The Peculiar Nature of the Guanidine Hydrochloride-Induced Two-State Denaturation of Staphylococcal Nuclease: A Calorimetric Study†

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Received April 26, 1999; Revised Manuscript Received June 10, 1999

ABSTRACT: This work determines the ratio of $\Delta H_{vH}/\Delta H_{cal}$ for staphylococcal nuclease (SN) denaturation in guanidine hydrochloride (GdnHCl) to test whether GdnHCl-induced denaturation is two-state. Heats of mixing of SN as a function of [GdnHCl] were determined at pH 7.0 and 25 °C. The resulting plot of ΔH_{mix} vs [GdnHCl] exhibits a sigmoid shaped curve with linear pre- and post-denaturational base lines. Extending the pre- and post-denaturational lines to zero [GdnHCl] gives a calorimetric ΔH (ΔH_{cal}) of 24.1 ± 1.0 kcal/mol, for SN denaturation in the limit of zero GdnHCl concentration. Guanidine hydrochloride-induced denaturation Gibbs energy changes in the limit of zero denaturant concentration ($\Delta G°_{N-D}$) at pH 7.0 were determined for SN from fluorescence measurements at fixed temperatures over the range from 15 to 35 °C. Analysis of the resulting temperature-dependent $\Delta G°_{N-D}$ data defines a van't Hoff denaturation enthalpy change (ΔH_{vH}) of 26.4 ± 2.8 kcal/mol. The model-dependent van't Hoff ΔH_{vH} divided by the model-independent ΔH_{cal} gives a ratio of 1.1 ± 0.1 for $\Delta H_{vH}/\Delta H_{cal}$, a result that rules out the presence of thermodynamically important intermediate states in the GdnHCl-induced denaturation of SN. The likelihood that GdnHCl-induced SN denaturation involves a special type of two-state denaturation, known as a variable two-state process, is discussed in terms of the thermodynamic implications of the process.

The interpretation of urea- or GdnHCl[1]-induced denaturation of staphylococcal nuclease (SN) is a highly controversial issue in protein folding (*1*−*6*). Shortle proposed that wt SN and most all SN mutant proteins undergo two-state denaturation induced by either guanidine hydrochloride (GdnHCl) or urea, and that the denatured ensembles of these SN proteins differ significantly from one another in terms of the surface area exposed on denaturation (*1*, *2*, *6*, *7*). Based largely on thermal denaturation data, Carra and Privalov proposed the contrasting view that urea or GdnHCl denatures wt SN and *m*- SN mutant proteins by a three-state mechanism ($N \rightleftarrows I \rightleftarrows U$), and that the unfolded ensembles (U) of these proteins are identical to one another (*3*−*5*).

To distinguish between the two opposing views, it is clear that the question of whether denaturant-induced denaturation of wt SN is two-state or multistate must be resolved. The most accepted test of whether a protein exhibits two-state denaturation is the ΔH ratio test. In this test, a model-independent parameter such as an enthalpy change for denaturation, determined by calorimetry (ΔH_{cal}), is compared to the ΔH_{vH} for denaturation determined by van't Hoff analysis using a two-state model (*8*). A ratio of $\Delta H_{vH}/\Delta H_{cal} = 1$ provides evidence that the model adequately represents the observed data. To determine the $\Delta H_{vH}/\Delta H_{cal}$ ratio, we report here the development of a calorimetric means to evaluate ΔH_{cal} for GdnHCl-induced denaturation and the determination of ΔH_{vH} from GdnHCl-induced denaturation under the same solution conditions using fluorescence-detected denaturation.

While the enthalpy ratio of unity is regarded as a definitive test of the adequacy of the two-state assumption (*4*), it should be recognized that the test also makes certain assumptions about the nature of the denatured ensemble. Namely, it assumes that the denatured state is an ensemble whose thermodynamic properties (e.g., enthalpy, proton inventory, etc.) do not change with denaturant concentration; i.e., it assumes the thermodynamic properties are "fixed" (*6*, *8*). This is a condition that indeed holds for such proteins as RNase A and α-chymotrypsin (*9*), but it is not believed to hold for SN proteins (*6*). In fact, the thermodynamic properties of the denatured ensembles of SN proteins are believed to "vary" with denaturant concentration (*2*, *6*). Taking into account the properties of "fixed" and "variable" denatured ensembles, the thermodynamic consequences of proteins that denature by a "fixed two-state" mechanism are distinctly different from those believed to exhibit "variable two-state" denaturation (*10*). These distinctions require a radically different interpretation of the thermodynamics of proteins that denature by "variable two-state" processes than the interpretation of protein denaturation to which the field is generally accustomed.

† Supported by U.S. Public Health Research Grant GM49760, The John Sealy Memorial Endowment Fund for Biomedical Research, and Amgen Inc.

