Pressure-Jump Small-Angle X-Ray Scattering Detected Kinetics of Staphylococcal Nuclease Folding

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ABSTRACT The kinetics of chain disruption and collapse of staphylococcal nuclease after positive or negative pressure jumps was monitored by real-time small-angle x-ray scattering under pressure. We used this method to probe the overall conformation of the protein by measuring its radius of gyration and pair-distance-distribution function $p(r)$ which are sensitive to the spatial extent and shape of the particle. At all pressures and temperatures tested, the relaxation profiles were well described by a single exponential function. No fast collapse was observed, indicating that the rate limiting step for chain collapse is the same as that for secondary and tertiary structure formation. Whereas refolding at low pressures occurred in a few seconds, at high pressures the relaxation was quite slow, ~1 h, due to a large positive activation volume for the rate-limiting step for chain collapse. A large increase in the system volume upon folding implies significant dehydration of the transition state and a high degree of similarity in terms of the packing density between the native and transition states in this system. This study of the time-dependence of the tertiary structure in pressure-induced folding/unfolding reactions demonstrates that novel information about the nature of protein folding transitions and transition states can be obtained from a combination of small-angle x-ray scattering using high intensity synchrotron radiation with the high pressure perturbation technique.

INTRODUCTION

The reversible folding of small, single-domain proteins often conforms reasonably well to a first order phase transition between the folded and unfolded forms. However, in order to analyze a protein system within this framework, it is important to investigate as many order parameters for the protein structure as possible, using a large number of experimental observables. For example, due to high sensitivity and rapid response times, the kinetics of protein folding is typically assessed by UV fluorescence, which reports on tertiary structure local to the intrinsic fluorophores of the protein (tyrosine and tryptophan), and circular dichroism (or laser T-jump IR), which provides an assessment of the global secondary structural properties. More detailed information can be obtained by quench-flow NMR, which can inform on both secondary and tertiary structure throughout the protein sequence. Recently, thanks to improvements in sensitivity, a number of studies have appeared of protein folding kinetics using stopped-flow small-angle x-ray scattering to ascertain the evolution of the degree of chain collapse (Semisotnov et al., 1996; Chen et al., 1998; Segel et al., 1999; Plaxco et al., 1999). This order parameter is of particular interest, because models of protein folding have been proposed in which rapid chain collapse could be followed by a rate-limiting step which involves a reorganization of the chain (Dill et al., 1995).

Like simple first-order phase transitions, protein folding is accompanied by a change in enthalpy and heat capacity, as well as by a change in volume. Thus, although the study of protein folding has been undertaken primarily using perturbation by temperature or chemical denaturants, a complete thermodynamic description of the folding and unfolding reactions requires the characterization of the response of the protein structure to pressure (see, for example, Bridgman, 1914; Kauzmann, 1987; Heremans, 1982; Silva and Weber, 1993; Silva et al., 1994; Gross and Jaenicke, 1994). This pressure response is governed by the volume changes (protein + solvent) associated with folding and unfolding, and their determination provides unique insight concerning issues of packing and hydration. Most proteins studied to date exhibit a decrease in specific volume upon unfolding (Royer, 1999; Frye and Royer, 1998; Mozhaev et al., 1996; Silva and Weber, 1993) such that the application of pressure results in a perturbation of the equilibrium toward the unfolded state. The underlying molecular basis for this decrease in system volume upon unfolding has been attributed to the combined effects of electrostriction of water molecules around newly exposed charged and polar moieties, the decrease in specific volume of hydrophobic residues upon transfer from a nonpolar protein interior to water, and the elimination of packing voids upon unfolding (Royer, 1999; Mozhaev et al., 1996). Though the relative contribution of each of these effects remains subject to debate, it is clear that they all involve an increase in the hydration of the protein. Thus pressure studies provide

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unique information concerning packing and hydration in the folding and unfolding process.

