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The kinetic basis for the stabilization of staphylococcal nuclease by xylose

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Abstract

The effect of xylose on the rates of folding and unfolding of staphylococcal nuclease (nuclease) have been investigated using fluorescence-detected pressure-jump relaxation kinetics in order to establish the kinetic basis for the observed stabilization of nuclease by this sugar (Frye KJ, Perman CS, Royer CA, 1996, *Biochemistry* 35:10234–10239). The activation volumes for both folding and unfolding and the equilibrium volume change for folding were all positive. Their values were within experimental error of those reported previously (Vidugiris GJA, Markley JL, Royer CA, 1995, *Biochemistry* 34:4909–4912) and were independent of xylose concentration. The major effect of xylose concentration was to increase significantly the rate of folding. The large positive activation volume for folding was interpreted previously as indicating that the rate-limiting step in nuclease folding involves dehydration of a significant amount of surface area. A large effect of xylose on the rate constant for folding provides strong support for this interpretation, because xylose, an osmolyte, stabilizes the folded state of proteins through surface tension effects. These studies further characterize the transition state in nuclease folding as lying closer to the folded, rather than the unfolded state along the folding coordinate in terms of the degree of burial of surface area. The image of the transition state that emerges is consistent with a dry molten globule.

Keywords: osmolytes; pressure; protein folding; staphylococcal nuclease; sugar stabilization

Sugars have been used as protein stabilizers for many years, but it was not until recently that the mechanism of stabilization was established (Timasheff, 1993). Timasheff and coworkers demonstrated by equilibrium dialysis that sugars are preferentially excluded from the surface of proteins (Timasheff, 1993). In other words, in a three-component solution of water, sugar, and protein, proportionally more water molecules, and fewer sugar molecules, are found at the surface of the protein than in the bulk. Addition of sugar to a protein solution therefore results in a net increase in free energy that is proportional to the amount of protein surface area that must be hydrated preferentially. Hence, the addition of sugars favors folding. Glycerol may be an exception, because in certain cases it binds to proteins at specific sites, and its effects may be stabilizing or destabilizing depending upon the observed property. The stabilization of proteins by sugars must involve modifications in either the rate of folding or unfolding or both, but the kinetic basis for this stabilization has never been demonstrated. We were interested in determining the effects of a sugar, xylose, on the folding and unfolding rate constants and deriving information about the degree of exposed surface area in the transition state.

In order to determine the effects of xylose on the rates of folding and unfolding of staphylococcal nuclease (nuclease), pressure-

induced unfolding was studied as a function of xylose concentration. High pressure leads to the unfolding of proteins because the volume of the protein–solvent system is smaller when the protein is in the unfolded state. Pressure-jump relaxation kinetics have revealed that this destabilization occurs due to a large positive activation volume for folding, leading to a significant decrease in the rate of folding upon increasing pressure. The rate of unfolding is also slowed by pressure, but to a much lesser degree (Vidugiris et al., 1995). Nuclease was chosen as our model protein because the wild type (WT) has been shown to be well represented by a simple two-state equilibrium by a number of methods (Schechter et al., 1970; Shortle & Meeker, 1986; Shortle et al., 1988, 1990; Eftink et al., 1991; Royer et al., 1993; Eftink, 1995; Vidugiris et al., 1995), although kinetic (Chen et al., 1992a, 1992b; Nakano et al., 1993), NMR (Fox et al., 1986; Evans et al., 1987, 1989; Alexandrescu et al., 1989, 1990; Loh et al., 1991; Jacobs & Fox, 1994), and calorimetric (Carra et al., 1994) evidence has revealed small populations of intermediates under certain conditions. Previous high-pressure studies of WT nuclease were well described by a two-state transition, except at low pressures, where little unfolding occurs (Royer et al., 1993; Vidugiris et al., 1995, 1996). Thus, the fluorescence signal of nuclease, which decreases in intensity upon unfolding, is representative of the unfolding of the entire protein even though it emanates from a single residue, tryptophan 140 (Shortle & Lin, 1985; Royer et al., 1993; Vidugiris et al., 1995). This is not the case for all proteins. Non-two-state behavior

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has been observed by comparing pressure effects on the NMR signals of individual residues of hen egg white lysozyme (Samarasinghe et al., 1992) and the NMR and fluorescence signals of arc repressor (Peng et al., 1993).

