Monitoring the Sizes of Denatured Ensembles of Staphylococcal Nuclease Proteins: Implications Regarding *m* Values, Intermediates, and Thermodynamics[†]

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ABSTRACT: Fluorescence and size-exclusion chromatography (SEC) are used to monitor urea denaturation of wild-type staphylococcal nuclease (SN) as well as the *m*+ and *m*− mutants A69T and V66W, respectively. It is found that the SEC partition coefficient, $1/K_d$, is directly proportional to the Stokes radii of proteins. From the Stokes radii, the denatured ensembles of the three proteins are found to be highly compact in the limit of low urea concentration and expand significantly with increasing urea concentration. The *m* values from fluorescence-detected denaturation of the SN proteins are generally considered to reflect the relative sizes of denatured ensembles. However, the rank order of *m* values of the SN proteins studied do not correspond to the rank order of denatured ensemble sizes detected by $1/K_d$, suggesting that *m* values reflect more than just surface area increases on denaturation. SEC provides two complementary ways to demonstrate the existence of intermediates in urea denaturation and illustrates that V66W undergoes a three-state transition. Fluorescence-detected urea denaturations of A69T and wt SN do not correspond with $1/K_d$-detected denaturation profiles, a result that would ordinarily mean that the transitions are non-two-state. However, this interpretation fails to recognize the rapidly changing size and thermodynamic character of the denatured ensembles of these proteins both within and outside of the transition zone. The implications of the changing sizes and thermodynamic character of the denatured ensembles for SN proteins are manifold, requiring a reconsideration of the thermodynamics of proteins whose denatured ensembles behave as those of SN proteins.

One of the more controversial issues in protein folding is the question of whether the effect of a protein mutation alters the denatured ensemble or the native ensemble of a protein. Staphylococcal nuclease was the first of several proteins for which it is now claimed that the principal effect of mutation is on the denatured rather than the native state ensemble (*1*−*4*). The basis of these claims is largely derived from comparisons of the *m* values for wt and mutant proteins, assuming that the *m* value reflects the amount of newly exposed surface area upon denaturation of the proteins with guanidinium chloride or urea (*1*). The *m* value, an experimentally determined parameter, represents the sensitivity of the protein to chemically induced denaturation, and it has been proposed that SN mutant proteins that have larger *m* values than wt SN (referred to as *m*+ mutants) expose more surface area in chemical denaturation than does wt protein, whereas *m*− mutant proteins (which have *m* values smaller than that of wt SN) expose a smaller denatured surface area when compared to denatured wt SN (*1*).

Intertwined with the issue of whether mutation is largely affecting the denatured ensemble are questions of whether the denaturations of wt and mutant SN proteins exhibit two-state or multistate behavior (*5*, *6*). Sorting out the various possibilities brings into play such basic problems as distinguishing a "compact denatured ensemble" that has residual structure from a "discrete intermediate state" existing between the native and a more fully denatured state (*5*−*7*). Many of these issues are focused in the opposing views of Shortle and Carra and Privalov concerning the denaturation of SN proteins (*6*, *8*, *9*). A major difference between these two views is that Shortle considers the denatured ensembles of *m*+, *m*−, and wt SN proteins as being dimensionally and thermodynamically different from one another while Carra and Privalov consider the final denatured states of SN proteins (i.e., the U state in their definition) as thermodynamically (and, presumably dimensionally) identical to one another.

This major difference in the views held by these investigators has considerable implications in terms of the approaches that have been taken to explain structure-energetic differences between wt and mutant proteins. Considering the unfolded states of wt and mutant SN proteins as being thermodynamically identical (Carra and Privalov's view) permits the unfolded state to serve as a reference state (*9*). For proteins that exhibit two-state behavior, this allows energetic denaturation differences determined for wt and mutant proteins to be evaluated in terms of structural differences between their native states. If however, the nonnative ensembles of wt and mutant proteins differ thermodynamically from one another, energetic differences in the denaturation of wt and

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mutant proteins cannot be solely explained in terms of structural differences between their native states. In short, in terms of structure-energetic analyses and the validity of these analyses, there is much at stake in the controversy between Shortle's view and that of Carra and Privalov.

Key to these issues is the dimensional size(s) of the denatured ensemble as a function of chemical denaturant concentration. Here, we use size-exclusion chromatography (SEC) to monitor the Stokes radii of wild-type, *m*+, and *m*− mutants of SN proteins as a function of urea concentration. Our objective is to determine how this measure of a protein's dimensions compares with other means of monitoring denaturation transitions and how these various methods correlate with molecular-level interpretations based upon *m* values. In using chemical denaturants such as urea, we have no way, a priori, of knowing whether we are dealing with a denatured state, an intermediate state, or a mixture of such states. For the discussion of urea-induced denaturation of SN proteins, we segregate protein species into two categories, one of which is the native ensemble and the other we term the nonnative ensemble. This latter category includes intermediate protein species as well as all denatured species regardless of their degree of structural compactness.

MATERIALS AND METHODS

Chemicals. 3-(*N*-Morpholino)propanesulfonic acid (MOPS) was purchased from Sigma, while sodium chloride was obtained from Mallinckrodt, tris base was from Fisher, and ultrapure urea was from ICN. Prior to use, urea solutions were treated with a mixed-bed ion-exchange resin (AG501-X8, Bio-Rad Laboratories) for at least 1 h to rid the solution of ions formed by decomposition of urea (*10*). Urea solutions were then filtered through 0.22 μM GV filter paper (Millipore Corp.), and the molar urea concentration ([urea]) was determined by measuring the refractive index [urea] = $117.66*\Delta n + 29.753\Delta n^2 + 185.56*\Delta n^3$, where Δn represents the difference between the refractive index of the urea solution and water (*11*).

