Testing the Correlation between ΔA and ΔV of Protein Unfolding Using *m* Value Mutants of Staphylococcal Nuclease[†]

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ABSTRACT: The application of hydrostatic pressure to aqueous protein solutions results in the unfolding of the protein structure because the protein−solvent system volume is smaller for the unfolded state. Contributions to this decrease in volume upon unfolding (ΔV_u) derive from altered interactions of the protein with solvent and are presumed to include electrostriction of charged residues, elimination of packing defects, and hydration of hydrophobic surfaces upon unfolding. If the contribution of hydrophobic surface area solvation to the observed volume change of unfolding were large and negative, as is generally assumed, then one would expect to find a correlation between the amount of surface area exposed on unfolding, ΔA_u, and the volume change, ΔV_u. In order to test this correlation, we have determined ΔV_u for two mutants of staphylococcal nuclease, A69T + A90S and H121P, whose unfolding by denaturant is, respectively, either significantly more (28%) or significantly less (28%) cooperative than that observed for wild-type (WT). This cooperativity coefficient or *m* value has been shown to correlate with ΔA_u. If, in turn, ΔV_u is correlated with ΔA_u, we would expect the m^+ mutant, A69T + A90S, to exhibit a ΔV_u that is more negative than WT nuclease, while the ΔV_u for the m^- mutant, H121P, should be smaller in absolute value. To verify the correlation between *m* value and ΔA_u for these mutants, we determined the xylose concentration dependence of the stability of each mutant at atmospheric pressure and as a function of pressure. The efficiency of xylose stabilization was found to be much greater for the m^+ mutant than for WT, consistent with an increase in ΔA_u, while that of the m^- mutant was found to be only slightly greater than for WT, indicating that other factors may contribute to the denaturant *m* value in this case. Regardless of the denaturant *m* value or the effect of xylose on stability, the volume changes upon unfolding for both mutants were found to be within error of that observed for WT. Thus, there does not appear to be a correlation between the volume change and the change in exposed surface area upon unfolding. We have previously shown a lack of pH dependence of the volume change, ruling out electrostriction as a dominant contribution to ΔV_u of nuclease. These studies implicate either compensation between polar and nonpolar hydration or excluded volume effects as the major determinant for the value of ΔV_u.

Thermodynamically, the conformation of many small globular proteins can be described by a simple two-state, reversible transition between folded and unfolded forms, exhibiting a high degree of cooperativity of folding. The application of hydrostatic pressure to aqueous protein solutions results in the reversible unfolding of such proteins because the protein−solvent system volume is smaller for the unfolded state. The microscopic contributions to this decrease in system volume, ΔV_u, upon unfolding are not well defined, although they are thought to involve hydrophobic hydration, electrostriction, and excluded volume. This study addresses the role of exposed surface area and hydrophobic hydration to the ΔV_u observed for staphylococcal nuclease (nuclease). We have previously demonstrated a pH independence of ΔV_u for nuclease, eliminating electrostriction as a major contributor (Royer et al., 1993).

When chemical denaturants, such as guanidinium chloride or urea, are used to denature these small, globular proteins, a sharp transition results due to the cooperativity of unfolding. Using the Linear Extrapolation Model (Tanford, 1970; Pace, 1986) to analyze the data, it has been found that the unfolding profiles can be described by a linear relation: $\Delta G_i = \Delta G° - m[\text{denaturant}]_i$, where ΔG_i is the free energy of unfolding at denaturant concentration, *i*, and *m* is the cooperativity coefficient or the *m* value. The degree of cooperativity has been shown in many cases to be correlated with the amount of surface area exposed upon unfolding, ΔA_u (Schellman, 1978; Shortle & Meeker, 1986; Shortle et al., 1988; Myers et al., 1995). Privalov and co-workers (Carra et al., 1994; Carra & Privalov, 1995) have concluded that, for nuclease, m^+ (*m* values greater than the wild-type) behavior is a result of mutational destabilization of the hydrophobic core or the β-barrel, consistent with an increase in ΔA_u corresponding to an increase in exposed surface area in the unfolded chain, while the unfolding of m^- mutants (*m* values less than the wild-type) can be more complex.