Staphylococcal nuclease (SNase) is a small, reversibly folding single-domain protein that has served as a model for the study of protein folding for some time. The folding of this protein seems to conform reasonably well to a two-state model at equilibrium, using chemical denaturants and differential scanning calorimetry, although certain solution conditions or mutations can result in the population of stable intermediates (Shortle and Meeker, 1986; Shortle et al., 1989; Shortle, 1995; Ionescu and Eftink, 1997; Carra et al., 1994; Carra and Privalov, 1995). Moreover, the folding kinetics of SNase are complex in both denaturant and pH-jump experiments, in part due to prolyl peptide bond isomerization in the native and denatured states (Chen et al., 1992a,b; Nakano et al., 1993; Ikura et al., 1997; Walkenhorst et al., 1997) and in part due to non-concomitant folding of the two subdomains (Walkenhorst et al., 1997). In contrast to denaturant- and pH-jump kinetics, pressure-jump fluorescence and Fourier transform infrared spectroscopy (FTIR) relaxation profiles of the folding/unfolding of SNase have been shown previously to exhibit identical mono-exponential kinetics, indicating that under pressure, both secondary structure formation (for both α-helix and β-sheet) and tertiary structure formation conform to a simple two-state model (Vidugiris et al., 1995; Panick et al., 1998, 1999) with the same rate-limiting step. Apparently, the non-native prolyl peptide bond isomers are not significantly populated at high pressures, in either the native or denatured states, and moreover, the subdomains fold cooperatively under these conditions. Thus, folding and unfolding kinetics using the pressure perturbation reflect directly the global folding process for this protein.

Previous equilibrium small-angle x-ray scattering (SAXS) studies by our groups demonstrated that increasing pressure in the range of 1 bar to 3.5 kbar results in a transition in the value of the radius of gyration, R_g, of SNase from ~18 to ~33 Å (Panick et al., 1998, 1999). However, the 10-min acquisition time in these previous high-pressure SAXS measurements precluded the detection of fast-forming collapsed intermediates, which have been proposed to occur in the folding process of some proteins (Dill et al., 1995; Chan and Dill, 1998). In order to ascertain whether chain collapse occurs before or concomitant with the rate-limiting step in folding of SNase as assessed by FTIR and fluorescence, we have carried out pressure-jump SAXS with a 1-s dead time performed at the high intensity APS synchrotron source at Argonne National Laboratory. To our knowledge, these measurements are the first of their kind.

MATERIALS AND METHODS

SNase was overproduced and purified as previously described (Panick et al., 1998). Briefly, recombinant staphylococcal nuclease with the sequence of nuclease A from the V8 strain of *Staphylococcus aureus* was obtained using the λ expression system in the *Escherichia coli* strain Arλ9 as described by Shortle and Lin (1985). The cells were grown according to the procedure described by Shortle et al. (1989), except that SB rather than MOPS media was employed. The protein purification was carried out according to the method described by Shortle and Meeker (1986) with modifications described by Frye et al. (1996).

Sample concentration was 1 weight % SNase in 50 mM bis-Tris, pH 5.5, at either 25 or 40°C. The high-pressure apparatus was as previously described (Panick et al., 1998; Woenckhaus et al., 2000). The high-pressure SAXS sample cell is equipped with flat diamond windows of 0.8 mm thickness (type IIa quality, Drukker, Cuijk, The Netherlands), and the optical pathlength of the sample cell is 1.5 mm (for details, see Woenckhaus et al., 2000). The pressure cell is equipped with a thermostatable jacket. The temperature accuracy is ±0.05°C by using a computer-controlled thermostat. SAXS measurements were made at the third generation synchrotron APS, Argonne National Laboratories, at the beamline 12-ID BESSRC-CAT (Seifert et al., 2000). After subtraction of the background scattering using the pure buffer solution data, taking into account the different absorption factors, data evaluation of the SAXS measurements was performed using the indirect Fourier-transformation method (Glatter, 1977, 1979; Müller and Glatter, 1982).

The pair distance distribution function $p(r)$, which depends on the molecular particle shape and on the intra-particle scattering distribution, is given by the indirect Fourier transform of the measured scattered intensity (Glatter and Kratky, 1982). For a particle of uniform electron density, it is given by

$$p(r) = \frac{1}{2\pi^2}\int_0^\infty I(Q)Qr\sin(Qr)\mathrm{d}Q \tag{1}$$

where Q is the magnitude of the scattering vector defined by

$$Q = \frac{4\pi}{\lambda}\sin\theta \tag{2}$$

(λ is the wavelength of the radiation, and 2θ is the scattering angle). The function $p(r)$ represents the frequency of vector length r connecting small volume elements within the volume of the scattering particle, that is, the protein molecule, with maximum dimension D_{max}. As the use of the Guinier approximation for the determination of the radius of gyration, R_g, might lead to errors caused by concentration or aggregation effects, we used the normalized second moment of the pair distance distribution function for calculation of R_g:

$$R_g^2 = \frac{\int_0^{D_{max}} p(r)r^2\mathrm{d}r}{2\int_0^{D_{max}} p(r)\mathrm{d}r} \tag{3}$$

As a first estimate of the cutoff distances employed in the indirect Fourier transform method of the SAXS analysis for calculation of R_g, values of $p(r) \approx 0$ and $D_{max} \approx 4R_g$ (with R_g obtained from the Guinier plots) were used.