Purification of SNase Proteins. The strains of *Escherichia coli* containing the λ/AR 120 expression system for expression of SNase proteins (wild type, A69T, V66W) were provided by Dr. David Shortle, and a strain of the T62P SN mutant was kindly provided by Dr. Wesley Stites. The procedure for expression and purification of SNase proteins was carried out according to Shortle with some modification (*12*). Cells collected by centrifugation were resuspended in 6 M urea containing 25 mM Tris-HCl and 5 mM EDTA, pH 8.1, and centrifuged again. The resulting pellet was resuspended in the same buffer which also contained 200 mM NaCl. After centrifugation of this suspension, ethanol precipitation of the supernatant was carried out twice for better separation of SNase proteins from impurities. The ethanol precipitate was recovered by centrifugation then dissolved in column load buffer (6 M urea, 25 mM Tris, pH 8.1, and 1 mM EDTA) and applied to an *S*-Sepharose (Sigma Inc.) column (2−5 mg of protein/mL of matrix) which had been preequilibrated with 10-bed volumes of column load buffer. After applying the protein solution, the column was further rinsed with 5−10 bed volumes of column load buffer followed by elution of the protein with 3−4 bed volumes of column load buffer containing 0.3 M NaCl. The eluate was dialyzed against 1 M NaCl at 4°C overnight and then dialyzed against Milli-Q purified water. The dialyzed solution was filtered through a 0.22 μM (Micron Separations) and then lyophilized.

Protein concentrations were determined by absorbance at 280 nm in 30 mM MOPS buffer, pH 7.00, using a molar extinction coefficient of 15 600 $M^{-1}cm^{-1}$ for wild-type SN, A69T, and T62P mutants and 21 100 M^{-1} cm^{-1} for V66W mutant (*13*). Protein purity was evaluated from the behavior of the protein on SDS−polyacrylamide gel electrophoresis with silver stain detection, from the symmetry of ion exchange (Resource-S column) and gel filtration chromatography elution profiles of the protein, with absorbance detection at 220 and 280 nm. By these criteria, all protein preparations were deemed to be pure.

Fluorescence Measurement. Intrinsic fluorescence of the tryptophan residue at position 140 (for wild type, A69Tand T62P) and tryptophan residues at positions 66 and 140 (for V66W) were monitored using a SPEX FluoroMax spectrofluorimeter, to observe urea-induced denaturation. Protein samples (50 μg/mL) and 8 M urea titrant solutions were separately prepared in 0.1 M NaCl and 30 mM MOPS buffer, pH 7.00. The protein solution (2.5 mL) in a 1.0 cm rectangular quartz fluorescence cuvette fitted with a stir bar and a water-tight cap and under constant rate of magnetic stirring was titrated by adding aliquots of urea titrant without withdrawing the cuvette from the sample chamber. The cuvette was thermostated at a fixed temperature 23.0 ± 0.1 °C using a circulating waterbath. After addition of each aliquot of urea titrant, a 10−30 min time interval elapsed before the equilibrium fluorescence intensity was ultimately recorded. A 0.8 mm slit width was used for excitation at 295 nm and emission was measured at 335 nm. Fluorescence data were corrected with respect to the fluorescence of a blank performed under the same experimental conditions and then expressed as F/F_o, where F is the corrected fluorescence as a function of urea concentration and F_o is the corrected fluorescence of the protein solution in the absence of urea.

Size-Exclusion Chromatography. The experiments were carried out using a Phenomenex Biosep SEC-S3000 HPLC gel filtration column 300 × 7.80 mm. SNase samples (50 μg/mL) were incubated before injection for 30−120 min in the same solution with which the column was equilibrated, viz., 0.2 M NaCl and 30 mM MOPS buffer (pH 7.00) at 23.0 ± 0.1 °C containing different concentrations of urea. All column runs were made at room temperature 23.0 ± 0.5 °C. The steel-jacketed column was operated with mechanical injection within a fully automated BioCad SPRINT HPLC system that allowed the elution volume to be repeated to within ±0.015 mL. Partition coefficients (K_d) were calculated with each equilibrating urea solution used in accordance with the equation

$$K_d = (V_e - V_o)/V_i$$

Here, V_e is the elution volume of protein sample, V_o is the void volume, represented by the elution volume of Blue Dextran, and V_i is the volume of solvent inside the gel available to low molecular weight compounds, evaluated as the difference between the elution volume of NADH and the elution volume of Blue Dextran.

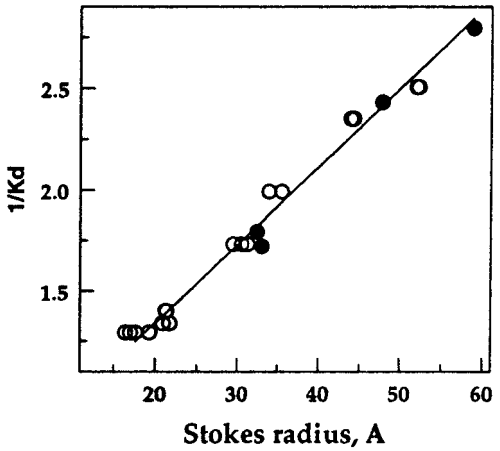

FIGURE 1: Dependence of the $1/K_d$ parameter of calibration proteins on their Stokes radius. (○) Native proteins, cyt-c, RNase A, chymotrypsinogen, myoglobin, ovalbumin, BSA, LDH, catalase; (●) Proteins unfolded with urea and DTT, RNase A, lysozyme, chymotrypsinogen, ovalbumin. The Stokes radii were taken from Uversky and Corbett and Roche (*14*, *15*).