We were interested in establishing whether a correlation exists between ΔA_u and ΔV_u, and consequently if hydrophobic hydration were a determining factor in the value of ΔV_u. We chose to determine the ΔV_u values for two mutants of nuclease, H121P and A69T + A90S (Figure 1), that exhibit strongly altered cooperativity values for unfolding by denaturant, 28% smaller and larger, respectively, relative to WT (Shortle et al., 1989). In order to verify that the changes in *m* values for these mutants arise from differences in ΔA_u, first we examined the effect of the osmolyte, xylose, on the stability of these proteins at atmospheric pressure and as a function of increasing pressure. Xylose is a colligative agent and as such stabilizes proteins through surface tension perturbation, i.e., since the unfolded state exposes more

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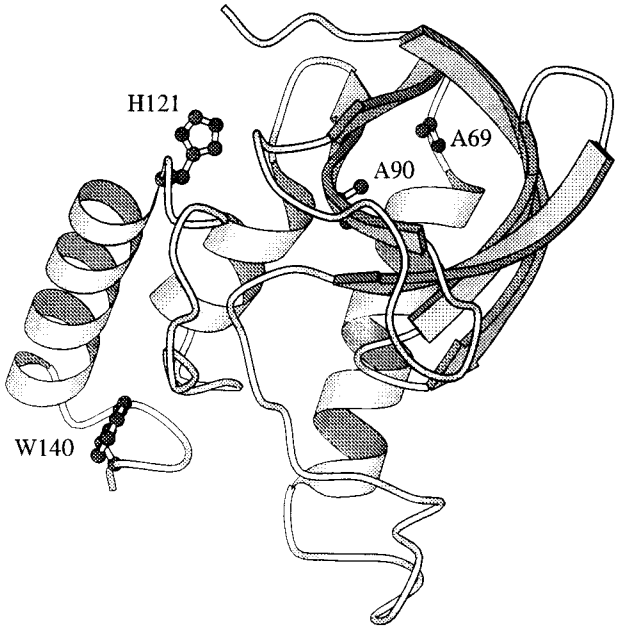

FIGURE 1: Three-dimensional structure of staphylococcal nuclease determined from X-ray crystallographic data (Hynes & Fox, 1991). The figure was drawn using the program Molscript (Kraulis, 1991). Histidine 121, alanine 69, alanine 90, and tryptophan 140 are shown in ball-and-stick presentation.

surface area than the folded state, these additives favor the folded state (Lee & Timasheff, 1981). A xylose *m* value (m_{xy}) can be defined as the extent of increase in free energy of unfolding, ΔG_u, as a function of xylose concentration. If the guanidine *m* values correlate with ΔA_u, then m^+ and m^- mutants should have m_{xy} values greater ($m_{xy}{}^+$) and smaller ($m_{xy}{}^-$), respectively, than wild-type (WT). We also precisely determined the values of ΔV_u for these mutants to evaluate the contribution of hydrophobic hydration to the volume change of unfolding.

MATERIALS AND METHODS

Protein Expression and Purification. WT nuclease was produced using the T7 expression system from the *Escherichia coli* strain BL21(DE3) as described by Alexandrescu et al. (1989) and Royer et al. (1993). Both the growth procedure and the protein purification procedure were carried out according to Wang et al. (1990) and Royer et al. (1993) except that the protein was stored in solution instead of lyophilized. The mutants were produced using the λ expression system in the *E. 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 media was used instead of MOPS media. The protein purification followed the method used in Shortle and Meeker (1989) except an additional extraction step was added without any salt and a Pharmacia Fast Flow S-Sepharose column was used alone as opposed to a CM-25 Sephadex column followed by a FPLC Mono S column. A 15% SDS−polyacrylamide gel was run to ensure the purity. Activity of the protein preparations was evaluated as described by Cuatrecasa et al. (1967) using sperm whale DNA and monitoring the increase in absorbance at 260 nm with the addition of protein due to the hydrolysis of nucleic acids.

