Protein Science (1994), *3*:944-951. Cambridge University Press. Printed in the USA.
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Thermodynamics of staphylococcal nuclease denaturation. I. The acid-denatured state

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(RECEIVED December 10, 1993; ACCEPTED March 16, 1994)

Abstract

Using high-sensitivity differential scanning calorimetry, we reexamined the thermodynamics of denaturation of staphylococcal nuclease. The denaturational changes in enthalpy and heat capacity were found to be functions of both temperature and pH. The denatured state of staphylococcal nuclease at pH 8.0 and high temperature has a heat capacity consistent with a fully unfolded protein completely exposed to solvent. At lower pH values, however, the heat capacity of the denatured state is lower, resulting in a lower ΔC_p and ΔH for the denaturation reaction. The acid-denatured protein can thus be distinguished from a completely unfolded protein by a defined difference in enthalpy and heat capacity. Comparison of circular dichroism spectra suggests that the low heat capacity of the acid-denatured protein does not result from residual helical secondary structure. The enthalpy and heat capacity changes of denaturation of a less stable mutant nuclease support the observed dependence of ΔH on pH.

Keywords: denatured state; molten globule; protein denaturation; structural intermediates; unfolding

The stability of the native state of a protein can be defined as the difference in free energy between the native state and a fully unfolded reference state. Determination of this energy is always complicated, however, by the question of to what extent a denatured protein is fully unfolded. In this paper we address how the characteristics of the denatured state can affect the thermodynamics of denaturation. Following Tanford (1968), we define the denatured state of a protein operationally, as that form of the protein which is observed after the breakdown of the cooperative native structure by a particular method of denaturation. Each method of denaturation may, if necessary, be considered as a distinct process, potentially yielding different products. We refer to a hypothetical extreme form of the protein, lacking all regular structure and approximating a random coil in a theta solvent (Miller & Goebel, 1968; Dill & Shortle, 1991), as the unfolded form.

In some cases, it has been shown that denatured proteins contain some amount of structure or compactness and therefore are not completely unfolded (Dobson, 1992; Ptitsyn, 1992). Tanford (1968) reviewed the properties of proteins denatured by various methods and concluded that denaturation by high concentrations of guanidine-HCl or urea generally yields the most complete unfolding. Denaturation by acid was in some cases not so thorough, giving a product Tanford considered to be far from a random coil. Nevertheless, for several proteins it has been found (Pfeil & Privalov, 1976; Privalov, 1979; Makhatadze & Privalov, 1992) that the enthalpy changes associated with denaturation do not depend upon the method of denaturation when all appropriate corrections for ionization or denaturant binding are taken into account. All methods of denaturation could thus be considered to yield the same product as far as thermodynamics is concerned. For several proteins studied at pH values far from their isoelectric points and at very low ionic strength (Privalov et al., 1989), the changes in thermodynamic parameters associated with denaturation indicated that the denatured product could reasonably be considered as a random coil. This finding, while having the advantage of greatly simplifying thermodynamic analysis of protein stability, seems to conflict with the opinion that the extent of protein denaturation is in many cases incomplete and depends upon the conditions utilized.

Many recent studies (Goto et al., 1990a, 1990b; Alonso et al., 1991; Buchner et al., 1991; Dryden & Weir, 1991; Fink et al., 1993) have focused on proteins under acidic conditions, following the observation at low pH of species that are often referred to as "molten globules." Intermediate forms of proteins are as yet poorly understood, but in its traditional definition (Ptitsyn, 1992) the molten globule is a compact form containing part of the secondary-structure content of the native protein but lacking specific tertiary interactions. The structural and thermodynamic distinction between these forms and the denatured state

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as defined above is often unclear (Haynie & Freire, 1993) and is a current point of interest.

Staphylococcal nuclease (SNase) is a widely studied model system for protein folding and has previously been the subject of calorimetric investigation (Calderon et al., 1985; Griko et al., 1988; Shortle et al., 1988; Tanaka et al., 1993). As part of a project to study the effects of mutations on the heat capacities, enthalpies, and entropies of proteins, we herein reexamine the thermodynamics of SNase denaturation using differential scanning calorimetry. We find that the heat capacity of the acid-denatured protein is anomalously low, with a consequent effect on the enthalpy of denaturation. The heat capacity change and configurational enthalpy change of denaturation thus depend upon pH. The structural origin of this dependence is unclear, but may be caused by partial clustering of nonpolar residues in the denatured state.

Other investigators (Nakano & Fink, 1990; Fink et al., 1993) have found partial structure in SNase at low pH and high salt concentrations and have called this form the "A-state." We believe that the SNase A-state is actually a different species from the denatured state studied herein and explore the issue further in the following paper in this issue (Carra et al., 1994).