The column was calibrated using proteins with Stokes radii taken from the literature as compiled by Uversky and Corbett and Roche (*14*, *15*). To produce randomly coiled denatured proteins, various proteins were incubated at 23 °C for 4 h in 30 mM MOPS (pH 7.00) containing 8.5 M urea, 0.2 M DTT, and 0.4 M NaCl and their elution volumes were determined using the same denaturing conditions. Under native conditions, proteins were chromatographed in 30 mM MOPS buffer (pH 7.00) containing 0.4 M NaCl. In Figure 1, $1/K_d$ is shown to be proportional to the Stokes radius (Sr) of proteins as described by the equation below; an equation we used for estimating the dimensions of native and denatured states of SNase proteins:

$$1/K_d = 0.573(\pm 0.115) + 0.0386(\pm 0.0031) \times \mathrm{Sr} \quad (1)$$

RESULTS

To select an appropriate parameter of SEC that is proportional to a dimensional property of proteins, a calibration curve relating $1/K_d$ to protein Stokes radii was prepared (the partition coefficient K_d is defined in the Materials and Methods). The data in Figure 1 illustrate a linear correlation of $1/K_d$ with Stokes radii covering the range of dimensions of the native and denatured states for the proteins used in this study. Some of the deviations in Figure 1 are due to the variability of Stokes radii reported in the literature as compiled by Uversky, and Corbett and Roche (*14*, *15*).

To assess the validity of using $1/K_d$ as a means to evaluate $\Delta G°$, m, and $C_{1/2}$ values of urea-induced denaturation, $1/K_d$ measurements of RNase A unfolding were performed as a function of urea concentration and the results are given in Figure 2. The solid line in the figure is the nonlinear least-squares fit of the data to a two-state model using the linear extrapolation method as described previously (*16*). Table 1 illustrates that the parameters $\Delta G°$, m, and $C_{1/2}$, evaluated for RNase A using $1/K_d$ to monitor the denaturation, are identical, within error, to literature values using absorbance as a monitor (*17*). These data show that the weighted average size of the molecular participants in denaturation can serve as a valid quantity in evaluating the thermodynamics of denaturation.

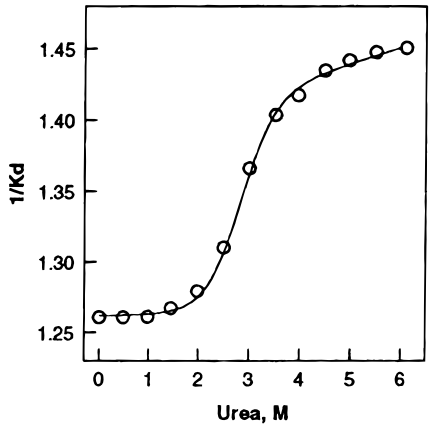

FIGURE 2: Urea-induced equilibrium denaturation of RNase A monitored by size-exclusion chromatography. Measurements were performed in 0.1 M β-Ala buffer (pH 3.0) and 0.421 M NaCl at 25 °C. The solid curve represents the result of the nonlinear least-squares best fit to the linear extrapolation model.

Table 1. Thermodynamic Parameters for the Urea-Induced Unfolding of wt and A69T SNases and RNase A

	parameters used for fitting	ΔG (kcal/mol)	m (kcal/mol M)	$C_{1/2}$ (M)
RNase A[a]	ΔA_{287} nm[b]	5.04 ± 0.15	1.67 ± 0.05	3.02 ± 0.13
	$1/K_d$	4.84 ± 0.63	1.72 ± 0.26	2.81 ± 0.32
wt SN	F/F_o	5.59 ± 0.05	2.31 ± 0.02	2.42 ± 0.03
	$1/K_d$	7.02 ± 0.50	2.83 ± 0.26	2.48 ± 0.15
A69T SN	F/F_o	2.57 ± 0.05	2.45 ± 0.04	1.05 ± 0.04
	$1/K_d$	3.78 ± 0.35	3.53 ± 0.25	1.07 ± 0.1

[a] Urea-induced denaturation for RNase A has been performed in 0.1 M β-Ala buffer (pH 3.0) and 0.421 M NaCl at 25 °C. [b] Data were taken from Yao and Bolen (*17*).

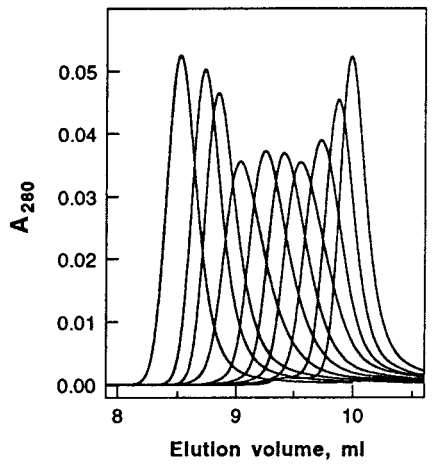

FIGURE 3: Elution profiles of SN mutant V66W protein in the presence of different urea concentrations (M): 0.0 M urea (the rightmost peak), 0.8, 1.2, 1.4, 1.6, 1.8, 2.2, 2.6, 3.0, and 4.5 (the leftmost peak).