For a two-state protein system, the measured R_g is an average radius of gyration of the species in solution (Segel et al., 1999):

$$R_g^2 = f_f R_{g,f}^2 + f_u R_{g,u}^2 \tag{4}$$

where f_f is the native fraction (folded state) and f_u the unfolded fraction ($f_f + f_u = 1$). Since R_g^2 maintains a linear dependence upon the fractional populations, R_g^2 is the relevant quantity to fit.

Pressure-jump fluorescence measurements were carried out as previously described (Vidugiris et al., 1995, 1996). The optical pressure cell used in these experiments was similar to that previously described, except that the windows were sapphire and the seal was a C-seal, rather than a Bridgman seal on all plugs. After pressure jumps spectra were acquired

every 6 to 130 s, depending upon the relaxation times, with an integration time of 6 s. The emission detection system was back illuminated UV/VIS (512 × 512 pixels) MTE-CCD detector. Upon unfolding, emission spectra shifts to the red followed the recorded loss in intensity. Intensity values are those obtained at the weighted average emission wavelength. Protein concentration was approximately 15 μM, and the buffer conditions were 10 mM bis-Tris, pH 5.5. A temperature controller bath was coupled to a brass thermostating jacket around the high-pressure cell.

The equilibrium fluorescence intensity profiles versus pressure were fit for the free enthalpy ΔG_u^o and volume change ΔV_u^o of unfolding under the particular solution conditions of each experiment, using

$$d(\Delta G_u^o)/dp = \Delta V_u^o \quad (5)$$

These fluorescence intensities were fit assuming a two-state model with a folded and an unfolded state as populated species, so that

$$\Delta G_u^o(p) = -RT \ln K_u(p) = -RT \ln[f_u(p)/f_f(p)] \quad (6)$$

Observable mapping was carried out by assigning adjustable fluorescence intensity plateau values to both the folded and unfolded states. The free enthalpy and volume change parameters were adjusted using a Levenberg-Marquardt nonlinear least squares algorithm until the minimum χ^2 value was attained.

RESULTS AND DISCUSSION

The rapid decrease of pressure for a solution of SNase at 25°C from, for example, 4000 bar (denaturing conditions) to 800 bar (native conditions) results in a relatively rapid decrease in the value of the radius of gyration, R_g, from near 29 to 18 Å (Fig. 1). For a random coil of the chain length of SNase (149 amino acids), one would expect a much larger value for R_g, 45 Å (Miller and Goebel, 1968), and thus the pressure-denatured state of SNase, like the urea-denatured state (Flanagan et al., 1992), is not completely random coil in nature. The observed pressure-jump relaxation profile for the decrease in R_g fits well to a single exponential decay with a time constant τ of 4.5 s. Pressure jumps from 4000 bar to ambient pressure conditions yield similar results. At 40°C the relaxation times are quite similar, near 5 s (data not shown). No burst phase is apparent in the profiles of R_g, demonstrating the absence of any significantly populated collapsed intermediates in the refolding reaction of SNase under pressure.

In contrast, a positive pressure jump at 25°C from 1500 bar (near-native conditions) to 3000 bar (fully denaturing conditions) results in a very slow relaxation of R_g from 20 to 31 Å (Fig. 2). Complete relaxation is observed only after 1 h. As for the negative pressure jumps, the positive pressure jump profile is well fit by a single exponential function, with a much longer time constant of τ = 14 min.