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[1] Abbreviations: LEM, linear extrapolation method; ΔH_{cal}, calorimetric enthalpy change; ΔH_{vH}, van't Hoff enthalpy change; SN, staphylococcal nuclease; GdnHCl, guanidine hydrochloride; ITC, isothermal titration calorimetry.

10.1021/bi9909400 CCC: $18.00
Published on Web 08/03/1999

The calorimetric measurements reported here are consistent with two-state GdnHCl-induced denaturation of wt SN, with a strong likelihood that the mode of denaturation is of the "variable two-state" type.

MATERIALS AND METHODS

The strain of *Escherichia coli* containing the lambda/AR 120 expression system for wild type SN was a gift from Dr. David Shortle, and the protein was overexpressed and purified using Shortle's method (*1*). The protein used in fluorescence measurements was further purified using a Pharmacia FPLC system equipped with a Source-S (Pharmacia) column. Protein concentrations were determined spectroscopically using $A_{280} = 0.93$ for a 1 mg/mL solution (*11*).

Ultrapure GdnHCl from Amresco was used without further purification. Concentrations of GdnHCl were determined from refractive index measurements at 25.0 ± 0.1 °C on a Milton Roy Tabletop refractometer (Abbe 3L) with temperature controlled by a waterbath. The relationship between the refractive index and the concentration of GdnHCl given here was from Pace, where, Δn represents the difference between the refractive index of the GdnHCl solution and the buffer solution (*12*):

$$[\text{GdnHCl}] = 57.147\Delta n + 38.68\Delta n^2 - 91.6\Delta n^3 \quad (1)$$

Isothermal Titration Calorimetry. Heats of mixing of SN as a function of GdnHCl concentration were determined at pH 7.0 and 25 ± 0.1 °C using an OMEGA Isothermal Titration Calorimeter (ITC) (MicroCal Inc.). Stock solutions of SN as well as a series of GdnHCl solutions with different concentrations were separately prepared in 25 mM phosphate buffer containing 0.1 M NaCl (PBS, pH 7.0). The ITC experiments were performed by four injections (using 6−10 μL of a 40 mg/mL stock solution of SN per injection) into the calorimeter cell (ca. 1.5 mL) containing a specified GdnHCl concentration. Control experiments involving injections of PBS buffer into GdnHCl solution and injections of stock SN solution into PBS buffer were also conducted. For each GdnHCl concentration, heats of mixing experiments for SN solution with GdnHCl solution (Q_s), PBS buffer with GdnHCl solution (Q_b), and SN solution with PBS buffer (Q_d) were separately measured. The net calorimetric heat of mixing of SN with GdnHCl (ΔH_{mix}) was obtained using eq 2:

$$\Delta H_{mix} = (Q_s - Q_b - Q_d)/P \quad (2)$$

where P represents the total moles of SN added in each injection and the other symbols are as described above. The final concentration of SN in the titration cell after four injections of SN solution was about 1 mg/mL.

Heats of mixing (ΔH_{mix}) of acid-denatured SN as a function of GdnHCl concentration were also determined at pH 3.0 and 25 ± 0.1 °C in 25 mM citrate buffer containing 0.1 M NaCl, using the same procedure as described above for pH 7.0.

Intrinsic Fluorescence Intensity Measurements. GdnHCl-induced denaturation of SN was monitored by tryptophan fluorescence emission (excitation at 295 nm, emission at 335 nm) using a SPEX FluoroMax spectrofluorometer. A volume of 2.50 mL of SN solution was delivered into a 1.0 × 1.0 cm quartz cuvette fitted with a water-tight cap. The cuvette was thermostated at the set temperatures (±0.1 °C) reported. The 50 μg/mL SN stock solution for fluorescence measurements was prepared in 25 mM phosphate containing 0.1 M NaCl buffer (PBS, pH 7.0) as was the 4 M GdnHCl titrant solution. The protein solution in the cuvette was titrated with the 4 M GdnHCl solution, using a PB600 dispenser (Hamilton Co.) equipped with two 100 μL Hamilton syringes. An aliquot of from 10 to 40 μL of the protein solution was removed from the cuvette, and an aliquot of the exact same volume of the 4 M GdnHCl solution was added to the cuvette, maintaining a total volume of 2.50 mL solution in the cuvette. Thorough mixing of the solution was achieved by magnetic stirring from the bottom with a "cross-shape" Teflon covered stirring bar. After each addition of GdnHCl, a time interval of from 10 to 30 min was observed before the equilibrium fluorescence intensity was recorded. A blank titration was performed in the same manner described above, except the PBS buffer replaced the protein solution. After each titration step involving equivolume removal of solution and addition of the GdnHCl titrant, the protein concentration in the cuvette was calculated. The observed equilibrium fluorescence intensity was corrected by subtraction of the blank, and the data were normalized in terms of fluorescence intensity per mole of protein (F). The fluorescence intensity at each GdnHCl concentration (F) divided by the initial fluorescence intensity at zero GdnHCl concentration (F_0) gives the fluorescence ratio (F/F_0), which was then plotted as a function of GdnHCl concentration.