Results

High-sensitivity differential scanning calorimetry allows the determination of both the enthalpy change of protein denaturation and the absolute heat capacities of the native and denatured states (Privalov & Potekhin, 1986). Because the heat capacity is the temperature derivative of the enthalpy function, integration of the peak in the heat capacity function gives the enthalpy change of denaturation. Denaturation by heat or acid is mostly reversible for SNase, allowing the application of equilibrium thermodynamics. Figure 1A shows the heat capacity function of SNase in buffers of pH values 3.0, 4.1, 7.0, and 8.0. The native state of SNase becomes less stable with decreasing pH from 8.0 to 3.0 (Cuatrecasas et al., 1968) and, as we can see in Figure 1A, the protein is in the denatured state at pH 3.0 over the whole temperature range measured. The dashed line represents the heat capacity of a hypothetical, fully unfolded and solvent-exposed nuclease. It has been calculated from the heat capacities of the individual amino acids, assuming simple additivity, using the method of Makhatadze and Privalov (1990). The heat capacity of the denatured SNase at pH 8.0 comes close to this calculated value and slightly exceeds it at high temperatures. This is evidence that denatured SNase at pH 8.0 and 90 °C is fully unfolded, considering that the heat capacity increase of protein denaturation has been found to be proportional to the exposure of buried nonpolar residues to solvent (Makhatadze & Privalov, 1990). With lower pH, however, the heat capacity of the denatured SNase is lower, decreasing by approximately 9 kJ K^{-1} mol^{-1} from pH 8.0 to 3.0. Figure 1B shows more heat capacity curves at additional pH values, with the same behavior evident. The curves at pH 8.5 and 2.1 were both obtained in phosphate buffer. The change in heat capacity with pH is therefore not due to the difference in buffers used over the range of pH values. Acid-denatured SNase by the criterion of heat capacity is not fully unfolded at high or low temperatures over the range from 15 to 100 °C. Incomplete exposure of nonpolar residues to solvent might be expected to result in such a lower heat capacity.

The heat capacity of the native state, on the other hand, shows no consistent variation with pH within an error in measurement of ±2 kJ K^{-1} mol^{-1}. From pH 8.5 to 4.1, the 4 histidines of the native protein will be titrated as will partially the 12 glutamate and 7 aspartate residues. The denatured state of SNase is not much more charged than the native state. At pH 4.1, it binds only about 1 more proton than the native state, as determined from the pH dependence of the denaturation temperature T_d (Table 1) and the following equation from Privalov et al. (1969):

$$\Delta n = \frac{\Delta H^{cal}}{2.303RT_d^2} \cdot \frac{dT_d}{d\mathrm{pH}}.$$

The heat capacity of the native state therefore functions as an internal control in these experiments, telling us that the heat capacity function of the protein is not very sensitive to ionization of side chains alone.

The heat capacity of the native and denatured states is also not very sensitive to ionic strength (Fig. 1C). At pH 4.1 in a 20 mM glycine-HCl buffer, the heat capacities of the native and denatured states change little with the addition of sodium chloride to 800 mM. The heat capacities of both states do show a slight decrease with increasing salt concentration, but this occurs in parallel and is within the error of the measurements. The

Table 1. *Thermodynamics of SNase denaturation*

pH	[NaCl] (mM)	T_d [a] (°C)	ΔH^{cal} [b]	$\Delta H^{fit}/\Delta H^{cal}$ [c]	ΔS [d]	Δn [e]	ΔC_p [f]
			Wild-type nuclease				
4.10	0	43.2	206	1.04	0.651	0.6	5.0
4.10	100	44.0	189	1.05	0.596	0.4	7.1
4.10	100	42.5	180	1.09	0.570	0.7	5.5
4.10	300	42.1	175	1.11	0.555	0.8	5.4
4.10	800	42.0	185	1.10	0.587	0.9	5.0
4.50	100	45.5	239	1.07	0.750	1.3	9.1
4.80	100	48.7	258	1.08	0.802	1.4	8.3
5.00	100	50.8	306	0.98	0.945	0.4	9.0
5.50	100	52.1	301	1.05	0.925	0.4	9.0
6.00	100	53.4	328	0.99	1.00	−0.1	8.2
7.00	100	54.1	324	1.10	0.99	0.1	9.0
7.50	100	54.0	341	1.07	1.04	0.0	9.6
8.00	100	53.5	350	1.06	1.07	0.1	9.7
8.50	100	53.8	348	1.06	1.06	–	11
			V66A nuclease				
5.00	100	36.3	160	1.21	0.517	0.4	6.9
6.00	100	41.2	225	1.13	0.716	0.1	8.8
7.00	100	42.2	248	1.14	0.786	0	8.2
8.00	100	42.4	252	1.13	0.799	–	8.4

[a] Errors are ±0.5 K.
[b] Calorimetric enthalpy of denaturation in kJ mol^{-1}. Results are given to 3 figures, but errors are approximately ±10%.
[c] Ratio of the ΔH of a fitted 2-state transition (Privalov & Potekhin, 1986) to the calorimetric ΔH.
[d] In kJ K^{-1} mol^{-1}.
[e] An approximate estimate of the number of protons taken up by the protein upon denaturation by heating. Numbers were calculated using the differences between each pH value and the next higher value and rounded to 1 decimal place.
[f] In kJ K^{-1} mol^{-1}. Errors are approximately ±2 kJ K^{-1} mol^{-1}.