High Pressure Experiments. High pressure experiments were carried out in a Vascomax high pressure cell using a high pressure generating system similar to that described by Paladini and Weber (1981). The pressure generator (High Pressure Equipment Co., Erie, PA) is connected to a high pressure gauge and the pressure cell through a series of metal tubing and high pressure valves. The steady-state fluorescence unfolding profiles were measured using an ISS spectrofluorometer (ISS, Urbana, IL). Exciting light from a xenon arc lamp at 295 nm was coupled to the pressure cell from the monochromator via a UV multifiberoptic bundle (Oriel Corp., Stratford, CT). The tryptophan emission was monitored at a 90° angle through a Corion 340 nm high pass filter in order to minimize scattering contributions which can be significant in the high pressure cell if emission is monitored at lower wavelengths. Monitoring the intrinsic tryptophan fluorescence with the emission cuton filter at the red edge of the emission spectra (340 nm) results in a percentage decrease in the fluorescence intensity upon unfolding of about 60% for the WT. This percentage decrease is smaller than the 90% decrease observed by Shortle and Meeker (1986) since these investigators monitored the fluorescence intensity at 325 nm which is on the blue edge of the emission spectra. Due to the red-shift of the emission spectra as the protein unfolds, a smaller decrease in signal is detected at an emission wavelength of 340 nm (Royer, 1996). Pressure jumps of 100−200 bar were made, and the fluorescence intensity was measured with a 20 s integration time. Equilibrium was reached after 20−25 min for all pressures, and those intensity values were used for the equilibrium unfolding profiles. The experiments were carried out at a concentration less than 10 μM (as determined by UV absorption at 280 nm and an extinction coefficient of 18 050 cm^{-1} M^{-1}) to avoid any aggregation which can occur in unfolding experiments at high concentrations. The buffer was 10 mM bis-Tris at pH 4.5 for the WT and pH 7 for the mutants since WT has a higher stability at pH 7.0 requiring a lower pH to destabilize the protein. A bis-Tris buffering solution was used because its pK_a is pressure insensitive (Neuman et al., 1973). The experiments were carried out in triplicate at 7−8 different xylose concentrations for each mutant and WT.

Data Analysis. The data were analyzed using the BIOEQS analysis program for the free energy and volume change of unfolding (Royer et al., 1990; Royer, 1993). The data were first analyzed in the unlinked or single curve mode to verify that there was no systematic difference in the volume change with increasing xylose concentrations. Next, each set of raw intensity data consisting of 7−8 xylose concentrations was globally analyzed by linking the ΔV_u across the set allowing its value and the ΔG_u and asymptotic intensity values for each individual curve to vary. An identical analysis was performed for each replicate set of xylose concentrations for both mutants. The volume and free energy changes recovered from the fits of each replicate set were averaged and standard deviations calculated. For purposes of visual comparison, the raw data from the unfolding profiles were then normalized to a scale from 0 to 100% native protein by calculating the percentage decrease in intensity at each pressure relative to the asymptotic intensity values recovered from the fits (Figures 3 and 4).

Since the WT is so much more stable than the mutants, reasonable fits could not be obtained allowing the asymptotic intensity values to float. Therefore, the WT data were analyzed globally by linking the ΔV_u across each data set

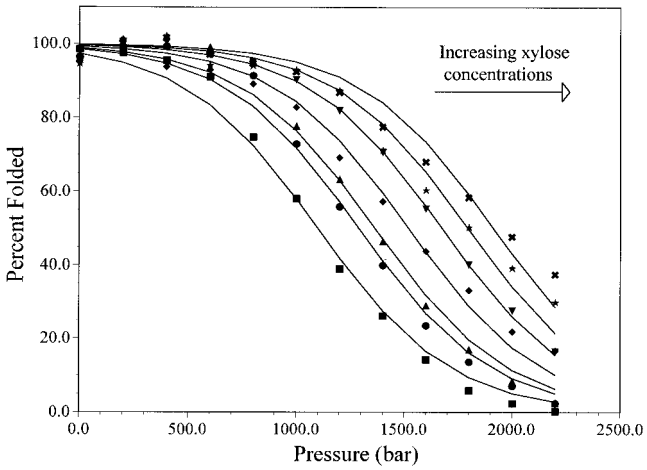

FIGURE 2: Unfolding profiles for WT nuclease as a function of pressure and mole fraction xylose at pH 4.5 and 21 °C. The symbols are data from a representative set, and the lines represent the global curve analysis fits to the data. (■) 0 mole fraction xylose, (●) 0.003 mole fraction xylose, (▲) 0.0062 mole fraction xylose, (◆) 0.0094 mole fraction xylose, (▼) 0.0127 mole fraction xylose, (×) 0.0161 mole fraction xylose, (∗) 0.0196 mole fraction xylose.