The fluorescence ratio measurements of SN denaturation by GdnHCl were performed at different fixed temperatures (15, 20, 25, 30, and 35 °C). Since denaturation was found to be slower at low temperature, the longest delay time (30 min) between addition of GdnHCl and fluorescence measurement was used for data gathered at 15 °C. The resulting fluorescence-detected denaturation profiles were analyzed using the nonlinear least-squares method described previously, giving $\Delta G°_{N-U}$, m, and C_m parameters at each temperature (*13*).

RESULTS

Determination of the Calorimetric Enthalpy (ΔH_{cal}) of SN Denaturation in GdnHCl. Heat of mixing (ΔH_{mix}) results of native SN (pH 7.0) and acid-denatured SN (pH 3.0) with GdnHCl at 25 °C are shown in Figures 1 and 2, respectively.

The data presented in Figure 1 demonstrate a distinct denaturation transition with a midpoint GdnHCl concentration (C_m) of 0.77 M. In the pre- and post-denaturation regions, ΔH_{mix} of native SN is shown to be linearly dependent on [GdnHCl]. To evaluate the model-independent calorimetric enthalpy (ΔH_{cal}) from these data, the linear post-denaturation base line was extended to zero concentration of GdnHCl, and the difference between the pre- and post-denaturation intercepts at zero GdnHCl concentration was determined. This difference represents the calorimetric enthalpy (ΔH_{cal}) for conversion of native SN to denatured SN at zero concentration of denaturant, and was found to be 24.1 ± 1.0 kcal/mol.

The rationale for linear extension of the post-denaturation base line in Figure 1 is the following: (1) the heat of mixing

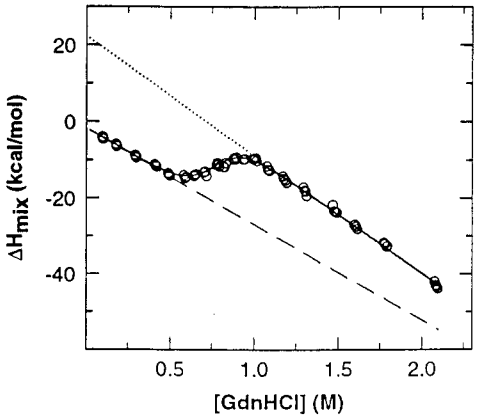

FIGURE 1: Heats of mixing (ΔH_{mix}) of SN with GdnHCl at 25.0 °C in 0.1 M NaCl and 25 mM phosphate buffer (pH 7.0). Final SN concentration is about 1 mg/mL. Open circles (○) represent experimental data, and the solid line is the nonlinear least-squares best fit of the data using the LEM. The dotted and dash lines are the LEM-determined extensions of post- and pre-denaturational base lines, respectively.

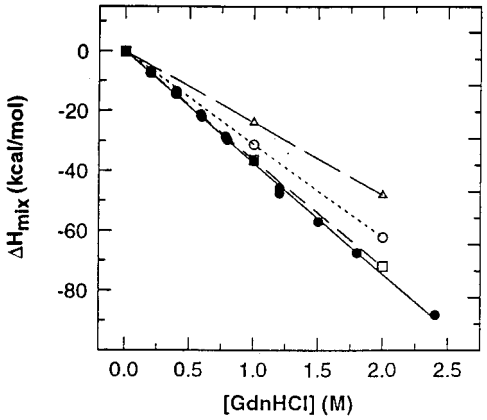

FIGURE 2: Heats of mixing (ΔH_{mix}) of pH 3 acid-denatured SN with GdnHCl (filled circles) at 25.0 °C in 25 mM citrate plus 0.1 M NaCl buffer. Final SN concentration following four additions of 6−10 μL of protein stock is about 1 mg/mL with a total calorimetric cell volume of 1.5 mL. Heats of mixing data for other proteins with GdnHCl were taken from Table 2 of Makhatadze and Privalov (*15*), and include reduced and carboxymethylated derivatives of RNase A (open circles), hen lysozyme (open squares), and bovine heart cytochrome *c* (open triangles). The solid lines are linear least-squares best fits to the data.

experiments of acid-denatured SN (pH 3.0) with GdnHCl at 25 °C give a linear plot of ΔH_{mix} versus [GdnHCl], (see Figure 2), suggestive of a linear dependence of ΔH_{mix} on [GdnHCl] with acid (0−2 M GdnHCl range); (2) the generally accepted linear extrapolation method provides a precedent using linear extensions of pre- and post-denaturation base lines to evaluate $\Delta G°_{N-D}$ (*14*); and (3) calorimetric titration of reduced and carboxymethylated (denatured) forms of RNase A, lysozyme, and cytochrome *c* all give linear ΔH_{mix} vs [GdnHCl] over the 0−2 M GdnHCl range (see Figure 2) (*15*).