Results

Xylose equilibrium

The equilibrium unfolding of nuclease WT is displaced to higher pressures with increasing xylose concentrations (Fig. 1), consistent with previous results (Frye et al., 1996). The degree to which the equilibrium free energy depends upon the xylose concentration is termed the xylose m -value. It was found to be 153 ± 34 , which is slightly larger than reported previously, but not as well determined (Frye et al., 1996). The observed change in volume upon unfolding is independent of xylose concentration. The results for the equilibrium free energy and volume change of unfolding at the various xylose concentrations are shown in Table 1.

Xylose kinetics

Representative kinetic traces of the pressure-jump relaxation profiles at 5% xylose for the denaturation of nuclease monitored by intrinsic tryptophan fluorescence can be seen in Figure 2. The timescale for reaching equilibrium increases systematically as a function of pressure, as was observed previously (Vidugiris et al., 1995). The relaxation profiles were fit as described in the Materials and methods for their characteristic relaxation times, τ . Plots of the natural logarithm of τ as a function of pressure for the four tested xylose concentrations are depicted in Figure 3A, B, C, and D. It can be seen that $\ln \tau$ increases linearly with pressure up to a break point that corresponds to the midpoint of the equilibrium unfolding curve, and then increases linearly with a much smaller slope. The values for the rate constants for folding were obtained from the intercept of the linear regression of the $\ln \tau$ plots below the midpoint (because k_f predominates in the sum in Equation 3), whereas those for unfolding are derived from the linear regression of the points above the midpoint. The activation volumes, ΔV_f^\ddagger and

Table 1. Comparison of equilibrium and kinetic changes in free energy and volume as a function of xylose concentration

Mole fraction xylose	ΔG_u (kcal/mol) (eq)	ΔG_u (kcal/mol) (kinetics)	ΔV_u (mL/mol) (eq)	ΔV_u (mL/mol) (kinetics)
0.0015	2.2 ± 0.2	2.4 ± 0.4	76.8 ± 4	89.8 ± 17
0.003	2.5 ± 0.2	2.4 ± 0.5	79.1 ± 4	83.6 ± 13
0.006	3.2 ± 0.3	3.0 ± 0.6	86.4 ± 7	92.8 ± 14
0.009	3.3 ± 0.4	$3.6 \pm 1.8/-0.8$	83.8 ± 8	102.1 ± 35

ΔV_u^\ddagger , obtained from the slopes of the plots of $\ln \tau$ versus pressure (Fig. 3) at each xylose concentration are within error of one another and within experimental error of those reported previously for nuclease (Vidugiris et al., 1995, 1996) (Table 2). The rate constants and activation volumes from the kinetic analysis were used to calculate equilibrium values for the change in free energy, ΔG_u , and volume, ΔV_u upon unfolding in order to compare them to those obtained from analysis of the equilibrium profiles. Their values, given in Table 1, are within error of one another, validating the two-state approximation. In Figure 4 are shown the plots of the rate constants of folding and unfolding, k_f° and k_u° , as a function of the mole fraction xylose. The linear fits to the data points reveal that the rate of folding increases significantly with increasing xylose, whereas that of unfolding may decrease slightly, although the effect is much smaller. Thus, we have found that xylose stabilizes nuclease primarily by increasing the rate of folding.

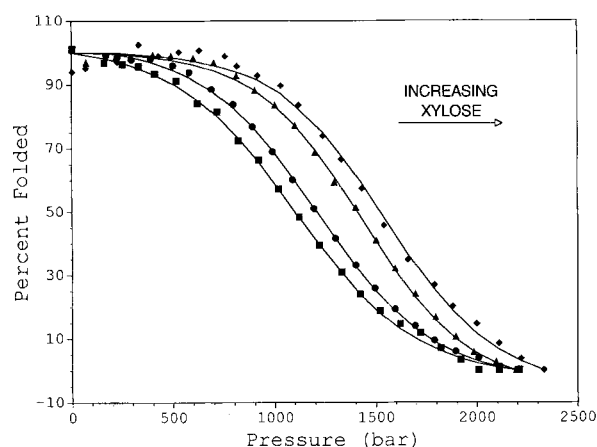


Fig. 1. Unfolding profiles for WT nuclease as a function of pressure and mole fraction xylose at pH 4.5 and 21 °C. Symbols are data from a representative set; lines represent the global curve analysis fits to the data. ■, 1.25% xylose; ●, 2.5% xylose; ▲, 5% xylose; ◆, 7.5% xylose.