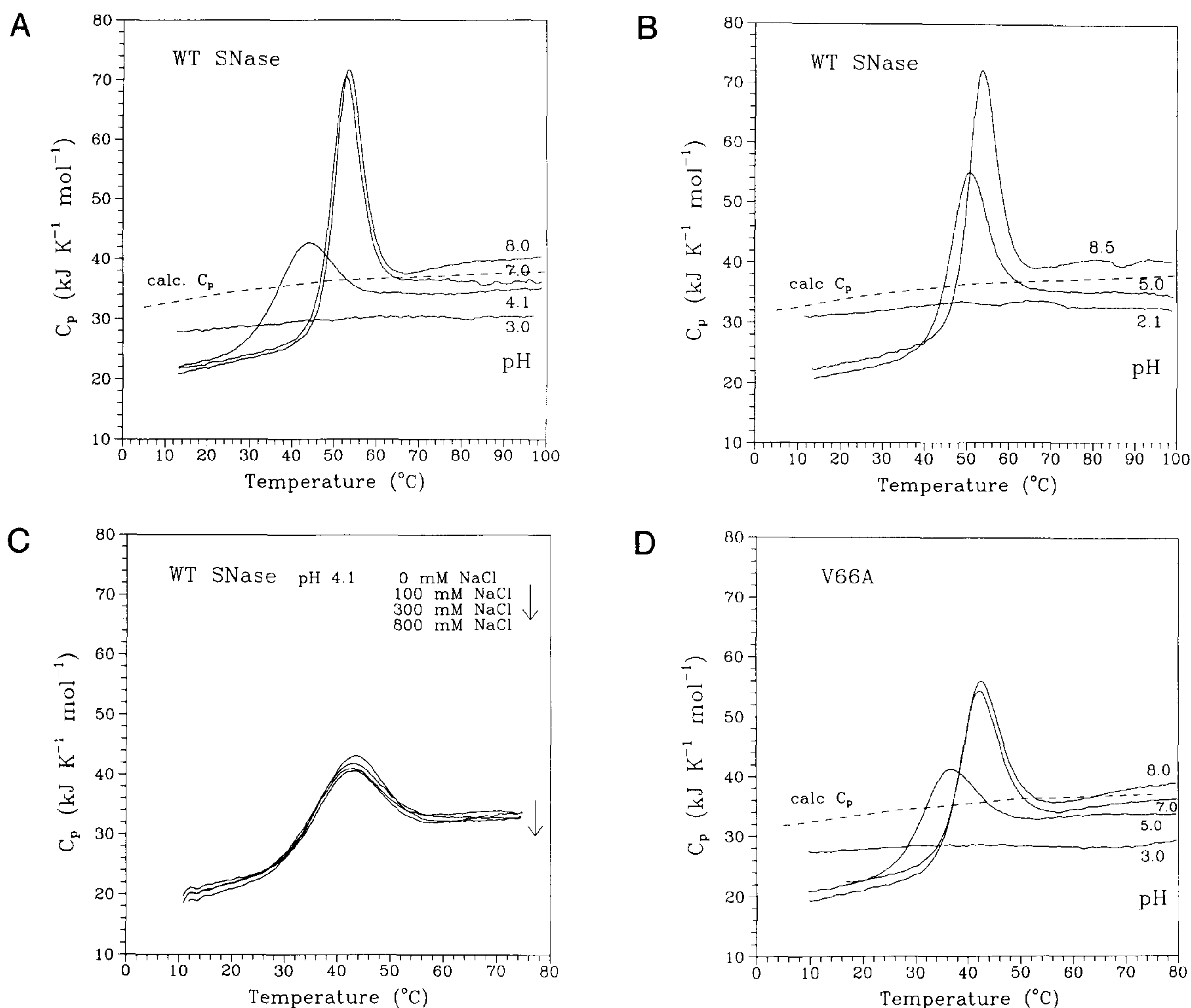

Fig. 1. Differential scanning calorimetry on wild-type and V66A nuclease. pH values are indicated on figure. Buffers used are listed in the Materials and methods. The dashed line indicates the calculated heat capacity of fully unfolded SNase (Makhatadze & Privalov, 1990). **A:** Wild-type SNase calorimetry at various pH values. **B:** Wild-type SNase at more pH values. **C:** Wild-type SNase at pH 4.1 with 20 mM glycine-HCl and varying concentrations of NaCl from 0 to 800 mM. The arrows on the figure indicate the order of curves corresponding to salt concentrations. **D:** Calorimetry on V66A mutant nuclease.

ΔH and T_d of the reaction also show only very small changes with salt concentration (Table 1).

Figure 1D shows differential scanning calorimetry of a mutant nuclease in which valine 66 has been replaced by alanine (Shortle et al., 1990). This valine residue is buried in the hydrophobic core of the wild-type protein (Loll & Lattman, 1989). The replacement of valine with alanine destabilizes the native state, lowering the T_d at each pH value. As with the wild-type protein, the heat capacity of the denatured state is close to the calculated unfolded value at pH 8.0 and 70 °C, but decreases with lower pH. The lower T_d of this mutant allows observation of the behavior of the denatured state's heat capacity function at lower temperatures than with the wild-type protein. At pH 8.0, the heat capacity of the denatured mutant protein is a little lower than the calculated unfolded value immediately after the denaturational peak and rises slightly with higher temperatures. This rise of the denatured state heat capacity at pH 8.0 with higher temperatures may result from increasing solvent exposure of nonpolar residues.