The partition coefficient, K_d, is derived from measurements of the elution volumes of the chromatographic peaks from SEC. Figure 3 provides an example of typical elution profiles of the $m-$ SN mutant (V66W) as a function of urea concentration from low urea concentration, where native protein is the dominant species, to high urea, where the denatured protein is the prevailing species. The chromatographic peaks are narrow at low urea concentration, become wider at the center of transitions, and ultimately narrow again in the posttransition regions. SNase proteins undergo inter-

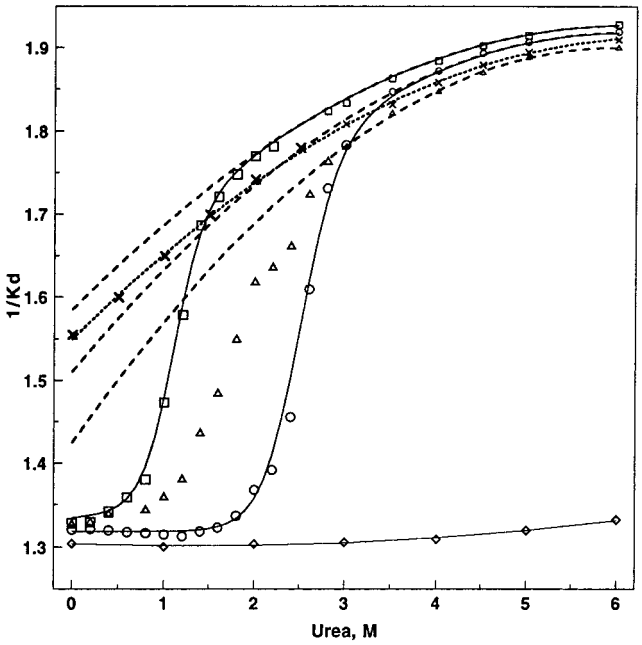

FIGURE 4: Urea-induced equilibrium denaturation of SNase wild-type (○), and SNase mutants A69T (□) and V66W (△) are presented as changes of $1/K_d$ vs concentration of urea. $1/K_d$ values for the T62P mutant of SNase (×) and for chymotrypsinogen (◇) are shown as a function of urea concentration. The solid curves represent the nonlinear least-squares best fits of the data to a two-state model. Dashed curves represent the $1/K_d$ values measured in the posttransition region and extrapolated to the pretransition region. The dotted curve is an extrapolation of $1/K_d$ values for T62P mutant as described in the text.

conversion between states (native, denatured, and intermediate) throughout the transition region (*18*), and for rapidly interconverting species, the rates of conversions between these states may be reflected in the square of the width of the chromatographic peak at 0.608 of the height, $*\sigma^2$, the variance of a Gaussian profile.

Demonstration of the validity of using $1/K_d$ to quantitatively evaluate thermodynamic parameters in urea denaturation in Figure 2 and the relationship of $1/K_d$ to Stokes radius (Figure 1), provides the backdrop for evaluation of the denaturation behavior of SN proteins using SEC. Figure 4 illustrates the dependencies of the $1/K_d$ parameters of four SNase proteins (wild-type, A69T, V66W, and T62P mutants) on urea concentration. With nondenaturing urea concentration, $1/K_d$ of wild type SN remains constant as urea is increased. This indicates that the Stokes radius of the native state is unaffected by urea concentration. Within the transition region, it is anticipated that the observed $1/K_d$ values represent a weighted average size contribution of each protein species (native, denatured, and intermediates) present at the particular concentration of denaturant. However, in the posttransition urea concentration range, the $1/K_d$ values are observed to continue to increase with increasing urea concentration. These results show that, unlike native SN, the nonnative ensemble of wt SN continues to expand significantly with increasing urea concentration.

To determine whether changes in permeation properties of the column that probably occur as a function of urea are affecting the $1/K_d$ parameter, we performed a control experiment monitoring $1/K_d$ values of chymotrypsinogen as a function of urea from 0 to 6 M. Chymotrypsinogen is highly resistant to urea denaturation and should remain in its native state at the pH of these experiments. It is observed in Figure 4 that $1/K_d$ for this protein is constant with urea concentration up to 5 M, a denaturant concentration which is getting near the onset of chymotrypsinogen denaturation. The lack of change in $1/K_d$ over the 0−5 M urea concentration range indicates that the $1/K_d$ is not affected by changes in permeation properties of column, and as expected, $1/K_d$ reflects essentially no change in Stokes radius of the native state of the protein.

In evaluating the denaturing transitions by SEC, it is necessary to extrapolate the posttransition baseline toward zero concentration of denaturant. The extrapolations of $1/K_d$ values for the denatured states of wt SN, A69T, and V66W illustrate considerable contraction of the denatured states upon approaching zero denaturant concentration (Figure 4). To explore the accuracy of such extrapolations, we used T62P SNase mutant protein, which is known to exist as nonnative species even at zero concentration of urea (*19*). In Figure 4, T62P appears to be quite compact in the absence of urea and expands significantly with increasing urea. The $1/K_d$ value for T62P in the absence of urea (1.555) lies between the $1/K_d$ values of nonnative A69T mutant and wt SN obtained from extrapolation of their respective $1/K_d$s from high urea to zero denaturant concentration. Thus, the extrapolations are reasonable, giving Stokes radii quite comparable to that of a very compact nonnative ensemble of an SN protein. The dotted curve in Figure 4 represents the extrapolation of $1/K_d$ values for T62P mutant using only the data in the range of high urea concentration (3−6 M), the range where the native ensembles of most SN proteins are not populated. The points used are indicated as smaller (×) symbols, and the $1/K_d$ vs [urea] dependence was determined by fitting a second degree polynomial to these points, then extrapolating the functional dependence to zero M urea. The agreement between the extrapolation (dotted line) and the actual data (large × symbols) in the 0−3 M urea concentration range suggests that a second degree polynomial is a reasonable mathematical function to use in extrapolations involving other SN proteins. For every extrapolation shown in Figure 4, data in the posttransition range are used to determine the parameters of a second-order polynomial and the extrapolations are made from the resulting functional dependence.