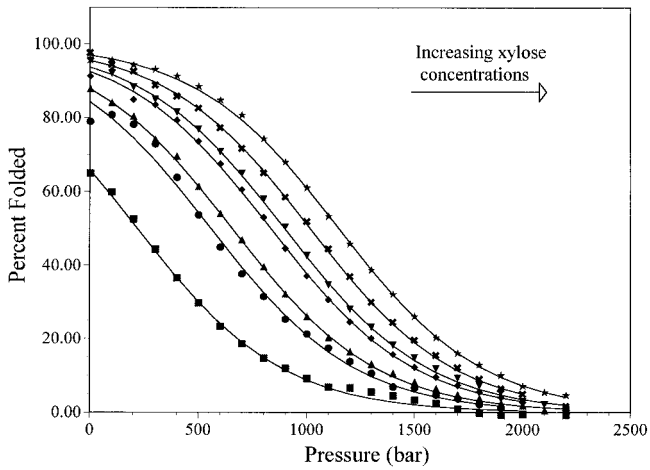

FIGURE 3: Unfolding profiles for H121P as a function of pressure and mole fraction xylose at pH 7.0 and 21 °C. The symbols are the data from a representative set, and the lines represent the global curve analysis fits to the data. (■) 0 mole fraction xylose, (●) 0.0062 mole fraction xylose, (▲) 0.0105 mole fraction xylose, (◆) 0.0127 mole fraction xylose, (▼) 0.0150 mole fraction xylose, (×) 0.0172 mole fraction xylose, (∗) 0.0196 mole fraction xylose.

allowing ΔG_u to vary for each xylose concentration. The asymptotic values were obtained from the averaged total intensity decrease observed for the pressure profiles at 0% xylose since these were the only profiles of WT that exhibited complete unfolding. The three experiments on WT protein completed without xylose yielded an average decrease in intensity upon unfolding of 57.7%. For experiments in the presence of xylose, the value of the intensity of the denatured state in each profile was fixed at a value 57.7% lower than the intensity observed at atmospheric pressure. The ΔV_u and ΔG_u for each data set were averaged and standard deviations calculated. Like the mutants, the raw data were also normalized for purposes of presentation (Figure 2).

RESULTS

Prior to testing the correlation between ΔV_u and ΔA_u, the verification of the hypothesis that there is a correlation between *m* values and the amount of surface area exposed upon unfolding (Shortle and Meeker, 1986; Shortle et al., 1988) was tested for the two mutants used in our study. The pressure-induced denaturation of WT nuclease, the m^- mutant (H121P), and the m^+ mutant (A69T + A90S) was conducted as a function of xylose. We monitored the decrease in intensity of the intrinsic fluorescence of the sole tryptophan residue in nuclease, tryptophan 140 (see Figure 1) upon unfolding in order to observe pressure denaturation (Shortle & Lin, 1985). It has been previously demonstrated that pressure, temperature, and chemical denaturant have similar effects on the NMR signals of the histidine $H^{\epsilon 1}$ protons of WT nuclease (Royer et al., 1993). The tryptophan fluorescence emission energy shifts red to 360 nm in all cases, and no ANS binding is observed to either the pressure or guanidine denatured forms of nuclease (data not shown). Thus, the manifold of pressure denatured states appears similar in many respects to that obtained by denaturant. In fact, the ΔG_u values obtained by pressure and guanidine are found to be within experimental error (Vidugiris et al., 1996).