The decrease in heat capacity of the denatured state with pH results in a dependence of the ΔC_p of denaturation on pH. In Figure 2, the ΔC_p values at T_d of the wild-type protein obtained from individual heat capacity curves are plotted versus pH. The ΔC_p values contain considerable scatter. This is not unusual because the ΔC_p of denaturation is a parameter difficult to measure with extreme accuracy, mostly due to error in extrapolation of the native and denatured state heat capacities to the T_d. The steepest dependence of ΔC_p on pH appears to be in the region of pH 4-5, where glutamate and aspartate residues may be titrating.

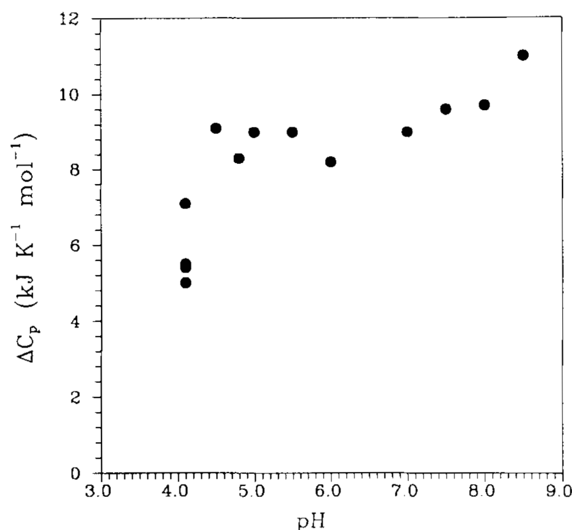

Fig. 2. ΔC_p values of wild-type SNase denaturation at various pH values. The ΔC_p is found by extrapolating the heat capacities of the native and denatured states to the T_d and subtracting the denatured value from the native value.

The ΔH versus T_d of the wild-type and V66A mutant proteins has been plotted in Figure 3 with data taken from Table 1. Variation of denaturation temperature has been achieved by varying pH, so this plot actually represents ΔH as a function of 2 different variables, T_d and pH. The slopes of lines fit to each set of data tell us the total variation of ΔH with T_d and pH over the experimental range for each protein. Interestingly, the values of these slopes, 13.4 kJ K^{-1} mol^{-1} for the wild type and

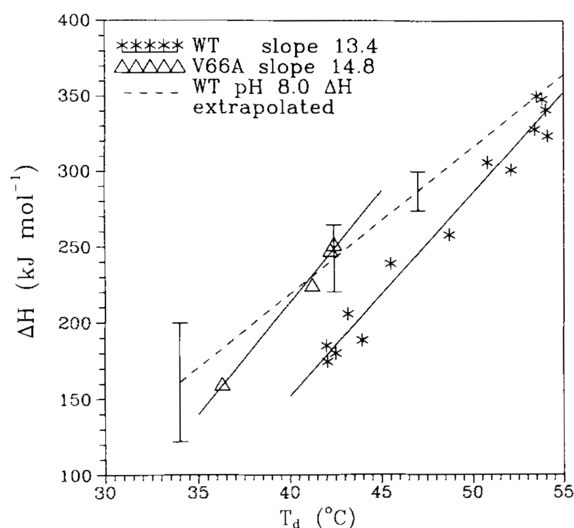

Fig. 3. ΔH of denaturation of wild-type and V66A nuclease at various denaturation temperatures. The solid lines are fitted to the data by linear least squares. The denaturation temperature is changed for each protein by varying the pH of the buffer. The dashed line represents the extrapolation of the wild-type pH 8.0 ΔH value to lower temperatures using the measured ΔC_p value of 9.7 ± 2 kJ K^{-1} mol^{-1}. Error bars on the dashed line represent the results of extrapolation using ΔC_p values of 7.7 or 11.7 kJ K^{-1} mol^{-1}.

14.8 kJ K^{-1} mol^{-1} for V66A, are equal to each other within an error of ±10%, but are significantly larger than the ΔC_p values obtained at a single pH from a scanning calorimetric curve (Fig. 2). The greater slope obtained by the plotting method of Figure 3 points to a dependence of ΔH on pH as well as T_d. Excluding enthalpy effects arising purely from ionization of side chains, a dependence of ΔH on pH has not been observed in previous calorimetric studies of this or other proteins (Privalov, 1979). The enthalpy change varies with pH due to differences in the energetics of the denatured state, resulting in a lower ΔH at more acidic pH. The conclusion reached above that the denatured state is not thermodynamically equivalent to a random coil at low pH, judging from the absolute values of the heat capacities, is also supported by the variation of ΔH with pH.