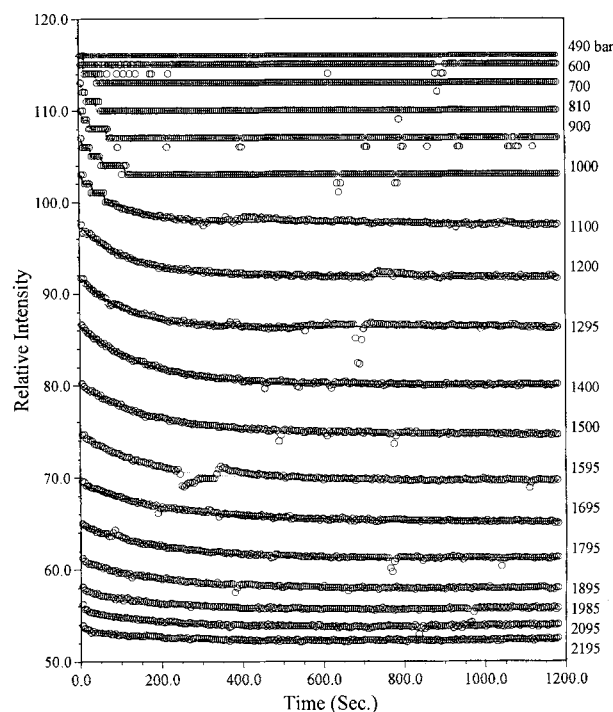


Fig. 2. Representative pressure-jump relaxation profile at 5% xylose for WT nuclease at pH 4.5 and 21 °C. Fluorescence intensity is plotted on the left side of the y axis and the final pressure from each pressure jump is on the right side. Lines through the points represent the results of the global curve analysis fits to the data.

Table 2. Rate constants and activation volumes of folding and unfolding as a function of xylose concentration

Mole fraction xylose	ΔV_f^\ddagger (mL/mol)	ΔV_u^\ddagger (mL/mol)	k_f° (s ⁻¹)	k_u° (s ⁻¹)
0.0015	99.4 ± 12.4	9.6 ± 4.4	0.54 ± 0.26	0.010 ± 0.003
0.003	95.9 ± 7.2	12.3 ± 5.7	0.91 ± 0.2	0.016 ± 0.008
0.006	103.0 ± 8.7	10.2 ± 5.5	1.9 ± 0.7	0.012 ± 0.006
0.009	115.1 ± 24.5	13.0 ± 10.5	3.0 + 8.0/-2.2	0.007 ± 0.010

Discussion

The energetic basis for the stabilization of proteins by osmolytes such as xylose is accepted generally to arise from a preferential exclusion of sugar from protein surfaces that increases the free energy of all states of the protein (Timasheff, 1993). This net increase in free energy for all of the states of the protein is necessarily proportional to the amount of protein surface area exposed in these states. States that expose the most surface area are destabilized to the greatest extent, as depicted in Figure 5. Therefore, the free energy of the unfolded state increases much more than that of the folded state, which is preferentially populated in the presence of xylose. The kinetic basis for this increase in the free energy of unfolding depends upon the effect of xylose on the transition state relative to its effect on the native and denatured states. This in turn depends upon the degree of exposure of surface area of the transition state relative to the native and denatured states. We have found that the kinetic basis for the increase in the ΔG of unfolding upon increasing xylose concentration is due primarily to an increase in the rate of folding, whereas the decrease in the rate of unfolding plays a less significant role. This indicates that a greater degree of surface area is buried between the unfolded and the

transition states than between the latter and the folded state, and suggests that the transition state in nuclease folding lies closer along the folding coordinate to the folded, rather than unfolded state in terms of amount of exposed surface area.