Figures 2, 3, and 4 show the normalized denaturation curves and fits for the WT, H121P, and A69T + A90S, respectively, as a function of xylose concentration. Due to

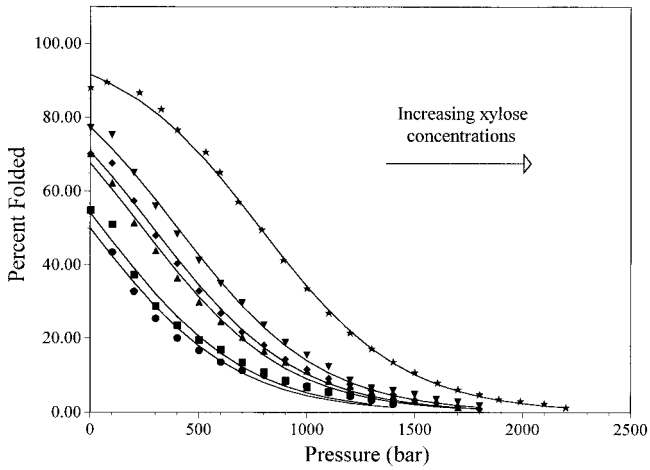

FIGURE 4: Unfolding profiles for A69T + A90S as a function of pressure and mole fraction xylose at pH 7.0 and 21 °C. The symbols are the data from a representative set and the lines represent the global curve analysis fits to the data. (■) 0 mole fraction xylose, (●) 0.003 mole fraction xylose, (▲) 0.0062 mole fraction xylose, (◆) 0.0094 mole fraction xylose, (▼) 0.0127 mole fraction xylose, (∗) 0.0196 mole fraction xylose.

the aforementioned surface area perturbation by this agent, as the xylose concentration is increased, the curves shift to higher pressures because the free energy of unfolding increases as a function of xylose concentration. Furthermore, since the mutants are partially unfolded at atmospheric pressure, addition of xylose causes the fluorescence intensity to increase at atmospheric pressure upon xylose-induced folding of the proteins.

Plotted in Figure 5 panels a and b are the free energies of unfolding as a function of mole fraction xylose for WT, H121P, and A69T + A90S from analysis of the pressure denaturation data and the atmospheric pressure data, respectively. If a correlation exists between *m* values and exposed surface area, the m^+ mutant should exhibit a larger slope than the WT and the m^- mutant should exhibit a smaller slope than the WT since stabilization by xylose is a function of surface area. The slopes of the ΔG_u vs [xylose] plots obtained from analysis of the pressure profiles and the

Table 1: Guanidine *m*-Values, Xylose *m*-Values and ΔV

	relative m_{gdn}[a]	m_{xy}[b] atmospheric	m_{xy}[b] pressure profiles (linked)	m_{xy}[b] pressure profiles (unlinked)	ΔV_u (ml/mol) (linked)	ΔV_u (ml/mol) (unlinked)
WT	1	nd[c]	73.3 ± 2.3	59.3 ± 11.3	-75.3 ± 1.3	-73.7 ± 5.9
H121P	0.72	82.8 ± 0.4	83.7 ± 0.4	81.0 ± 13.5	-70.5 ± 2.8	-69.9 ± 9.3
A69T + A90S	1.28	88.9 ± 0.1	88.6 ± 0.1	99.0 ± 14.4	-78.0 ± 8.8	-81.8 ± 10.6

[a] Shortle et al. (1989). m_{gdn} in kcal/mol per M. [b] m_{xy} in kcal/mol per mole fraction xylose. [c] nd, not determined.