From Figure 3 it is clear that the ΔH of the V66A protein is higher than that of the wild-type protein at equal T_d values. At a T_d of about 42 °C, the ΔH of V66A is approximately 70 kJ mol^{-1} higher than that of the wild-type protein (Table 1). Point mutations of different proteins are often observed to result in large changes in the enthalpy of denaturation, for reasons that are not at all clear (Sturtevant, 1993; Tanaka et al., 1993). Fortunately, in this case, an explanation for the higher ΔH of V66A is readily available in the dependence of ΔH on pH. In fact, when we compare the ΔH of V66A at 42 °C with that of the wild-type protein at the same temperature, we are comparing denaturation at pH 8.0 for the mutant with denaturation at pH 4.1 for the wild-type protein. The difference in ΔH between the 2 cases arises from differences in the enthalpies of the denatured state due to pH. From this line of reasoning, we could expect that if the enthalpy functions of the wild-type and mutant proteins are fundamentally the same and appear different only because of changes in pH, we could predict the ΔH values for the mutant protein at any pH from the wild-type ΔH values. This is done in Figure 3 by extrapolating the ΔH of the wild-type protein at pH 8.0 (dashed line) to lower temperatures using the ΔC_p value obtained at that pH from a single calorimetric curve. Some uncertainty is inevitable because of extrapolation using the relatively imprecise ΔC_p values and is shown by the error bars. Nevertheless, the ΔH of the mutant at pH 8.0 is fairly well predicted by extrapolation of the wild-type protein's ΔH at pH 8.0 to lower transition temperatures.

Ionization of side chains upon denaturation can produce an enthalpy change that will depend on pH. The enthalpy of ionization of side chains is, however, compensated for by the enthalpy of transfer of protons from buffer molecules if the magnitudes of these quantities are comparable (Privalov, 1979). In the range of pH values used in these calorimetric experiments, ionization of glutamate, aspartate, or histidine residues may occur upon denaturation. The enthalpy of ionization of glutamate and aspartate is relatively small, being less than −5 kJ mol^{-1} (Izatt & Christensen, 1976), and will be mostly compensated for by the similar enthalpies of carboxyl groups on the glycine and acetate buffers used from pH 4.0 to 5.0. From pH 5.0 to 8.0, the dependence of T_d on pH is small (determined from the data in Table 1), the number of protons gained upon denaturation is less than 1, and the ionization enthalpy of side chains is therefore negligible. Histidine residues have a larger enthalpy of ionization of approximately 30 kJ mol^{-1} (Izatt & Christensen, 1976) and will not be effectively compensated for by the buffer. However, the 4 histidine residues of SNase (Foggi strain) have pK values from 5.5 to 6.8 in the native state (Alexandrescu et al.,

1988), while the T_d of SNase and the enthalpy of denaturation are most sensitive to pH in the region of pH 4. Acid-induced denaturation of SNase at 25 °C occurs with a pH midpoint of 3.9 (Epstein et al., 1971). Therefore, the uptake of a proton by the protein upon denaturation should be assigned instead to a glutamate or aspartate residue, as has been concluded previously (Shortle et al., 1988). For these reasons, we have decided that no adjustment of the calorimetrically measured ΔH for side-chain ionization is justifiable.

An aggregation of the denatured state of the protein that depends on pH might be proposed as an explanation for the observed dependence of ΔH and ΔC_p on pH. Equilibrium analytical ultracentrifugation on acid-denatured nuclease at pH 2.1, however, indicates no appreciable aggregation (data not shown), which was also found by Epstein et al. (1971). We find that the ratio of the enthalpy of a fitted 2-state transition to the calorimetric enthalpy is close to 1 (Table 1), which means that the cooperative unit of SNase denaturation is a monomer (Privalov & Potekhin, 1986) and rules out aggregation of either the native or denatured states during the denaturational transition. In the range of 1-3 mg/mL, we do not observe a significant protein concentration dependence of the T_d or $\Delta H^{fit}/\Delta H^{cal}$ ratio (Table 2). At higher protein concentrations, however, aggregation may be extensive, as was found by Tanaka et al. (1993) (see Discussion). Aggregation of denatured proteins is also generally highly salt dependent far from the isoelectric point, which for SNase is pH 9.62 (Heins et al., 1967). We do not observe such a large dependence of ΔH or ΔC_p on salt concentration (Fig. 1C).

Figure 4 shows circular dichroism spectra of native and denatured SNase in the peptide region. Circular dichroism is highly sensitive to secondary-structure content, especially of alpha-helix, and may be applied cautiously to the question of structure in the denatured state. Denatured SNase in 6 M guanidine-HCl has an ellipticity at 222 nm of 0, whereas the acid- or heat-denatured protein has an ellipticity of about $-5{,}000$ deg cm^2 dmol^{-1}. This value is typical of the denatured proteins studied by Privalov et al. (1989), which were not considered to contain residual structure. The significance of this residual ellipticity is not clear. It may not be assigned to the presence of regular secondary structure in the heat-denatured state because the ellipticity of denatured conformations is not well understood. If we nevertheless compare the ellipticities at 222 nm of the heat-denatured protein at pH 4.1 versus pH 8.0, we find that they are the same (Fig. 4A). The heat capacity difference between the protein at acidic versus neutral pH in the denatured state is therefore not apparently due to a difference in alpha-helix content. There is also no appreciable difference in ellipticity at 222 nm between 4 °C and 90 °C at pH 2.1 (Fig. 4B). The denatured state at acid pH shows no apparent change in alpha-helix content upon heating and seems to contain no regular alpha-helical secondary structure.