Fersht and coworkers (Serrano et al., 1992) have drawn similar conclusions for barnase based on the effects of denaturant on the rates of unfolding. Our previous interpretation (Vidugiris et al., 1995) of the large positive activation volume for nuclease folding was that the rate-limiting step involves dehydration of a significant amount of surface area, and that the transition state is largely dehydrated. The present results on the effect of xylose on the rate constants provides additional evidence for this interpretation. The resulting picture of the transition state in nuclease folding, and perhaps generally, is consistent with what Finkelstein and Shakhnovich (1989) have termed a dry molten globule, with dehydration constituting the rate-limiting step. Dehydration of protein surfaces manifests itself by a large difference in the heat capacity (ΔC_p) of the different states involved. In fact, Fersht and coworkers (Oliveberg et al., 1995) determined the ΔC_p of activation in barnase folding, implicating an enthalpic, as well as entropic contribution to the activation energy. Such a picture is in contrast to the increasingly popular folding funnel theory (Dill et al., 1995; Socci et al., 1996), in which the barrier to folding consists entirely of the loss in configurational entropy. Further experimental as well as theoretical work is needed to reconcile these inconsistencies.

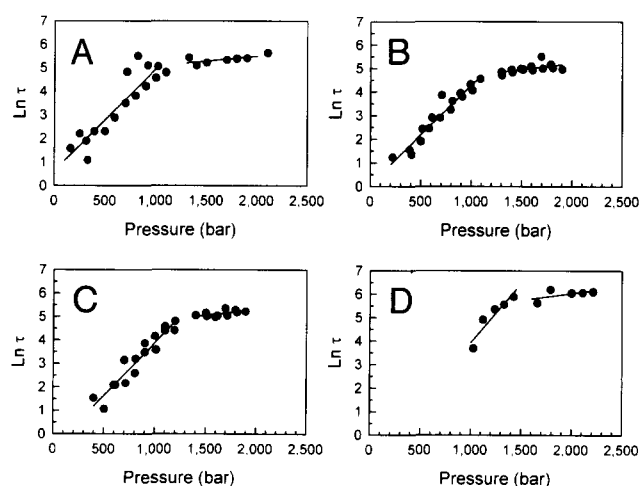


Fig. 3. Natural logarithm of the relaxation time, $\ln \tau$, as a function of pressure. Relaxation times are those obtained from the global analysis of the relaxation data according to a single exponential decay model as described in Materials and methods. Lines through the points represent the results of linear regression analysis of the points above and below the midpoint of the transition as described in the text. **A:** 1.25% xylose. **B:** 2.5% xylose. **C:** 5% xylose. **D:** 7.5% xylose.

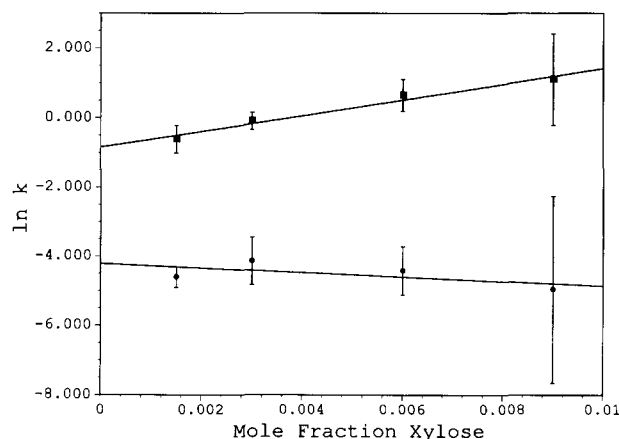


Fig. 4. Natural logarithm of the rate of folding and unfolding, $\ln k_f^\circ$ and $\ln k_u^\circ$, as a function of xylose concentration. Rate constants are those obtained from the linear plots of the relaxation time as a function of pressure as described in Materials and methods. Lines represent linear regression analysis of the points. ■, $\ln k_f^\circ$; ●, $\ln k_u^\circ$.

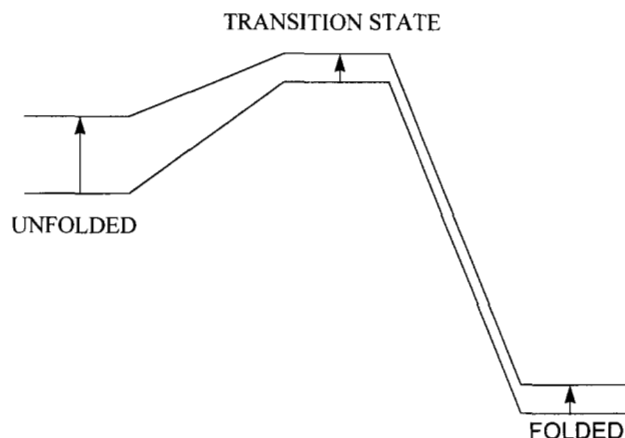


Fig. 5. Free energy diagram of folded, unfolded, and transition states for xylose stabilization of nuclease that emerges from the data presented.