Using the extrapolated curves to represent the respective non-native ensemble Stokes radii as a function of urea, nonlinar least-squares analyses were performed for wt SN and A69T assuming two-state behavior and the linear extrapolation method (*16*). The thermodynamic parameters obtained for A69T and wt SNase using both $1/K_d$ and intrinsic fluorescence to monitor the denaturations are shown in Table 1.

Figure 5 presents experimental data on urea denaturation of wt SN and mutants A69T and V66W monitored by changes of intrinsic fluorescence. The fluorescence-monitored denaturations exhibit single symmetrical transitions for all three proteins. These data were analyzed assuming two-state behavior and the linear extrapolation method with the solid lines in Figure 5 representing the fits and the data in Table 1 giving the parameters derived from the fits.

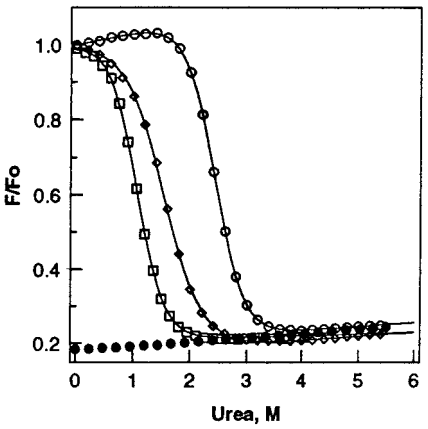

FIGURE 5: Fluorescence ratio (F/F_o) of SN proteins as a function of urea concentration for wild type (○), A69T (□), V66W (◇) and T62P (●). The solid curves represent the results of the nonlinear least-squares best fits of the data using the linear extrapolation method.

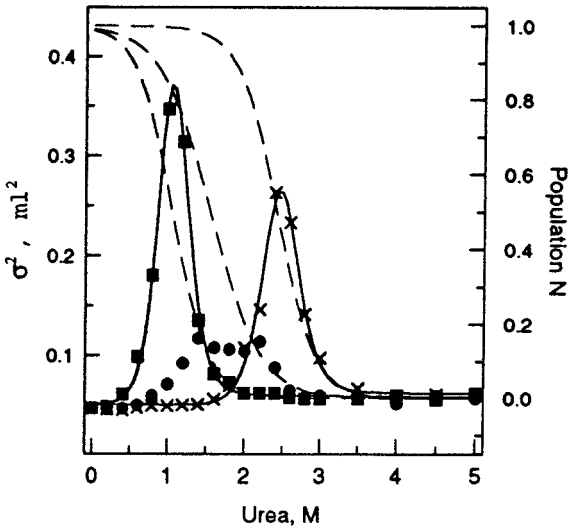

FIGURE 6: Dependences of the square of variance of the chromatographic peak versus urea concentration for SN wt (×), A69T (■), V66W (●). Solid lines represent the result of nonlinear least-squares best fit of the data to eq 36 published by Hilser and Freire (*18*). Dashed lines show population of the native state versus concentration of urea calculated from the primary data presented in Figure 5 using the two-state model of denaturation.

In contrast to the symmetrical denaturation profile for V66W detected by fluorescence as shown in Figure 5, the denaturing transition for V66W monitored by SEC is nonsymmetrical, suggesting non-two-state behavior. The possibility of the existence of a stable intermediate in the denaturation of V66W mutant also becomes obvious when $*\sigma^2$ of the elution profiles in Figure 3 is plotted as a function of urea concentration as shown in Figure 6. Both of the parameters monitored by SEC, $1/K_d$ and $*\sigma^2$, represent different aspects of the denaturation process. $1/K_d$ is proportional to the weighted average of Stokes radii of all states interconverting at a particular concentration of urea, whereas $*\sigma^2$ also reflects the rates of interconversions of the participating protein species. Figure 6 shows that wt SN and A69T mutant exhibit single sharp symmetrical profiles of $*\sigma^2$ vs [urea], and the maxima in $*\sigma^2$ correspond to the $C_{1/2}$ values of the transitions for the two proteins. In contrast, the urea dependence of $*\sigma^2$ for V66W mutant exhibits two maxima, indicating the presence of an equilibrium intermediate. The first maximum corresponds to the urea concentration where approximately 50% of fluorescence signal is lost, whereas the second maximum occurs with less than 10% of the trailing edge of the fluorescence remaining. Hence, the second transition of the V66W mutant detected by size-exclusion chromatography is not at all evident in the fluorescence-detected denaturation profile.