Table 2. *Effect of protein concentration*[a]

[Protein] (mg mL^{-1})	T_d (°C)	ΔH^{cal} (kJ mol^{-1})	$\Delta H^{fit}/\Delta H^{cal}$ [b]
0.97	41.5	203	1.08
2.51	44.0	189	1.05
2.59	42.5	180	1.09
3.61	43.4	233	1.01
4.76	41.9	173	1.25

[a] Data for wild-type SNase at pH 4.1, 20 mM glycine-HCl, 100 mM NaCl. Enthalpies have errors of approximately ±10%, T_d of ±0.5 K.
[b] Ratio of the ΔH of a fitted 2-state transition (Privalov & Potekhin, 1986) to the calorimetric ΔH.

Discussion

Almost 20 years ago, Privalov and Khechinashvili (1974) found that the enthalpy of denaturation of several proteins, if corrected for the ionization effect, does not depend on pH. This conclusion was based on the fact that plots of the enthalpy change of denaturation versus denaturation temperature across a range of pH values are linear and have a slope identical to the heat capacity change observed from a scanning calorimetry curve obtained at one pH. If there were an intrinsic dependence of ΔH

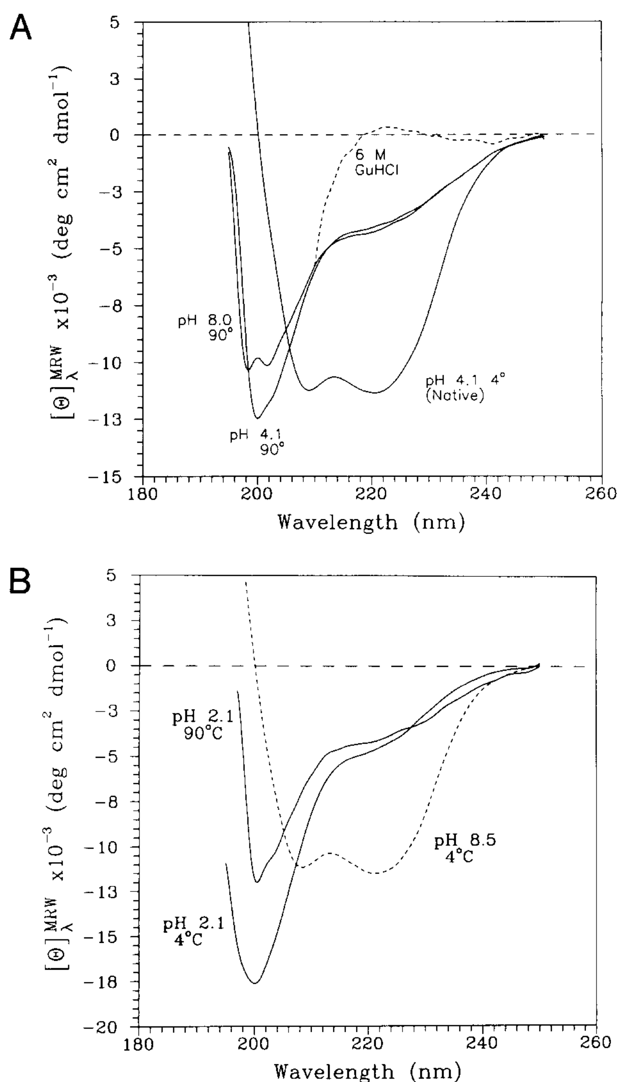

Fig. 4. Circular dichroism of the wild-type nuclease. The experimental conditions are indicated in panels **A** and **B**.

on pH, this would have shown itself as a disagreement between the temperature dependence of ΔH measured at various pH values and the heat capacity increase measured at a constant pH. In further support, the calorimetric enthalpy of denaturation obtained from titration with acid at a constant temperature was the same as the enthalpy change predicted for that temperature from differential scanning calorimetry (Privalov, 1979). The observed independence of ΔH on pH greatly facilitated analysis of denaturation as a first-order phase transition between 2 macroscopic states: the native and the denatured.

In this paper we find that SNase presents an exception to the above rule of independence of enthalpy on pH. In our hands, SNase at pH 8.0 denatures to a form that is fully exposed to solvent and is thermodynamically equivalent to a completely unfolded protein. With lower pH, however, the heat capacity and enthalpy changes of denaturation decrease in a way that is not explainable only by changes in denaturation temperature or ionization enthalpies of side chains. The lower heat capacity of the denatured protein at lower pH suggests partial exposure of nonpolar side chains to solvent. In contrast to the proteins studied by Privalov and Khechinashvili (1974) and Makhatadze and Privalov (1993), for SNase the temperature dependence of the enthalpy function measured over a range of pH values is significantly greater than the ΔC_p obtained from any 1 calorimetric curve. This clearly indicates that the enthalpy of denaturation should be considered as a function not only of transition temperature but also of pH.

