of the relaxation time increased with increasing final pressures (Figure 1). We note that some complexity in the profiles was evident at low pressures prior to bulk of the unfolding transition and is consistent with the complex unfolding that has been observed for nuclease by other methods at atmospheric pressure (Chen *et al.*, 1992a,b; Nakano *et al.*, 1993). This complexity may involve prolyl peptide bond isomerization as was shown for the complexity at atmospheric pressure, and this possibility is currently under investigation. The fact that the relaxation profiles are single-exponential decay functions at pressures in the range of the global unfolding transition as monitored by both NMR and fluorescence indicates that the pressure dependence of any intermediate steps is such that they are complete by 600 bar.

Above 600 bar the system behaves as a simple two-state system undergoing small perturbations near equilibrium. For such systems, the observed relaxation time τ at a given pressure is represented by the inverse of the sum of the two individual rate constants for the forward and backward

Table 1: Pressure-Jump Relaxation Kinetic Parameters

pressure range (bar)	activation volumes (mL/mol)	reaction rates at 1 atm (s^{-1})
<1500	$\Delta V^{\ddagger}_f = +92 \pm 4$	$k^{\circ}_f = 1.2 \pm 0.2$
>1500	$\Delta V^{\ddagger}_u = +20 \pm 3$	$k^{\circ}_u = 0.014 \pm 0.004$

reactions, unfolding and folding

$$\tau = 1/(k_u + k_f) \quad (3)$$

and as such should be independent of the sign of the perturbation (Eigen & de Maeyer, 1963). At pressures lower than the equilibrium midpoint (<1500 bar), the folding rate predominates in the sum, while above the midpoint, the unfolding rate predominates. It is clear from simple inspection of the plot of ln τ vs pressure (Figure 3) that the slope of the line is much larger below the midpoint (1500 bar) than above. Linear regression analysis according to eq 2 of the plot of ln τ vs pressure (Figure 3) at pressures below 1500 bar yielded an activation volume for folding of +92 mL/mol from the slope and a folding rate constant at atmospheric pressure of 1.2 s^{-1} from the intercept (Table 1), consistent with the time scales for these reactions at atmospheric pressure (Chen *et al.*, 1992a,b; Nakano *et al.*, 1993). Above 1500 bar, where the unfolding rate predominates, the activation volume for the unfolding transition was only +20 mL/mol, and the unfolding rate constant at atmospheric pressure was 0.014 s^{-1} (Table 1). The ratio of the rate constants at atmospheric pressure corresponds to an equilibrium constant of 85 for the folding equilibrium, consistent with the fact that the protein is predominantly folded prior to pressurization. Given the standard deviation on the regression, this leads to a free energy of unfolding at atmospheric pressure of 2.6 ± 0.3 kcal/mol. This value is consistent, within error, with the free energy of unfolding at atmospheric pressure obtained from the analysis of the equilibrium unfolding profile in Figure 2 (3.2 ± 0.3 kcal/mol), as expected for a two-state unfolding. Also as expected for a two-state transition, the difference between $\Delta V^{\ddagger}_f$ and $\Delta V^{\ddagger}_u$ (+72 mL/mol) was found to be equivalent within experimental error to the equilibrium volume change ΔV_f for folding of nuclease (+77 mL/mol) obtained from the fit of the equilibrium unfolding and refolding profiles (Figure 2). These values for the free energies and volume changes are smaller than those (3.75 kcal/mol and 92 mL/mol) obtained in previous studies (Royer *et al.*, 1993). However, with sufficient equilibration, the present results are quite reproducible. Differences between them and earlier work most likely arise from incomplete equilibration in the earlier studies.

Thus, the volume of the protein−solvent system in the transition state is significantly larger than in the unfolded state and somewhat larger than in the folded state. The fact that the activation volume for folding $\Delta V^{\ddagger}_f$ is greater than that of unfolding $\Delta V^{\ddagger}_u$ means that high pressure unfolds proteins because the rate of folding is slowed significantly more by pressure than that of unfolding. What image of the structure of the transition state do these volume differences provide? Pressure-induced unfolding arises from a combination of increased solvation and decreased excluded volume in the unfolded state. Thus, it is reasonable to interpret the large increase in volume between the unfolded and transition states as resulting from collapse of the unfolded

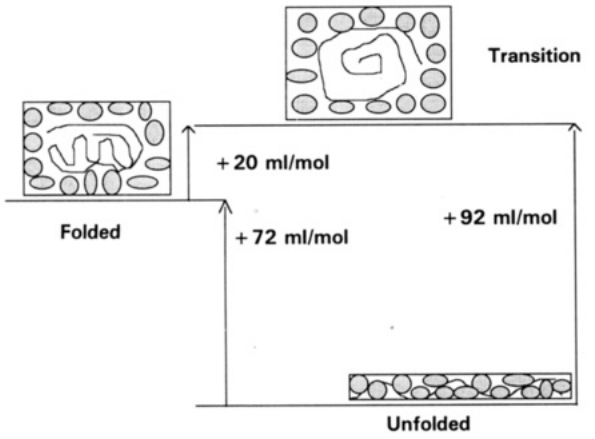

FIGURE 4: Schematic representation of the physical basis for the observed volume changes. Small ovals represent water molecules; the polypeptide chain is represented by a dark line. In the unfolded state, the polypeptide chain is efficiently hydrated and includes few solvent-excluded voids. Thus the system volume is lowest in this state. In the transition state, the chain is collapsed to a state that excludes water molecules, but it is not as well packed as the folded state. Therefore, the system occupies the largest volume in the transition state.

polypeptide chain to a loosely packed globule from which solvent is excluded. The decrease in volume observed between the transition state and the folded state most likely arises from more efficient packing in the final folded structure (Figure 4). Although these volume relationships were predicted 25 years ago (Lumry & Biltonen, 1968), the present results are the first to provide their clear demonstration and quantification. This molten globule-like picture of the transition state is consistent with that inferred recently from differential energetics of wild-type and mutant chymotrypsin inhibitor 2 (Otzen *et al.*, 1994). Our results are consistent with a model in which hydrophobic collapse with concomitant desolvation is the rate-limiting step in the folding of the polypeptide chain and solvent-excluded expansion of the folded state is the rate-limiting step in protein unfolding.

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