DISCUSSION

There is considerable controversy about denaturant-induced transitions involving SN and *m*+ and *m*− SN mutants. The view of urea- or GdnHCl-induced denaturation of SN and SN mutant proteins expressed by Shortle is (1) that *m*+, wt, and most *m*− mutant SN proteins undergo two-state denaturation, (2) the *m* value represents the amount of surface area newly exposed on denaturation, with *m*+ mutants having the most surface area exposed, followed in order of decreasing amount of denatured state surface area exposed by wt SN and *m*− SN mutants respectively, (3) that the nonnative species are considered to be "compact denatured ensembles" without discrete intermediates, and (4) that point mutations cause these compact ensembles to differ in size and continuously expand as a function of denaturant concentration (*1*, *20*, *21*). In contrast to Shortle's view, Carra and Privalov have proposed a very different interpretation of solvent-induced denaturation of SN proteins (*5*, *6*, *9*). Based mostly on DSC experiments in the absence of denaturants, their view of urea and GdnHCl-induced denaturation of SN proteins is (1) *m*− and wt SN proteins denature by a three-state mechanism (N ⇌ I ⇌ U) while *m*+ SN proteins are closer to exhibiting two-state denaturation, (2) the compact denatured ensemble cited by Shortle is considered by Carra and Privalov to contain a discrete intermediate, (3) an energy barrier exists between the intermediate and the unfolded state, a barrier that leads to a cooperative (first-order) transition from I ⇌ U, (4) CD and fluorescence measurements on denaturant-induced transitions of SN proteins fail to detect the I ⇌ U transition, and (5) the U state is thermodynamically identical for all SN proteins and therefore can serve as a reference state. A discussion of these different viewpoints with respect to the SEC data is presented below.

Distinguishing between a Compact Denatured Ensemble and an Intermediate State. On the basis of calorimetric data, Carra and Privalov suggested that the unfolding of *m*− mutants is less cooperative relative to the wild-type, because *m*− mutants have a stable intermediate state occurring within the transition (*5*, *6*, *9*). Our fluorescence-detected urea-induced denaturation experiment with the *m*− mutant V66W is quite symmetrical, and fitting the data to a two-state model gives no evidences of an intermediate species (Figure 5). [GdnHCl-induced denaturation of V66W is not symmetrical (*7*).] However, if instead of fluorescence, SEC is used to monitor the denaturation, two transitions are clearly detected, making it necessary to use at least a three-state model to explain the data. Evidence of the two transitions come both from monitoring the weighted average Stokes radius ($1/K_d$) of the protein species (see Figure 4) or by monitoring $*\sigma^2$ of each of the chromatographic peaks (Figure 6). The $1/K_d$ and $*\sigma^2$ data show that it is possible to detect an "intermediate" that is distinguished from a "compact denatured

ensemble" if they are separated by an energy barrier. This three-state model for denaturation of *m*− SN proteins was predicted by Carra and Privalov, and the SEC data show that reliance on fluorescence and CD alone gives problems with analysis of at least V66W denaturation and perhaps other *m*− staph nucleases as well (*9*). We hasten to add that while these data support the view of Carra and Privalov for *m*− mutants, they provide no positive support for Carra and Privalov's contention that wt SN should exhibit three-state denaturation. In fact, for wt SN denaturation, the $1/K_d$ detected transition is consistent with two-state behavior (Figure 4), and no hint of an intermediate is evident from measurements of $*\sigma^2$ derived from the chromatographic peaks (see Figure 6). At best, if an intermediate species is present, its further denaturation involves something other than a first-order transition.

While the fluorescence and $1/K_d$ data require modifications of Shortle's view in regard to at least certain *m*− mutants, it does not discount more novel aspects of his proposal having to do with the nature and sizes of the compact denatured ensembles.

Does Protein Mutation Alter the Denatured Ensemble and Does the Denatured Ensemble Gradually Expand with Added Denaturant? The Stokes radius of wt SN denatured in 6 M urea (36 Å, calculated using eq 1) is considerably smaller than that of a random-coil-protein of the same chain length (50 Å), a result showing that the ensemble of species in 6 M urea is "highly compact" despite the high concentration of urea (*22*). For this reason and the fact that there is no experimental evidence from SEC or the literature that a discrete intermediate exists for wt SN, we will drop the term "nonnative ensemble" for these proteins in favor of the term "denatured ensemble" or "compact denatured ensemble".

Figure 4 shows that the Stokes radii ($1/K_d$) of *m*+, *m*−, and wt SN denatured proteins differ measureably from one another even in 6 M urea and that dimensional differences between the denatured ensembles increase as urea concentration decreases from 6 M urea. These results are consistent with Shortle's view that the denatured ensembles of *m*+, *m*−, and wt SN proteins differ in surface areas exposed (denatured ensemble size) and is not consistent with Carra and Privalov's model that the U state (which should be reached at high urea concentration) should be essentially identical for all SN species. Carra and Privalov performed urea gradient gel electrophoresis on wt SN along with *m*+ and *m*− SN mutant proteins and found that the electrophoretic mobilities of the different proteins within the initial native and final denatured states are not distinguishable by this method (*6*). However, urea gradient gels have a gradient of acrylamide opposing the urea gradient and this reduces the ability to distinguish differences in sizes. SEC is better designed to detect size differences and the results presented here clearly show denatured ensemble size differences between *m*+, *m*−, and wt SN throughout the transitions and extending to high denaturant concentration. On this count, Shortle's view of significant variation in the surface areas of the denatured ensembles is borne out by the data while Carra and Privalov's expectation that the denatured ensembles are identical at high denaturant concentration is not observed.

The best prototype of a denatured ensemble we have studied is SN mutant protein T62P, which has essentially no catalytic activity in dilute buffer solution and has the same fluoresence characteristics in the absence of denaturant as urea-denatured *m*+, *m*−, and wt SN proteins (Figure 5) (*19*). This lack of fluorescence, virtual lack of catalytic activity, and low $1/K_d$ indicates that T62P is a compact denatured ensemble that becomes exceedingly compact at zero urea concentration. With the use of the osmolyte, trimethylamine *N*-oxide, we have shown that T62P can be forced to fold (cooperatively) to a species with 90% of the activity of wt SN (*19*). It is also noteworthy that T62P in 0 M urea expands with increasing urea concentration without giving any evidence of a first-order (cooperative) transition, indicative of an intermediate (see Figure 4).