Materials and methods

Protein purification

Staphylococcal nuclease from the nuclease A sequence from the V8 strain of *Staphylococcus aureus* was produced in *Escherichia coli* and purified as described by Alexandrescu et al. (1989) and Royer et al. (1993). Purified protein was stored in concentrated solutions. The final concentration used for experiments was less than 10 μM (as determined by UV absorption at 280 nm and an extinction coefficient of 18,050 $\text{cm}^{-1} \text{M}^{-1}$) in order to prevent aggregation, which can occur at higher protein concentrations. Bis-Tris buffer (10 mM bis Tris, pH 4.5) was used because it exhibits no pressure dependence of the pK_a . The experiments were conducted at four different xylose concentrations (1.25%, 2.5%, 5%, 7.5%) at 21 $^{\circ}\text{C}$.

Pressure-jump experiments

The high-pressure generating system used was similar to that described by Paladini and Weber (1981). Pressure-jump relaxation experiments were performed in a Vascomax high-pressure cell. A UV multifiber bundle (Oriol Corp., Stratford, Connecticut) with focusing optics coupled the exciting light at 295 nm from the monochromator to the pressure cell. Tryptophan emission was monitored at 90 $^{\circ}$ to the excitation light 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 then rapidly reopening the valve. Fluorescence intensity data were acquired with 5-s integration times, which are fast relative to the relaxation time scales. The pressure jumps were small enough that the adiabatic heating of the sample amounted to no more than 0.3 $^{\circ}\text{C}$, which, at the sample temperature of 21 $^{\circ}\text{C}$, would not perturb the folding equilibrium of nuclease significantly. In addition, 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). All pressure runs were >95% reversible.

Data analysis

The pressure-jump fluorescence intensity relaxation profiles were fitted to a single-exponential decay using the time-domain fluo-

rescence global analysis program described by Beechem et al. (1991). The intensity of fluorescence at time t , after the pressure jump $I(t)$ was taken to be an exponentially decaying function:

$$I(t) = I_0 e^{-t/\tau}, \quad (1)$$

where I_0 is the intensity prior to the jump and τ is the relaxation time.

The 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 ΔV^{\ddagger} for the formation of the transition state (Gladstone et al., 1941):

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

For a simple, two-state system undergoing small perturbations near equilibrium, 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 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). We have fitted the values of $\ln \tau$ across the pressure range above and below the midpoint, respectively, for the values of unfolding and folding rate constants at atmospheric pressure (k°_f and $(k^{\circ})_u$), and their individual activation volumes ΔV^{\ddagger}_f and ΔV^{\ddagger}_u , using linear analysis as described previously (Vidugiris et al., 1995).

The values of the intensities 25 min after the jump were taken as the equilibrium intensities, and these equilibrium profiles were fitted using the BIOEQS analysis program for the equilibrium free energy ΔG and volume change ΔV of the unfolding (Royer et al., 1990; Royer & Beechem, 1992; Royer, 1993) according to the relation:

$$d(\Delta G)/dp = \Delta V. \quad (4)$$

The BIOEQS program calculates data points at each pressure from the current parameters, ΔV , ΔG , and the asymptotic values of the folded and unfolded state intensities, I_f and I_u , using the equilibrium constant, K_{eq} , which is calculated from the intensities measured at each pressure, I_p :

$$\Delta G = -RT \ln K_{eq} = -RT \ln (I_f - I_p)/(I_p - I_u), \quad (5)$$

and minimizes on the basis of the χ^2 difference between observed and calculated data. Although the intensity values were fit to obtain the relevant parameters, both data and fits have been normalized in Figure 1 for reasons of comparison.

Estimates of the experimental error of the equilibrium volume changes and free energies were obtained from rigorous confidence limit testing of these two recovered parameters, as described by Beechem (1992). Error estimates on rate constants and activation volumes were obtained from linear error analysis.

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