Determination of the van't Hoff Enthalpy (ΔH_{vH}) of SN Denaturation in GdnHCl. The data presented in Figure 3 show fluorescence ratio-detected GdnHCl-induced denaturation transitions of SN at pH 7.0 at fixed temperatures in the range from 15 to 35 °C. All the transition curves appear to have a symmetrical sigmoid shape characteristic of an apparent "two-state" cooperative process. The dotted lines

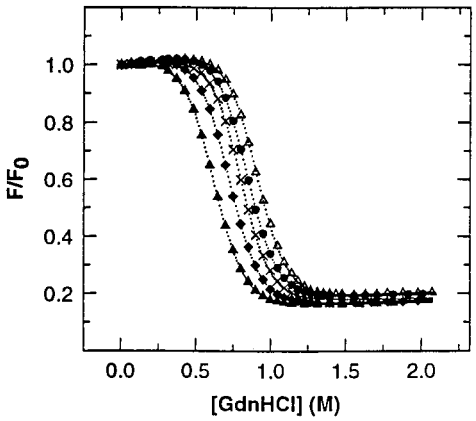

FIGURE 3: Fluorescence ratio (F/F_0) of SN in 25 mM phosphate plus 0.1 M NaCl (pH 7.0) as a function of GdnHCl concentration at 15.0 °C (open triangles), 20 °C (filled circles), 25.0 °C (crosses), 30.0 °C (filled diamonds), and 35.0 °C (filled triangles). Dotted lines are the nonlinear least-squares best fits of the data at different temperatures using the linear extrapolation method. The SN concentration is around 50 μg/mL. The wavelengths of excitation and emission are 295 and 335 nm, respectively.

Table 1: GdnHCl-Induced Denaturation of wt SN in 25 mM Phosphate plus 0.1 M NaCl, pH 7.0, at 15, 20, 25, 30, and 35 °C

temp (°C)	$\Delta G°_{N-D}$ (kcal/mol)	m (kcal mol^{-1} M)	C_m (M)
15.2	5.10 ± 0.04	5.65 ± 0.04	0.90
20.0	4.79 ± 0.02	5.72 ± 0.02	0.84
25.0	4.77 ± 0.04	6.00 ± 0.04	0.80
30.0	4.15 ± 0.03	5.78 ± 0.04	0.72
35.0	3.61 ± 0.02	5.88 ± 0.03	0.61

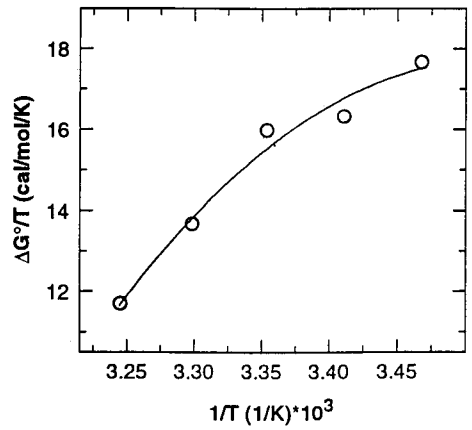

FIGURE 4: Plot of $\Delta G°_{N-D}/T$ versus $1/T$ to determine the van't Hoff ΔH of SN denaturation in GdnHCl at 25 °C and pH 7.0. Open circles represent $\Delta G°_{N-D}$ values obtained from Table 1, and the solid line represents the nonlinear least-squares best fit of the data using eq 3.

in Figure 3 are nonlinear least-squares fits of the transitions to the "two-state" model using the LEM (*13*). As a function of temperature, the fitted parameters derived from the fittings are listed in Table 1 and include $\Delta G°_{N-D}$ (the denaturation Gibbs energy change in the limit of zero GdnHCl concentration), C_m (the GdnHCl concentration at the midpoint of the transition), and m (the sensitivity of denaturation to GdnHCl concentration, d ΔG/d[GdnHCl]).

Figure 4 shows a plot of $\Delta G°_{N-D}/T$ versus temperature, and a van't Hoff type analysis was performed to evaluate the ΔH_{vH} of SN denaturation at 25 °C by means of eq 3. Nonlinear least-squares analysis of the data to eq 3 gives

Table 2: GdnHCl-Induced LEM Parameters of SN Denaturation at 25 °C and pH 7.0

	$\Delta G°_{N-D}$ (kcal/mol)	m (kcal mol^{-1}/M)	C_m (M)
calorimetry	5.02 ± 0.44	6.50 ± 0.52	0.77 ± 0.09
fluorescence	4.77 ± 0.04	6.00 ± 0.04	0.80 ± 0.01

$$\Delta G_{N-D}(T)/T = [\Delta H_{vH}(25\ °C) - \Delta C_p 298.15]/T - \Delta C_p \ln(T) + \text{const} \quad (3)$$

with $\Delta C_p = 1.8 \pm 1.0$ kcal/(mol·deg) and $\Delta H_{vH}(25\ °C) = 26.4 \pm 2.8$ kcal/mol as fitting parameters. Within error, ΔH_{cal} and ΔH_{vH} are found to be identical, and a ratio of $\Delta H_{vH}/\Delta H_{cal}$ (1.10 ± 0.12) near unity is obtained.