A second-order polynomial accurately describes the urea dependence of $1/K_d$ for T62P, suggesting that second-order polynomials can be used to extrapolate the Stokes radii of the denatured ensembles of *m*+, *m*−, and wt SN into their transition zones. These extrapolated lines (Figure 4) indicate that the Stokes radii of the denatured ensembles of these SN proteins are significantly different from one another and continuously contract as urea concentration is decreased. The results with T62P provide strong evidence in favor of Shortle's view of a compact denatured ensemble devoid of a first-order transition.

A key aspect of Shortle's view originates from the concept that the *m* value for protein denaturation is directly a reflection of the change in surface area on going from the native ensemble to the denatured ensemble. While this is a view held by many, for specific wt and mutant proteins, there is hardly any direct quantitative data on this issue (*23*−*25*).

Does the m Value Correlate with the Dimensional Changes in Going from the Native to the Denatured State? Judging from the *m* values determined from fluorescence-detected urea denaturation experiments (Table 1), and the association of *m* values with surface area newly exposed on denaturation, the expected relative Stokes radii of the denatured ensembles of SN proteins ranks A69T as the largest, followed in order of decreasing size by wt and V66W, respectively. The proportionality of $1/K_d$ to the Stokes radius, however, provides a more direct means to evaluate the size differences between the native state ensemble (A69T, V66W, and wt SN all have identical Stokes radii for the native state) and their denatured ensembles. Assuming spherical geometry for native and nonnative ensembles, the relationship $[1/K_d\ (\text{denatured})]^2 - [1/K_d\ (\text{native})]^2$ will be proportional to the newly exposed surface area in the native to denatured transition.

We used three ways to assess the dimensions of the denatured ensembles of SN proteins: one that depends on extrapolation of the dimensions of the denatured ensemble to the midpoint of the transition (the extrapolated value of $1/K_d$ at $C_{1/2}$ is used to represent the size of the denatured ensemble), a second that assesses the dimensions when 1% native protein remains (evaluated at the end of the transition), and a third that evaluates the dimensions of the ensemble of species at the highest urea concentration, 6 M. Table 2 shows that the rank order of size for the nonnative ensembles depends on the conditions under which the sizes are assessed. It is important to note that the *m* value is determined in the transition zone so the rank order obtained in Figure 4 at the midpoint of the transition or at the end of the transition should reflect the same relative surface area changes derived

Table 2. Relative Order of the *m* Values and $\Delta 1/K_d$[a] Values for SN Proteins

m (kcal/mol mol)	A69T (2.45) > wt (2.31) > V66W (1.78)
$\Delta 1/K_d$ at $C_{1/2}$[b]	wt (0.451) > A69T (0.373) > V66W (0.321)
$\Delta 1/K_d$ transition[c]	wt (0.530) > V66W (0.471) > A69T (0.468)
$\Delta 1/K_d$ up to 6 M[d]	A69T (0.608) > wt (0.599) > V66W (0.581)

[a] $\Delta 1/K_d = 1/K_{d\ denatured} - 1/K_{d\ native.}$ $1/K_d$ for native state was taken constant for all three proteins and for all three ways of estimation $\Delta 1/K_d$. [b] $1/K_d$s for the denatured states were taken at positions of $C_{1/2}$ values using baselines extrapolated to the transition region. [c] $1/K_d$s for the denatured states were taken at concentration of urea immediately after transition, where population of native states is 1% according to fluorescence data. [d] $1/K_d$s for the denatured states were taken at 6 M urea.

from the *m* values. This, however, is not what is observed. The quantity $\Delta 1/K_d$ indicates that the denatured ensemble of wt SN exposes more surface area than A69T or V66W, and the rank order at the end of the transition shows A69T as the SN protein having the smallest increase in newly exposed surface area! Only at 3.5 M urea and higher does the rank order of denatured ensemble sizes correspond to that predicted from *m* values, but this is well outside the concentration zone where *m* is measurable.

From plots of *m* values vs ΔCp, Myers et al. show that *m* is roughly proportional to surface area exposed in the denatured state (*23*). However, in their plots, SN is found to deviate significantly from the correlation line (J. Myers, personal communication). The lack of correlation that we find between the *m* value and the dimensions of the denatured ensembles in the transition zone indicates that the *m* value of SN proteins is more complex than simply being a measure of the increase in newly exposed surface area. This is a conclusion drawn earlier by Carra and Privalov (*6*).

The assumption that the *m* value quantitatively reflects the newly exposed surface area of the denatured state is a basic premise of Shortle's view that is not supported by the SEC data. Carra and Privalov have made the point that cooperativity of a transition (reflected by the *m* value) arises from a number of sources including the interaction Gibbs energy between subdomains that can be changed by mutation (*6*, *9*). The findings by Myers et al. of a correlation between *m* values and surface areas exposed on denaturation is important, but the fact that there are significant deviations from the correlation indicates that other factors may come into play that can alter the rank order of presumed sizes of nonnative ensembles in a family of mutated proteins (*23*).

Fixed and Variable Models of Protein Denaturation. SEC-detected urea denaturation of V66W mutant represents a clear example of non-two-state behavior, and while there is positive evidence that *m*+ and wt SN denature according to the two-state model, this has not been totally proven (*26*). Though they had little or no evidence based on data from chemically induced denaturation, Carra and Privalov asserted that a discrete intermediate (I) is involved in urea and guanidine hydrochloride denaturation of wt SN by means of two first-order (two-state) transitions ($N \rightleftharpoons I \rightleftharpoons U$), with the U state, a state that all SN mutant proteins are supposed to achieve at high denaturant concentration, being thermodynamically identical for all SN proteins (*6*). Because the denatured ensembles of wt and mutant SN proteins are viewed by Carra and Privalov as thermodynamic reference states, the thermodynamic character of the U ensemble in their model should be categorized as "fixed" (*21*).