More than 40 min after a positive pressure jump from 1000 to 3000 bar at 25°C, the pair distribution function $p(r)$ of SNase (Fig. 3) evolves from a symmetrical bell-shaped peak indicative of a nearly spherical molecule under native conditions to a more complex distribution at high pressure, resembling that for an ellipsoidal particle. The maximal real-space dimension of the pressure-denatured state is near 110 Å, which is smaller than would be expected for a random coil chain, yet twice that observed for the native state, indicating significant disruption of the protein structure under pressure. No evidence for an intermediate form is apparent in the time-evolution of the pair distribution function, consistent with the single exponential profiles for the evolution of R_g.

In simple two-state kinetics, the pressure dependence of the experimentally determined relaxation times τ can be fit to the following function (Eigen and de Maeyer, 1963):

$$\ln \tau(p) = \ln[k_u(p) + k_f(p)]^{-1} \quad (7)$$

where

$$k_u(p) = k_{u0}\exp[-(p - p_0)\Delta V_u^{\ddagger}/(RT)] \quad (8)$$

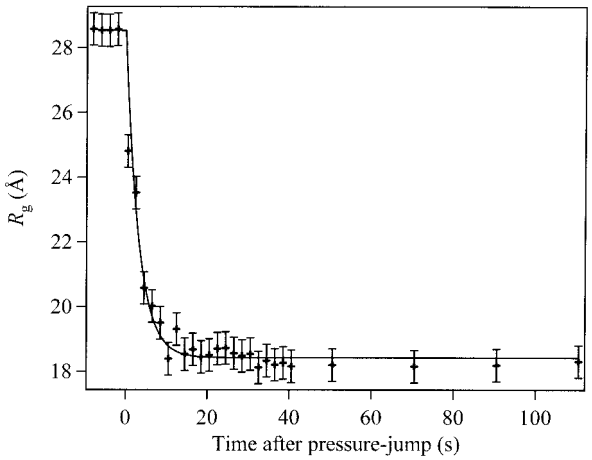

FIGURE 1 Time evolution of the radius of gyration R_g after a negative pressure jump from 4000 bar denaturing conditions) to 800 bar (native conditions). The fit of data to a single exponential decay function (*line*) yields a time constant of 4.5 s.

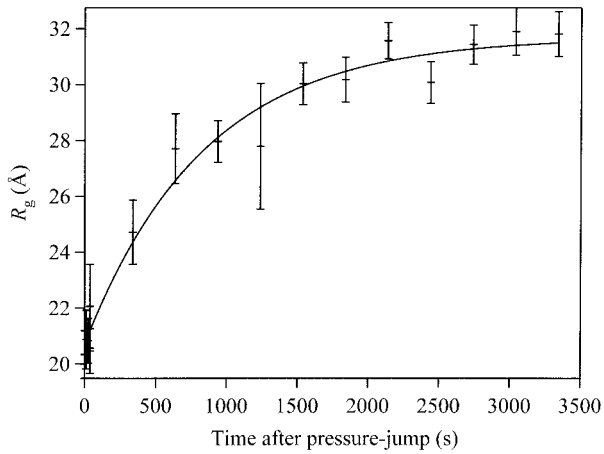

FIGURE 2 Time dependence of the increase in R_g after a positive pressure jump from 1500 bar (near-native conditions) to 3000 bar (denaturing conditions). The fit of the data to a single exponential function (*line*) yields a time constant of 14 min.

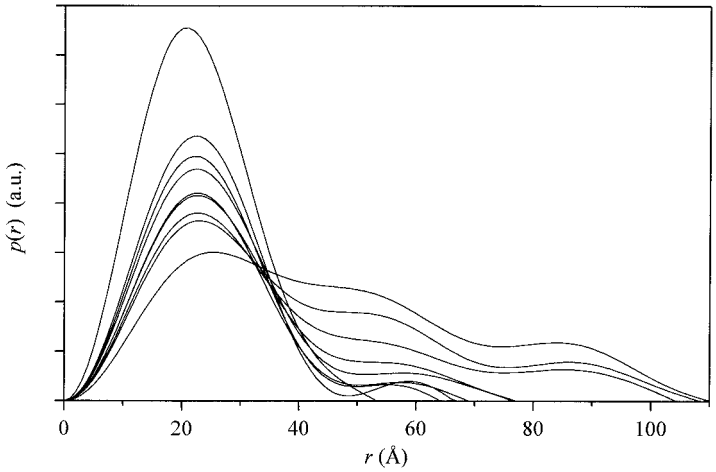

FIGURE 3 Pair distribution function $p(r)$ for SNase at 5-min intervals after a pressure jump from 1000 bar (native conditions) to 3000 bar (denaturing conditions). The first scan was acquired 5 s after and the last 40 min after the pressure jump.

and

$$k_f(p) = k_{f0}\exp[-(p - p_0)\Delta V_f^{\ddagger}/(RT)] \quad (9)$$

where k_{f0} and k_{u0} are the rate constants for folding and unfolding at atmospheric pressure (p_0) and $\Delta V_f^{\ddagger}$ and $\Delta V_u^{\ddagger}$ are the activation volumes for folding and unfolding.