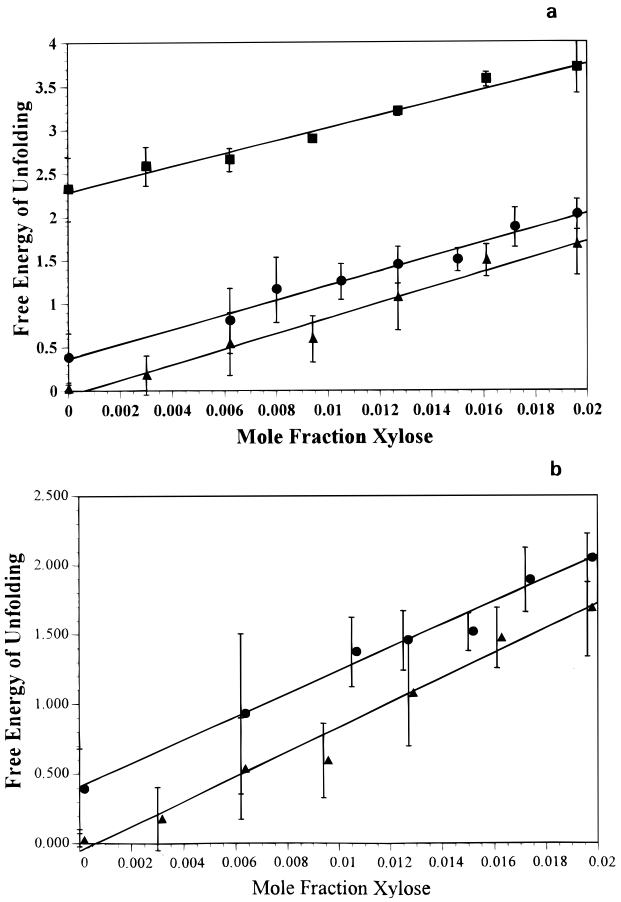

FIGURE 5: Free energy of unfolding vs mole fraction xylose. At the xylose concentration used here the activity coefficient is negligible and mole fraction is used since it is independent of temperature and pressure. (a) From the pressure unfolding profiles. (b) From the intensity values at atmospheric pressure. (■) WT at pH 4.5; (●) H121P and (▲) A69T + A90S, both at pH 7.0. The lines represent linear fits to the data.

stability atmospheric pressure are reported in Table 1. Since the WT is completely folded in absence of xylose, it is not possible to calculate a xylose *m* value for the WT from the atmospheric pressure data alone. Regardless of the method for obtaining the free energy of unfolding (the pressure unfolding profiles with linked or unlinked ΔV_u values or atmospheric pressure), the slopes for both the m^+ mutant and the m^- mutant are larger than that obtained for WT. In the case of the m^+ mutant, the m_{xy} value is 21% larger than that of WT, similar to the 28% difference observed for the cooperativity of unfolding by guanidine. This observation is consistent with a surface area effect for the increased cooperativity of unfolding of this mutant compared to WT. On the other hand, the correlation between ΔA_u and *m* value does not appear to hold for the m^- mutant. Its m_{xy} value is 14% larger than that of WT and only 6% smaller than that of the m^+ mutant. While calorimetric studies (Carra et al., 1994), CD and fluorescence melt comparisons (Ramsey & Eftink, 1995), and pressure-jump relaxation studies (Vidugiris et al., 1995) indicate WT nuclease equilibrium unfolding profiles are well described by a two-state model, Creighton and Shortle (1994) have shown that the altered *m* values measured for solvent denaturation of certain m^- mutants of nuclease may be due to the presence of a partially folded state which becomes significantly populated. Such complexity for m^- mutants has also been noted from calorimetric studies (Carra et al., 1994; Carra & Privalov, 1995). The m^- mutant used here, H121P, was too unstable for calorimetric studies; however, its CD melt profiles exhibited non-two-state character (data not shown). Thus, we conclude that the m^+ mutant exposes more surface area in the unfolded state than does the WT. However, we cannot conclude that the m^- mutant exposes less surface area. Rather, its altered *m* value likely arises from the population of intermediates.

Given that the m^+ mutant exposes more surface area upon unfolding than WT nuclease, we were interested in ascertaining whether this would correlate with a larger negative volume change upon unfolding of this mutant by pressure. The values of ΔV_u from the linked and unlinked analysis of the pressure unfolding of WT nuclease and the two mutants were averaged together for each replicate set of xylose concentrations for each protein and are given in Table 1 with their standard deviations. No trend was observed with xylose concentration in the value of ΔV_u recovered from the unlinked analysis. The value obtained for WT in these studies (75.3 mL/mol linked and 73.7 mL/mol unlinked) is identical within error of that obtained in earlier work (Vidugiris et al., 1995, 1996). Regardless of the method of analysis (linked or unlinked), the ΔV_u values are within experimental error for the three proteins, differing from one another by less than 10%, nearly 3-fold less than the 28% deviations in the denaturant *m* values. For example, the ΔV_u of the m^+ mutant is only 3% larger than that of WT, while the guanidine *m* value and xylose *m* value differ by 28% and 21%, respectively. Thus, the volume change upon unfolding does not appear to correlate with the amount of surface area exposed upon unfolding.