Multivariability Test. The multivariability test is frequently used to determine whether "two-state" behavior can be excluded as a possible model for denaturation (*8*). Two-state behavior requires that all observables, each of which may be sensitive to different characteristics of native or denatured protein, must change in unison as the fraction of native or denatured protein changes with denaturant concentration. The nonlinear least-squares fitted values for $\Delta G°_{N-D}$, m and C_m were obtained from analysis of the two sets of data by means of the LEM using eq 4 (13):

$$I = \{(I°_N + m_N[C]) + (I°_D + m_D[C]) \exp[-(\Delta G°_{N-D} - m[C])/(RT)]\}/\{1 + \exp[-(\Delta G°_{N-D} - m[C])/(RT)]\} \quad (4)$$

Here I is an observable that can be either ΔH_{mix} or the fluorescence ratio; [C] is the molar concentration of the denaturant (GdnHCl); $I°_N$ and $I°_D$ represent the intercepts and m_N and m_D the slopes of the linear pre- and post-denaturation base lines. Table 2 shows that within error the two observables (ΔH_{mix} and fluorescence) track identically with one another, a result consistent with two-state behavior.

DISCUSSION

The primary goal of this work has been to determine the ratio of $\Delta H_{vH}/\Delta H_{cal}$ for GdnHCl-induced denaturation of SN to test whether the denaturation can be described as two-state. Key to this determination is the evaluation of the model-independent calorimetric enthalpy (ΔH_{cal}) from heat of mixing data. There are two critical questions involved in evaluation of ΔH_{cal}: (1) is the heat of mixing of denatured protein linear with GdnHCl concentration, and (2) is the observed enthalpy difference between the pre- and post-denaturation intercepts in the limit of zero GdnHCl concentration equal to the denaturant-independent enthalpy change for the transition from native to denatured protein?

With respect to the first question, experimental support for linear extension of ΔH_{mix} of SN in the post-denaturation region to 0 M GdnHCl comes from heats of mixing experiments with acid-denatured SN (pH 3.0). SN acid denatures at low pH (pH 3) and is substantially unfolded (about 80% unfolded in terms of native secondary structure and fully unfolded by near-UV CD) (*16*). In this sense, acid-denatured SN may serve as a model of GdnHCl-denatured SN, and the linear dependence of ΔH_{mix} with 0−3 M GdnHCl observed in Figure 2 provides a rationale for extension of the post-denaturation base line in Figure 1 to zero concentration of denaturant. Moreover, Makhatadze and Privalov have conducted calorimetric studies of the interactions of urea and GdnHCl with three proteins (RNase A, lysozyme, and cytochrome *c*) (*15*). These proteins have been forced to exist as denatured ensembles in buffer solution because of reduction and carboxymethylation of their disulfide bonds. Makhatadze and Privalov's ΔH_{mix} data, also shown in Figure 2, are observed to be linear over the 0−2 M GdnHCl concentration range, the same GdnHCl concentration range encompassed by the heat of mixing data in Figure 1 (*15*). Thus, in cases that involve denatured proteins in buffer solution, ΔH_{mix} is found to be a linear function of GdnHCl in the concentration range of interest (0−2 M). These data provide a strong rationale for linear extension of the post-denaturation ΔH_{mix} data to zero denaturant concentration.

The second question deals with whether the observed enthalpy difference between pre- and post-denaturation intercepts in the limit of zero GdnHCl is equal to the denaturant-independent calorimetric enthalpy change (ΔH_{cal}) for the transition from native to denatured protein. Because the denatured state of a protein has more surface area exposed to solvent than does the native state, the number of denaturant molecules interacting with the denatured state is expected to be greater than that interacting with the native state. In Figure 1, this is reflected in the smaller slope observed for the GdnHCl interaction with the native state compared with the denatured state. In the transition region, the ΔH_{mix} values of native and denatured proteins can be obtained by extending the post- and pre-denaturation base lines into the transition zone. The difference between native and denatured ΔH_{mix} values includes not only the N ⇄ D conformational enthalpy change in the limit of zero GdnHCl concentration (ΔH_{Cal}) but also the denaturant concentration-dependent GdnHCl "binding" heats ($Q*_{b,D}$ and $Q*_{b,N}$) due to any conversion of native to denatured protein in the mixing experiment (see eq 5):

$$\Delta H_{mix,D} - \Delta H_{mix,N} = \Delta H_{cal} + (Q*_{b,D} - Q*_{b,N}) \quad (5)$$