The values of $k_u(p)$ and $\Delta V_u^{\ddagger}$ are constrained as follows:

$$k_u(p) = k_f(p)K_u(p) \quad (10)$$

and

$$\Delta V_u^{\ddagger} = \Delta V_f^{\ddagger} - \Delta V_f^{\circ} \quad (11)$$

using the values of the equilibrium constant for unfolding and the equilibrium volume change upon folding, $K_u(p)$ and ΔV_f° ($1.5 \cdot 10^{-3}$ and 81 mL/mol, respectively), obtained from the fit of the equilibrium fluorescence high pressure unfolding profile (Fig. 4). We have shown previously good agreement between the equilibrium pressure-induced unfolding profiles obtained from fluorescence and FTIR (Panick et al., 1999).

The fit to Eq. 7 of the pressure dependence of the relaxation times obtained from the SAXS data (Fig. 5, *triangles*) yields a large positive value for $\Delta V_f^{\ddagger}$ of 89 ± 10 mL/mol. The agreement between the present results, based on observation of chain collapse, with those obtained by fluorescence ($\Delta V_f^{\ddagger}$ = 99 ± 12 mL/mol, repeated here for comparison, Fig. 5, *open circles*) and FTIR (Panick et al., 1998) demonstrates that chain collapse depends upon the same rate-limiting step as that for secondary structure formation and tertiary structural contacts in the vicinity of the tryptophan residue of SNase. No rapidly formed collapsed intermediates are apparent, indicating that in the case of pressure denatured SNase, as for Protein L (Plaxco et al., 1999), folding does not involve prior condensation of the chain.

Refolding of SNase from high guanidine hydrochloride concentrations exhibits a complex kinetics (Carra and Privalov, 1995; Chen et al., 1992a,b; Nakano et al., 1993; Ikura et al., 1997). Even for SNase mutants in which all of the proline residues have been replaced, NMR evidence (Walkenhorst et al., 1997) indicates the population of a collapsed intermediate in the β-barrel subdomain upon refolding from high guanidine hydrochloride, to which tryptophan fluorescence is insensitive. However, refolding from the pressure-denatured state is shown here by small-angle x-ray scattering, at least within the accuracy of the experiment, to proceed without the population of any collapsed intermediates, and previous pressure-jump FTIR studies showed no evidence for rapid formation of β-type structure (Panick et al., 1998). Thus, the folding landscape appears to be much smoother under pressure. Moreover, an increase in specific volume associated with the rate-limiting step for chain collapse implicates a significant degree of dehydration associated with this process.

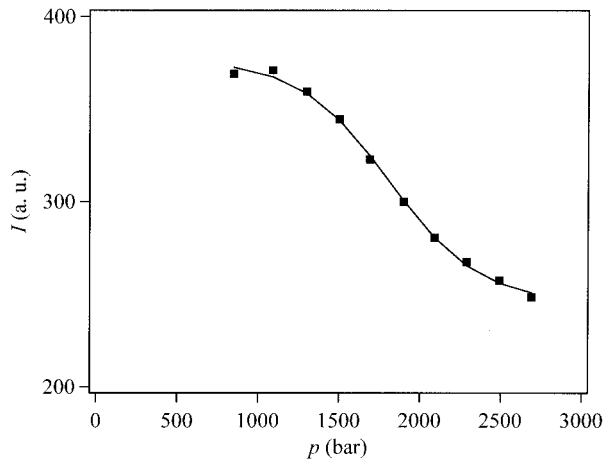

FIGURE 4 Equilibrium pressure induced unfolding profile of SNase as observed by fluorescence intensity in 50 mM bis-Tris buffer, pH 5.5, at 21°C. The line through the points represents the fit to the data with the ΔG_u° = 15.9 kJ/mol and the ΔV_u° = − 81.6 mL/mol as described in the text.