From Table 1, it is shown that fluorescence- and $1/K_d$-detected urea denaturation of wt SN and (*m*+ mutant) A69T give denaturation parameters that are not equivalent. Because these two observables do not track identically with one another, the traditional rules dictate that neither A69T nor wt SN can be considered as exhibiting two-state behavior (*27*). However, such a conclusion fails to acknowledge that the dimensions and thermodynamic character of the compact denatured ensembles are changing dramatically and continuously both inside and outside of the transition zone. This behavior forces consideration that SN proteins are fundamentally different from well-behaved proteins such as RNase A. The fundamental difference in denaturation of RNase A compared to SN proteins lies in the thermodynamic behavior of their respective denatured ensembles. The thermodynamic character of the denatured ensemble of RNase A does not change with denaturant concentration, i.e., it exhibits "fixed" thermodynamic behavior (*17*, *21*), while the thermodynamic characters of the denatured ensembles of SN proteins are continuously changing ("variable"), especially in the transition zones.

It is important to point out the relationship between dimensional changes in a denatured ensemble and the changes in thermodynamic character as the denaturant concentration increases. Polymer theory and experiment hold that coiled polymers will expand their dimensions as the solvent becomes a better solvent. Thus, regardless of whether a denatured ensemble is classified as "fixed" or "variable" its dimensions will increase as the solvent is made better by adding denaturant. What distinguishes a protein that exhibits "fixed" two-state behavior from one that exhibits "variable" two-state behavior are the differences in the *thermodynamic characters* of the denatured ensembles of the two proteins. Though the dimensions of the denatured ensemble will change with urea concentration, this does not mean that the thermodynamic character of a denatured ensemble will necessarily change. The difference in thermodynamic characters of "fixed" and "variable" denatured ensembles can be determined experimentally by taking advantage of the fact that the thermodynamic character of an ensemble is strongly linked to proton uptake/release brought about by increasing the denaturant concentration. With proteins that exhibit "fixed" character of their denatured ensemble, the net proton uptake/release observed with the denatured ensemble at the end of the denaturing transition is not altered with increasing concentration of denaturant. Thus, the thermodynamic character is said to be unchanging or "fixed" [e.g., see Figure 3 of Bolen and Santoro (*28*), and Figure 4 of Yao and Bolen (*17*) for examples of "fixed" thermodynamic behavior]. By contrast, with a protein that exhibits "variable" behavior of its denatured ensemble, the net proton uptake/release of the ensemble at the end of the denaturing transition continues to change ("vary") with increasing denaturant concentration, and this is the case with staphylococcal nuclease (Yang and Bolen, unpublished results) (*29*). The classification of "fixed" and "variable" behavior of denatured ensembles has been discussed in an important paper by Dill and Shortle, who proposed that the multiple variability test is not inviolable for proteins whose denatured ensembles exhibit "variable" rather than "fixed" behavior (*21*).

The existence of "variable" denatured ensembles poses conceptual problems in evaluating denaturation thermody-

namics of such proteins, problems we are not well equipped to deal with at this point. Our view is that it is difficult to deny that some proteins exhibit "variable" thermodynamic behavior in their denatured ensembles, and it is important to develop a sound framework to understand the implications these properties present to protein folding.

SUMMARY

The SEC data presented here show that $1/K_d$ provides evidence of intermediate species of denaturation that is not detected by fluorescence monitoring. These results are consistent with Carra and Privalov's view that *m*− SN mutant proteins should exhibit three-state denaturation and that Shortle's view involving *m*− must be modified, at least with respect to the *m*− mutant V66W. Our $1/K_d$ data strongly indicate that the denatured ensembles of an *m*+, an *m*−, and wt SN proteins dramatically change their dimensions as a function of urea concentration within their respective transition ranges and are distinguishable by size even at 6 M urea. These results are consistent with Dill and Shortle's view of "variable" behavior of the denatured ensembles of wt and SN mutant proteins and are not consistent with the view of Carra and Privalov that the denatured/unfolded ensembles are "fixed" and thermodynamically identical to one another. While it is commonly assumed that the magnitudes of the *m* values for SN proteins are proportional to the newly exposed surface areas on denaturation, this assumption is not supported by SEC measurements of the sizes of the denatured ensembles of SN proteins.

While many of the assumptions and interpretations of the denaturations of SN proteins by Shortle as well as Carra and Privalov are not borne out by our measurements, there is one critical aspect of Shortle's view that is supported. That crucial aspect is the variable dimensional characteristics exhibited by the denatured ensembles of SN proteins. The implications of this "variable" behavior are manifold: (1) calling into question the validity of solvent-induced denaturation Gibbs energy changes for these proteins, (2) denying the use of the denatured ensemble as a reference state for interpreting structure-based thermodynamic changes for such proteins, (3) requiring alterations in the interpretation of the linear extrapolation method and the interpretation of the nature of the denatured state in low denaturant concentration, and (4) requiring a distinction between proteins that exhibit "fixed" two-state behavior from those exhibiting behavior (i.e., "variable") like that of SN proteins. It is likely that a number of proteins exhibit this behavior, making it a general problem of importance in protein folding [see references cited in Shortle (*8*)] (*3*, *4*, *30*). This is a position long held by Shortle.

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