The enthalpy function of the mutant protein V66A helps confirm the above conclusion. At the same denaturation temperature, the ΔH of V66A is much greater than the ΔH of the wild-type protein (Fig. 3). This effect is easily explained by the difference in pH used in each case. Because the mutant is less stable than the wild-type protein, it denatures at pH 8.0 at approximately the same temperature as the wild-type at pH 4.1. The denatured state of the mutant at the higher pH is more fully solvent-exposed than that of the wild type at the lower pH, judging by the absolute heat capacities. Furthermore, if we use the ΔH and ΔC_p obtained for the wild-type protein at pH 8.0 to extrapolate backward to lower denaturation temperatures (Fig. 3), we find that the ΔH of the mutant at pH 8.0 lies close to that line. The larger enthalpy changes of the V66A mutant are therefore not inexplicable. The enthalpy function of this mutant is not fundamentally different from that of the wild-type protein when the pH is taken into account. Indeed, rationalization of a greater enthalpy change for a less stable mutant would be very difficult without this consideration. A plot of ΔH versus T_d for the mutant protein L25A (Tanaka et al., 1993) looks similar to that for V66A (Fig. 3), also giving greater ΔH values than wild type at the same T_d. It is possible that the same explanation for this behavior may be applied to both mutants.

The cooperative unit of a protein's denaturation can be found from the ratios of the enthalpy of a fitted 2-state transition to the calorimetrically measured enthalpy of denaturation (Privalov & Potekhin, 1986). From Table 1, we can see that this ratio averages 1.06 ± 0.04 for the wild-type protein and 1.15 ± 0.04 for V66A. The fact that this ratio is significantly greater than 1 in the case of V66A points toward a small degree of oligomerization or aggregation of the mutant protein in either the native or the denatured state.

A dependence of ΔC_p and ΔH on pH has not been previously observed for SNase nor, as far we know, for any other protein. The reasons for this most likely involve experimental procedures and accuracy of instrumentation. In the calorimetric study of Tanaka et al. (1993), the ratios of the fitted 2-state and calorimetric enthalpies were found to be significantly greater than 1, and a protein concentration dependence of T_d was found. The authors interpreted this as evidence of partial aggregation or oligomerization in the denatured state. These effects were observed over a large protein concentration range from approximately 1 to 20 mg/mL. In order to avoid protein aggregation in this study, we limited ourselves to a narrow concentration range of 2–3 mg/mL for most experiments and find that the ratios of the fitted and calorimetric enthalpies are close to unity for the wild-type protein (Tables 1, 2), which indicates that neither the native nor the denatured state is significantly aggregated during the melting transition. The studies of Griko et al. (1988) and Shortle et al. (1988), which also used relatively low protein concentrations, found that the ratios of these enthalpies were close to 1. Two previous studies (Calderon et al., 1985; Tanaka et al., 1993) did not take into account light scattering in determination of protein concentrations, which may produce an underestimation of the calorimetric enthalpy and an overestimation of the ratio of enthalpies. Extensive aggregation of the denatured state at high protein concentrations might also preclude observation of the effects of pH on enthalpy and ΔC_p reported here. We do not observe any significant dependence of T_d on protein concentration over the range of 1–3 mg/mL protein (Table 2). A comprehensive review of previous calorimetric results on SNase can be found in Tanaka et al. (1993).

Lower protein concentrations could be used in this work because of the availability of a new scanning microcalorimeter developed in this laboratory at The Johns Hopkins University. This calorimeter has an exceptionally stable baseline and permits measurement of absolute values of heat capacities at low protein concentrations more reliably than previous instruments.

Other factors may also contribute to the differences between this and other investigations. Two previous studies (Calderon et al., 1985; Griko et al., 1988) of the thermodynamics of SNase denaturation used a derivative of SNase containing an additional 6 amino acids at the N-terminus that are not present on the protein we used. Choice of buffers may also affect the degree of unfolding of the denatured state, although our observation of the pH dependence of ΔC_p when phosphate is used as a buffer at pH 2.1 or 8.5 (Fig. 1B) shows that differences in buffer composition do not explain the observed effect. Differences in ionic strength also do not account for this observation (Fig. 1C).

The denatured protein at low pH has a lower enthalpy than the unfolded protein. As was shown by Makhatadze and Privalov (1993), the enthalpy of transfer of nonpolar residues from a protein's interior to the solvent is positive above 25 °C. Clustering of nonpolar residues in the denatured state may be expected to have an associated enthalpy, and the disruption of this clustering will show a positive enthalpy change. This enthalpy difference under 1 particular condition can be estimated from the difference in ΔH between the mutant protein V66A at pH 8.0 and the wild-type protein at pH 4.1 (Fig. 3), where denaturation occurs in both cases with a T_d of approximately 42 °C. The $\Delta\Delta H$ amounts to approximately 70 kJ mol^{-1}. Disruption of clustering of nonpolar residues with increasing temperature has also been proposed for the denatured form of unreduced lysozyme (Evans et al., 1991).