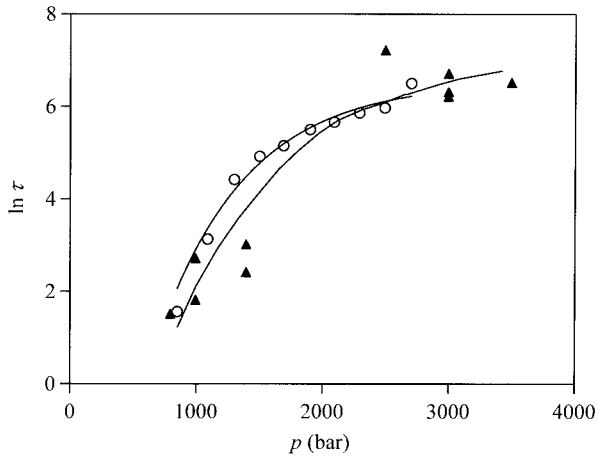

FIGURE 5 Comparison of the pressure-jump relaxation results from SAXS and fluorescence. Dependence of the natural logarithm of the relaxation time from fits of the SAXS (▲) and fluorescence (○) data as a function of pressure.

At 40°C, the relaxation times for the R_g profiles measured under high pressure (denaturing) conditions were found to be approximately 10-fold faster than those observed at 25°C (~25 s, data not shown). This is consistent with a decrease in the value of $\Delta V_f^{\ddagger}$ with increasing temperature (also observed in fluorescence relaxation experiments; Panick et al., 1999), since at 40°C, the effect of pressure on the folding rate constant is much smaller than it is at 25°C. The change in specific volume of activation with temperature ($d\Delta V_f^{\ddagger}/dT$) corresponds to the difference in the coefficient of thermal expansion between the transition state and the unfolded state, $\Delta\alpha$, and a negative value indicates that the thermal expansion of the transition state is smaller than that of the unfolded state. A decrease in thermal expansivity in the transition state indicates that significant structural constraints are introduced at the rate-limiting step.

As a further means of characterizing the properties of the transition state one can calculate a $\Phi(V)$ value, reminiscent of the mutational Φ values used for characterizing the amino acid residues that participate in the structure of the transition state (Fersht et al., 1992; Fersht, 1999). The $\Phi(V)$ value represents a measure of the degree of likeness of the transition and native states in terms of specific volume and can be written as the ratio of the activation and equilibrium volume changes for folding:

$$\Phi(V) = \frac{\left(\frac{\partial \Delta G_f^{\neq}}{\partial p}\right)}{\left(\frac{\partial \Delta G_f^{o}}{\partial p}\right)} = \frac{\Delta V_f^{\neq}}{\Delta V_f^{o}} \qquad (12)$$

In the case of SNase, we show here that for the transition as monitored by chain collapse, this value is approximately unity (~89/81), indicating that the transition state is highly native-like. The molecular basis for the decrease in system volume upon unfolding of proteins remains to be fully elucidated. Investigations of *m*-value and cavity mutants of SNase (Frye et al., 1996; Frye and Royer, 1998) suggest that packing defects may contribute more significantly to the value of ΔV_u^o than electrostriction and hydrophobic hydration. The fact that the rate-limiting step for chain collapse of SNase involves a large increase in system volume suggests that in the transition state, the packing of the protein interior is quite similar to that of the native state, and that this rate-limiting step involves significant dehydration. In the case of SNase, chain collapse depends upon the same rate-limiting step as does the formation of the secondary structure and tertiary contacts.

This study of the time dependence of chain collapse in pressure-induced folding/unfolding reactions demonstrates that novel information about the nature of protein folding transitions and transition states can be obtained from a combination of high sensitivity SAXS with the high pressure perturbation. The stage is now well set for work addressing more complex questions, such as the study of the folding reaction of oligomers and protein complex formation (see, e.g., Mohana-Borges et al., 1999).

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REFERENCES

Bridgman, P. W. 1914. The coagulation of albumen by pressure. *J. Biol. Chem.* 19:511–512.

Carra, J. H., E. A. Anderson, and P. L. Privalov. 1994. Three-state thermodynamic analysis of the denaturation of Staphylococcal nuclease mutants. *Biochemistry.* 33:10842–10850.