DISCUSSION

The goal of the present work was to evaluate the contribution of exposed surface area to the ΔV of unfolding using cooperativity mutants of staphylococcal nuclease. It was necessary to establish that the difference in cooperativity of unfolding of these mutants stemmed from differences in exposed surface area in the unfolded state. It appears from the effects of xylose on the stability of the nuclease mutants studied here that a correlation between *m* value and surface area exposed upon unfolding exists for the m^+ mutant. The percentage difference in m_{xy} between the WT and m^+ mutant is about 21%, close to the 28% difference in guanidine *m*

values. However, this correlation does not appear to hold for the m^- mutant; the m_{xy} value for H121P is actually slightly larger than the that of WT, rather than smaller. The percentage difference in m_{xy} between the m^- and m^+ mutants is about 6%, whereas the guanidine m values differ by a factor of 1.6 (Shortle et al., 1989). The small difference observed in m_{xy} between the m^- and m^+ mutants leads us to conclude that a correlation between guanidine m value and surface area for the m^- mutant is unlikely.

The m^+ mutants are located in the hydrophobic β-barrel (core I) of the protein, while the m^- mutants are found in a second cluster (core II) (Shortle et al., 1990). The m^- mutants enhance the segregation of the core I residues into an independent cluster, mostly through indirect effects, while the m^+ mutants reduce the stability of core I interactions (Shortle, 1995). Since m^+ mutations reduce the stability of this independent core, they result in an increase in exposed surface in the unfolded state and the correlation between m value and ΔA_u holds. In contrast, m^- mutants can either stabilize this independent cluster or destabilize core II resulting in an increase in the number of states populated between the native and denatured states. In such cases the correlation between m value and ΔA_u may not apply because the two-state approximation is not valid. We believe this to be the case for the m^- mutant studied here, H121P.

Since surface area, ΔA_u, and m value do correlate for the m^+ mutant, A69T + A90S, it provides a clear test of the correlation between ΔA_u and ΔV_u. We have clearly demonstrated that the volume change for this mutant is within experimental error ($\leq$10%) of that obtained for WT. The ΔV_u for the m^- mutant was found to be nearly identical to that of WT as well. Thus, we conclude that for staphylococcal nuclease unfolding there is no correlation between ΔV_u and ΔA_u.

It has long been assumed in high pressure biochemistry that the exposure of hydrophobic surface area results in a decrease in system volume (Nemethy & Scheraga, 1962; Weber & Drickamer,1983). However, it has been noted that the volume changes observed upon pressure-induced protein unfolding are much smaller than expected from model compound studies (Dill, 1990). More recently, alternative explanations for ΔV_u have been proposed. Prehoda and Markley (1996) have proposed compensation between a positive contribution from hydrophobic hydration and a negative contribution of elimination of excluded volume upon unfolding. On the other hand, the observed volume change could result from compensating positive and negative contributions from nonpolar and polar hydration as suggested by Chothia and co-workers (1994) and Breslauer and co-workers (Harpaz et al., 1994; Chalikian & Breslauer, 1996).

Identical values for ΔV_u for WT nuclease and the m^+ mutant, A69T + A90S, provide strong evidence that hydrophobic hydration does not make a large, negative contribution to the volume change of protein unfolding. The change in total accessible surface area upon unfolding for WT nuclease, assuming a completely solvent-exposed chain in the unfolded state, was calculated to be 12 058 Å^2 and can be broken down to ΔASA_{polar} = 3593 Å^2 and $\Delta ASA_{nonpolar}$ = 8465 Å^2 (Myers et al., 1995). Although the majority of ΔASA is due to the nonpolar residues, their positive contributions to ΔV_u may be offset by a larger negative contribution from the polar groups and backbone. If compensation between exposure of polar and nonpolar surfaces gives rise to the relatively small values observed for the volume changes upon unfolding, then we must conclude that this compensation remains essentially unchanged for the mutants used in this study as compared to nuclease WT, since the volume changes were found to be essentially identical. Another possibility is that exposed surface area, polar or nonpolar, contributes very little to the ΔV_u. In either case, the excluded volume of the folded chain due to imperfect packing should contribute significantly to the value of ΔV_u. This hypothesis is currently under investigation.

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