The question is how $Q*_{b,D}$ and $Q*_{b,N}$ depend on GdnHCl as mixing heats $\Delta H_{mix,D}$ and $\Delta H_{mix,N}$ are extrapolated back to zero denaturant concentration. From the data in Figures 1 and 2, it is clear that the magnitude of denaturant binding to native or denatured states ultimately reduces to zero as the denaturant concentration goes to zero. Thus, as $\Delta H_{mix,D} - \Delta H_{mix,N}$ is extrapolated back to zero denaturant concentration, $Q*_{b,D}$ and $Q*_{b,N}$ both vanish in the limit of zero GdnHCl, leaving $\Delta H°_{N-D}$, the model-independent SN denaturation enthalpy change in the limit of zero denaturant concentration.

Using intrinsic fluorescence, the denaturation free energy change of SN in the limit of zero GdnHCl concentration ($\Delta G°_{N-D}$) was determined under the same solution conditions as in the calorimetric measurements, but over a range of different set temperatures. Because a "two-state" model was used in estimating $\Delta G°_{N-D}$ by the LEM, a quantity evaluated in the limit of zero GdnHCl concentration, the van't Hoff denaturation enthalpy change is, by definition, a model-dependent thermodynamic parameter. The van't Hoff (26.4 ± 2.8 kcal/mol) and calorimetric (24.1 ± 1.0 kcal/mol) ΔH values give a ratio ($\Delta H_{vH}/\Delta H_{cal} = 1.10 \pm 0.12$) near unity, a result consistent with two-state denaturation.

The Controversy over GdnHCl-Induced Denaturation of SN. The issue of whether GdnHCl-induced denaturation of SN is two-state is the crux of a long-running debate on the peculiar properties of GdnHCl denaturation of SN and SN mutant proteins (*2, 4*). Dill and Shortle have claimed that GdnHCl denaturation of SN is two-state but that the denatured ensemble varies as a function of denaturant concentration; i.e., their model is said to exhibit "variable two-state" character and can be written as $N \rightleftarrows D_{(variable)}$ (*6*). In this model, the denatured ensembles for wt and mutant SN proteins can be quite different from one another both physically and thermodynamically, a concept very different from conventional views of denaturation. One reason this model is controversial is that it is at odds with the common practice in structural energetics of assuming that the denatured states of wt and mutant protein are thermodynamically equivalent. Carra and Privalov proposed an alternative view of GdnHCl denaturation of SN in which the denatured states of wt and mutant SN proteins are thermodynamically identical to one another, a model that is consistent with the assumption commonly applied in structural energetics (*4*).

Carra and Privalov have provided an excellent review of the differences between their position and that of Dill and Shortle (*4*). The model of Carra and Privalov involves a (first-order) two-state conversion of the native state (N) to an intermediate state (I), followed by a (first-order) two-state conversion of the intermediate to the unfolded state (U). That is, they view the overall GdnHCl denaturation process as involving an intermediate species that consists mechanistically of two consecutive first order events, $N \rightleftarrows I \rightleftarrows U$. Carra and Privalov prefer to think of Dill and Shortle's denaturation model [$N \rightleftarrows D_{(variable)}$] as a (first-order) two-state conversion of the native state (N) to a compact denatured state (C), followed by a one-state (higher order) transition of C to a less compact ensemble (D). They formulate Dill and Shortle's model as $N \rightleftarrows C \rightleftarrows D$, with the $C \rightleftarrows D$ event being a denaturant concentration dependent noncooperative process that has no activation barrier between C and D. In short, $C \rightleftarrows D$ represents interconversions within the [$D_{(variable)}$] ensemble of species, and the formulations, $N \rightleftarrows D_{(variable)}$ and $N \rightleftarrows C \rightleftarrows D$ are thermodynamically equivalent.

The key difference between the model of Carra and Privalov ($N \rightleftarrows I \rightleftarrows U$) and of Dill and Shortle [$N \rightleftarrows D_{(variable)}$] is that $N \rightleftarrows I \rightleftarrows U$ is multistate while $N \rightleftarrows D_{(variable)}$ is two-state (*2, 4, 6*). Lumry et al. have shown that multistate behavior will always result in a $\Delta H_{vH}/\Delta H_{cal} < 1$ (*8*), and what we have found with SN is that the enthalpy ratio is slightly greater than unity. Thus, the enthalpy ratio test clearly mitigates against Carra and Privalov's $N \rightleftarrows I \rightleftarrows U$ model. Lumry et al. have also shown that a $\Delta H_{vH}/\Delta H_{cal}$ ratio of unity is the hallmark of two-state behavior, and Carra and Privalov claim that a ratio equal to unity is definitive evidence that a reversible denaturation exhibits two-state character (*4, 8*). The enthalpy ratio we have obtained ($\Delta H_{vH}/\Delta H_{cal} = 1.10 \pm 0.12$) is identical to the $\Delta H_{vH}/\Delta H_{cal} = 1.10$ ratio obtained by Carra and Privalov from thermal denaturation measurements of SN in the absence of chemical denaturant (*17*). Based on the enthalpy ratio, Carra and Privalov concluded that thermal denaturation of SN is two-state (*4*), and the $\Delta H_{vH}/\Delta H_{cal}$ ratio slightly >1 is attributed by them to a small amount of protein−protein association in either the native or the denatured state (*17*). Given the fact that the $\Delta H_{vH}/\Delta H_{cal}$ ratios for thermal and GdnHCl-induced denaturation of SN are identical, GdnHCl-induced SN denaturation exhibits two-state behavior according to Carra and Privalov's own criteria.