Carra, J. H., and P. L. Privalov. 1995. Energetics of denaturation and *m* values of staphylococcal nuclease mutants. *Biochemistry.* 34: 2034–2041.

Chan, H. S., and K. A. Dill. 1998. Protein folding in the landscape perspective: chevron plots and non-Arrhenius kinetics. *Proteins Struct. Funct. Genet.* 30:2–33.

Chen, H. M., V. S. Markin, and T. Y. Tsong. 1992a. pH-induced folding/unfolding of staphylococcal nuclease: determination of kinetic parameters by the sequential-jump method. *Biochemistry.* 31:1483–1491.

Chen, H. M., V. S. Markin, and T. Y. Tsong. 1992b. Kinetic evidence of microscopic states in protein folding. *Biochemistry.* 31:12369–12375.

Chen, L., G. Wildegger, T. Kiefhaber, K. O. Hodgson, and S. Doniach. 1998. Kinetics of lysozyme refolding: structural characterization of a non-specifically collapsed state using time-resolved X-ray scattering. *J. Mol. Biol.* 276:225–237.

Dill, K. A., S. Bromberg, K. Yue, K. M. Fiebig, D. P. Yee, P. D. Thomas, and H. S. Chan. 1995. Principles of protein folding: a perspective from simple exact models. *Protein Sci.* 4: 561–602.

Eigen, M., and L. de Maeyer. 1963. Relaxation Methods in Techniques in Organic Chemistry. Weissberger, A., ed. Wiley, New York. 895–1054.

Fersht, A. R., A. Matouschek, and L. Serrano. 1992. The folding of an enzyme. I. Theory of protein engineering analysis of stability and pathway of protein folding. *J. Mol. Biol.* 224:771–782.

Fersht, A. 1999. Structure and Mechanism in Protein Science. W. H. Freeman and Company, New York.

Flanagan, J. M., M. Kataoka, D. Shortle, and D. M. Engelman. 1992. Truncated staphylococcal nuclease is compact but disordered. *Proc. Natl. Acad. Sci. USA.* 89:748–752.

Frye, K. J., C. S. Perman, and C. A. Royer. 1996. Testing the correlation between ΔA and ΔV of protein unfolding using *m*-value mutants of staphylococcal nuclease. *Biochemistry.* 35:10234–10239.

Frye, K. J., and C. A. Royer. 1998. Probing the contribution of internal cavities to the volume change of protein unfolding under pressure. *Protein Sci.* 7:2217–2222.

Glatter, O., and O. Kratky. 1982. Small Angle X-ray Scattering. Academic Press, New York.

Glatter, O. 1977. Data evaluation in small-angle scattering: calculation of the radial electron density distribution by means of indirect Fourier transformation. *Acta Physica Austriaca.* 47:83–102.

Glatter, O. 1979. The interpretation of real-space information from small-angle scattering experiments. *J. Appl. Crystallogr.* 12:166–175.

Gross, M., and R. Jaenicke. 1994. Proteins under pressure. *Eur. J. Biochem.* 221:617–630.

Heremans, K. 1982. High pressure effects on proteins and other biomolecules. *Annu. Rev. Biophys. Bioeng.* 11:1–21.

Ikura, T., G. P. Tsurupa, and K. Kiwajima. 1997. Kinetic folding and cis/trans prolyl isomerization of staphylococcal nuclease: a study by stopped-flow absorption, stopped-flow circular dichroism, and molecular dynamics simulations. *Biochemistry.* 36:6529–6538.

Ionescu, R. M., and M. R. Eftink. 1997. Global analysis of the acid-induced and urea-induced unfolding of staphylococcal nuclease and two of its variants. *Biochemistry.* 36:1129–1140.

Kauzmann, W. 1987. Thermodynamics of unfolding. *Nature.* 325: 763–764.

Miller, W. G., and C. V. Goebel. 1968. Dimensions of protein random coils. *Biochemistry.* 7:3925–3935.

Mohana-Borges, R., J. L. Silva, J. Ruiz-Sanz, and G. de Prat-Gay. 1999. Folding of a pressure-denatured model protein. *Proc. Natl. Acad. Sci. USA.* 96:7888–7893.

Mozhaev, V. V., K. Heremans, J. Frank, P. Masson, and C. Balny. 1996. High pressure effects on protein structure and function. *Proteins Struct. Funct. Genet.* 24:81–91.