The failure of Carra and Privalov's multistate model in terms of the enthalpy ratio test leaves open the question of whether the view of Dill and Shortle is viable. To better appreciate the issues implied by Dill and Shortle's model, it is useful to describe SN denaturation by chemical denaturants in these authors' terms. Dill and Shortle proposed some time ago that "state-distinguishable" properties of the denatured states of SN proteins (e.g., Stokes radius) vary with denaturant concentration, and they classified proteins whose denatured ensembles behaved this way [$N \rightleftarrows D_{(variable)}$] as exhibiting "variable two-state " behavior (*6*). Dill and Shortle also proposed a class of proteins whose state-distinguishable denatured ensemble properties do not vary with denaturant concentration, referring to these proteins as exhibiting "fixed two-state" behavior. The thermodynamic framework for proteins exhibiting "fixed two-state" behavior was established long ago (*8*), and in the ensuing years essentially all two-state denaturations of proteins have been evaluated by implicitly assuming "fixed two-state" behavior. Denaturations that exhibit "variable two-state" behavior are conceptually quite different from "fixed two-state" behavior (*10*). And as shown below, the thermodynamic consequences of "variable two-state" behavior differ markedly from the consequences that hold for "fixed two-state" protein denaturation.

In an effort to understand the dimensional characteristics of denatured wt and mutant SN proteins, we recently evaluated the Stokes radii of wt and three mutant SN proteins as a function of urea concentration (*10*). Consistent with Dill and Shortle's view, we found that the denatured ensembles of the SN proteins differ dimensionally from one another, and, most importantly, we determined that the dimensions of their denatured ensembles change significantly in the transition zone. For example, the Stokes radius of the wt SN denatured ensemble at the beginning of the urea-induced transition at pH 7 is 29.2 Å, and is increased to 32.8 Å by the end of the transition (evaluated from the data in Baskakov and Bolen) (*10*). Assuming spherical geometry, this represents a 40% increase in volume and a 25% increase in surface area of the denatured ensemble, all of which occurs within the transition zone. The fact that the denatured ensemble expands significantly as a function of increased urea concentration in the transition zone strongly suggests that the thermodynamic character of the denatured ensemble might also be changing.

To illustrate better what is meant by the thermodynamic character of a denatured ensemble, let us consider the GdnHCl-induced denaturation transitions as monitored by ΔH_{mix} and fluorescence in light of the fact that the SN-denatured ensemble expands significantly with GdnHCl in the transition zone. Figure 5 gives plots of the fraction of denatured SN induced by GdnHCl as monitored by fluorescence and ΔH_{mix}. In the lower GdnHCl concentration range (near the beginning of the transition), the denatured ensemble is compact, and the associated denaturation enthalpy change will be of a certain value. (For the sake of this discussion, the enthalpy of the denatured ensemble is taken as a measure of its thermodynamic character.) But with increasing GdnHCl concentration, the denatured ensemble increases in size (one-

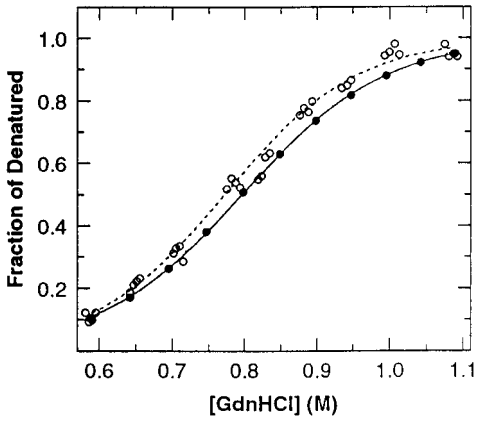

FIGURE 5: Fraction of denatured SN induced by GdnHCl and detected by calorimetry (open circles) and fluorescence (filled circles). The solid lines represent the nonlinear least-squares fit of the ΔH_{mix}-monitored and fluorescence-monitored transitions expressed in terms of fraction denatured, using the parameters given in Table 2. All results shown originate from GdnHCl-induced denaturation data on SN at 25 °C and pH 7.0.