Müller, K., and O. Glatter. 1982. Practical aspects in the use of indirect Fourier transformation methods. *Makromol. Chem.* 183:465–479.

Nakano, T., L. C. Antonino, R. O. Fox, and A. L. Fink. 1993. Effect of proline mutations on the stability and kinetics of folding of staphylococcal nuclease. *Biochemistry.* 32:2534–2541.

Panick, G., R. Malessa, R. Winter, G. Rapp, K. J. Frye, and C. A. Royer. 1998. Structural characterization of the pressure-denatured state and unfolding/refolding kinetics of staphylococcal nuclease by synchrotron small-angle x-ray scattering and Fourier-transform infrared spectroscopy. *J. Mol. Biol.* 275:389–402.

Panick, G., G. J. A. Vidugiris, R. Winter, and C. A. Royer. 1999. Exploring the temperature-pressure phase diagram of staphylococcal nuclease. *Biochemistry.* 38:4157–4164.

Plaxco, K. W., I. S. Millett, D. J. Segel, S. Doniach, and D. Baker. 1999. Chain collapse can occur concomitantly with the rate-limiting step in protein folding. *Nat. Struct. Biol.* 6: 554–556.

Royer, C. A. 1999. *In* High Pressure Molecular Science, R. Winter, and J. Jonas, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands. 473–495.

Segel, D. J., D. Eliezer, V. Uversky, A. L. Fink, K. O. Hodgson, and S. Doniach. 1999. Transient dimer in the refolding kinetics of cytochrome c characterized by small-angle x-ray scattering. *Biochemistry.* 38: 15352–15359.

Seifert, S., R. E. Winans, D. M. Tiede, and P. Thiyagarajan. 2000. Design and performance of a ASAXS instrument at the advanced photon source *J. Appl. Crystallogr.* 33:782–784.

Semisotnov, G. V., H. Kihara, N. V. Kotova, K. Kimura, Y. Amemiya, K. Wakabayashi, I. N. Serdyuk, A. A. Timchenko, K. Chiba, K. Nikaido, T. Ikura, and K. Kuwajima. 1996. Protein globularization during folding: a study by synchrotron small-angle x-ray scattering. *J. Mol. Biol.* 262: 559–574.

Shortle, D., and B. Lin. 1985. Genetic analysis of staphylococcal nuclease: identification of three intragenic global suppressors of nuclease-minus mutations. *Genetics.* 110:539–555.

Shortle, D. 1995. Staphylococcal nuclease: a showcase of *m*-value effects. *Adv. Protein Chem.* 46:217–247.

Shortle, D., and A. K. Meeker. 1986. Mutant forms of staphylococcal nuclease with altered patterns of guanidine hydrochloride and urea denaturation. *Proteins Struct. Funct. Genet.* 1:81–89.

Shortle, D., A. K. Meeker, and S. L. Gerring. 1989. Effects of denaturants at low concentrations on the reversible denaturation of staphyloccocal nuclease. *Arch. Biochem. Biophys.* 272:103–113.

Silva, J. L., and G. Weber. 1993. Pressure stability of proteins. *Annu. Rev. Phys. Chem.* 44:89–113.

Silva, J. L., X. Peng, and J. Jones. 1994. High-pressure NMR study of the dissociation of *arc* repressor. *Biochemistry.* 33:8323–8329.

Vidugiris, G. J. A., J. L. Markley, and C. A. Royer. 1995. Evidence for a molten globule-like transition state in protein folding from determination of activation volumes. *Biochemistry.* 34:4909–4912.

Vidugiris, G. J. A., D. M. Truckses, J. L. Markley, and C. A. Royer. 1996. High-pressure denaturation of staphylococcal nuclease proline-to-glycine substitution mutants. *Biochemistry.* 35:3857–3864.

Walkenhorst, W. F., S. M. Green, and H. Roder. 1997. Kinetic evidence for folding and unfolding intermediates in Staphylococcal nuclease. *Biochemistry.* 36:5795–5805.

Woenckhaus, J., R. Köhling, R. Winter, P. Thiyagarajan, and S. Finet. 2000. High pressure-jump apparatus for kinetic studies of protein folding reactions using the small-angle synchrotron x-ray scattering technique. *Rev. Sci. Instrum.* 71:3895–3899.