state expansion), and the thermodynamic character (enthalpy) of the denatured ensemble changes coordinately with the expansion of the denatured ensemble. This process has small but significant enthalpy contributions resulting from disruption of some intramolecular interactions within the compact denatured ensemble and exposure of additional protein fabric to solvent. In this particular case, the small enthalpy contribution due to expansion leads to a somewhat larger ΔH per mole of protein near the high denaturant concentration end of the transition than occurs at the beginning of the transition. Thus, throughout the denaturation transition, the ΔH per mole of denatured protein becomes increasingly more endothermic. An important consequence, then, of the changing dimensions of the "variable" denatured ensemble in the transition zone is that the denaturation ΔH is not a constant, but varies with denaturant concentration. This is in marked contrast to "fixed two-state" behavior in which the thermodynamic character of the denatured ensemble does not change with denaturant concentration, and the denaturation ΔH at the beginning of the transition is the same as the ΔH at the end of the transition.

Tryptophan 140 located in the carboxy-terminal helix gives rise to the fluorescence in SN, and the change in this observable as a function of [GdnHCl] is compared with the ΔH_{mix}-detected transition in Figure 5. Carra and Privalov have argued that denaturation exposes the tryptophan to solvent, greatly diminishing the fluorescence, and the diminished fluorescence is essentially independent of the existence of residual structure in the denatured ensemble (*4*). Thus, in Figure 5 we have two observables: one (ΔH_{mix}) that should be sensitive to the expansion of the denatured ensemble in the transition, and one (fluorescence) that has been argued (*4*) as being insensitive to the denatured ensemble expansion. Inspection of the data in Figure 5 shows that the calorimetric data progressively deviate from the fluorescence data, with the deviation (manifested in a sharper transition) being greatest at the high denaturant concentration end of the transition. This is a result one would expect of a "variable two-state" system.

We should note that although the deviation of the ΔH data in Figure 5 is progressive, the fitted data are not outside the error limits of the measurements (see Table 2. However, in terms of the steepness of the transition, it is clear from the comparison of the fitting parameters in Table 2 that the *m* value for the calorimetric data is greater than that for the fluorescence data, with the error limits of the *m* values just barely overlapping. The error analysis does not permit an unequivocal claim that GdnHCl-induced denaturation of SN exhibits "variable two-state" behavior, but given the fact that the size of the denatured ensemble is changing markedly in the transition zone (*10*), the calorimetric and fluorescence data clearly suggest that "variable two-state" behavior is plausible. The noncoincidence of two observables that monitor the same denaturation transition (the multivariability test) (*8*) is conventionally taken to mean that the denaturation process is not two-state. This is a good test that is absolutely correct for "fixed two-state" behavior, but will not necessarily hold for "variable two-state" denaturation.

The differences between variable and fixed two-state behavior result in markedly different interpretations of the Gibbs energy change ($\Delta G°_{N-D}$) for denaturation of SN in the limit of zero denaturant concentration obtained using LEM. At a denaturant concentration near the beginning of the SN denaturation transition, the equilibrium is between the native state and a compact denatured state, while at the end of the transition the equilibrium is between the native state and a more expanded and thermodynamically different denatured state. This situation means that with a "variable two-state" denaturing protein, use of the linear extrapolation method will result in a $\Delta G°_{N-D}$ in which the denatured ensemble represented by the putative $N \rightleftarrows D$ equilibrium in the limit of zero denaturant concentration bears little relationship either structurally or thermodynamically with the denatured species in the transition zone. This contrasts with $\Delta G°_{N-D}$ obtained from a protein exhibiting "fixed two-state" character, because in the "fixed two-state" case the denatured ensemble has the same thermodynamic character in the limit of zero denaturant concentration as it does in the transition zone (*9, 10, 18*).

If GdnHCl denaturation of wt SN is "variable two-state", then the $\Delta H_{vH}/\Delta H_{cal}$ ratio must be interpreted differently from the way it is usually interpreted. The van't Hoff and calorimetric enthalpy changes derived for a protein exhibiting "variable two-state" behavior are not reflecting the enthalpy change for one reaction; rather they represent derived quantities from a continuum of reactions that occur in the transition zone (*10*).

Enthalpy is only one of several measures of the thermodynamic character of a denatured ensemble. We have found that the linkage of proton uptake/release that accompanies changes in the denatured ensemble is a much more sensitive index of the thermodynamic character of the ensemble than enthalpy, and provides an excellent means of distinguishing "fixed" from "variable" behavior in the denatured ensemble (Yang and Bolen, unpublished results). Clearly, a protein that exhibits "variable two-state" denaturation will require interpretation based on a distinctly different paradigm from the "fixed two-state" concept that virtually all interpretations of denaturations have been based. The extent to which "variable two-state" behavior is exhibited by proteins is not known, because few have looked for this type of behavior. Shortle gives a listing of a number of proteins that are similar in behavior to SN and are likely to exhibit "variable" behavior (*7*).

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BI9909400