The fact that a lower heat capacity is observed at low pH, where electrostatic repulsion between positive charges on the protein should be high, is puzzling. Apparently this charge-charge repulsion is not a dominant force. We can additionally speculate that hydrophobic clustering of residues is facilitated by a decrease in the number of negative charges on SNase as the protein is titrated from pH 8.0 to 2.0. In this pH range the net positive charge on the protein will increase as histidine, glutamate, and aspartate residues are titrated (Fig. 5), but the number of individual charges of either sign will decrease as glutamate and aspartate residues are neutralized. The uncharged forms of glutamate and aspartate residues are naturally less hydrophilic than the charged forms and can be more easily removed from water. Thus, a decrease in the number of negative charges on the protein due to the lowering of pH would decrease the protein's hydrophilicity, facilitating the removal of some residues from water.

Incomplete unfolding may in fact be fairly general for SNase under denaturational conditions that are not extreme. A thermally denatured mutant SNase (K78C) has been found to exist partially in a compact form at pH 7.2 and 60 °C using fluorescence energy transfer measurements (Wu et al., 1994). SNase at 25 °C and pH 7.8 in moderate concentrations of guanidine-HCl (Shortle & Meeker, 1989; James et al., 1992) is also compact, as is a fragment of the protein in the absence of guanidine (Flanagan et al., 1992, 1993; Gittis et al., 1993; Shortle & Abeygunawardana, 1993). These results, along with those of this study, suggest that the form of the denatured state in equilibrium with the native state under physiological conditions, sometimes called D_0 (Dill & Shortle, 1991), is not thermodynamically a random coil. The enthalpy, entropy, and free-energy changes of SNase folding under a biologically relevant condition would then be the differences between the native state and an incompletely unfolded state.

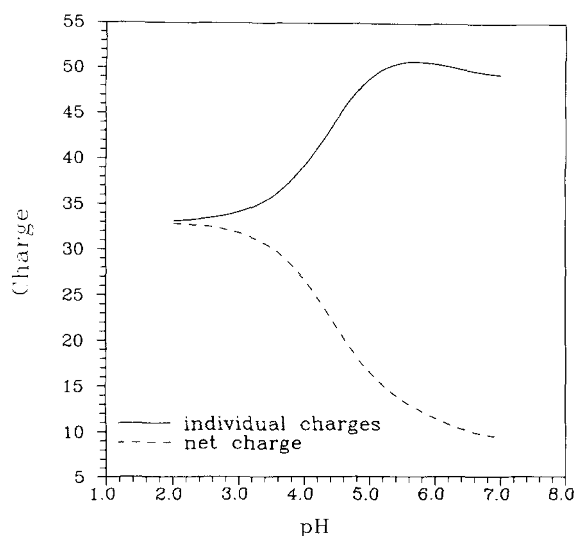

Fig. 5. Charges on nuclease as a function of pH. The number of individual charges (solid line) and the net charge (dashed line) were calculated assuming simple independent titration of residues in the denatured state. Seven aspartate and 12 glutamate residues were assigned a standard pK value of 4.4; 4 histidine residues, a pK of 6.0; and the C-terminus, a pK of 3.1. Twenty-three lysine residues, 5 arginine residues, and the N-terminus were assumed to be always charged in the pH range 7.0–2.0.

Materials and methods

Protein preparation and buffers

SNase A (Foggi strain) and the mutant nuclease V66A were purified from overproducing strains of *Escherichia coli* kindly provided by Drs. David Shortle and Alan Meeker. Purification was performed essentially as described by Shortle and Meeker (1989), except that the final step of FPLC Mono-Q chromatography was carried out without the presence of urea. Protein preparations were >95% pure as judged by SDS-PAGE and Mono-Q chromatography. Protein concentrations were determined as done by Griko et al. (1988), with correction for light scattering, using an A_{280} value of 0.93 cm^{-1} for a 1-mg/mL solution. The structure of SNase is stabilized by calcium ions at pH values between 5 and 7 (Calderon et al., 1985). The protein preparations used here contained no added calcium, and EDTA to 1 mM was added when necessary to ensure the absence of free calcium. Buffers used were: at pH 2.1, 20 mM Na phosphate, 100 mM NaCl; from pH 3.0 to 4.5, 20 mM glycine-HCl, 100 mM NaCl; from pH 4.8 to 6.0, 20 mM NaOAc, 100 mM NaCl, 1 mM EDTA; and from pH 7.0 to 8.5, 20 mM Na phosphate, 100 mM NaCl, 1 mM EDTA. NaCl concentrations were in some cases varied as indicated in the tables.

Circular dichroism

Circular dichroism measurements were performed on a Jasco J-710 spectropolarimeter (Japan Spectroscopic Company) with a jacketed 0.05-cm circular cell connected to a water bath for temperature control. Protein concentrations were kept close to 0.4 mg/mL. The molar ellipticity $[\theta]$ was calculated using an average molecular weight per amino acid residue of 112.5 Da. Noise in the data was smoothed using the Jasco J-710 software.

Microcalorimetry

Differential scanning calorimetry was performed using a new microcalorimeter built at The Johns Hopkins University by V. Plotnikov and P. Privalov from a DASM-1M prototype. The cell volume was 1.3 mL and the scanning rate was 1 K/min. Protein concentrations were between 2 and 3 mg/mL except where otherwise indicated. Data were analyzed as described by Privalov and Potekhin (1986). The partial specific volume used in the calculation of the partial specific heat capacity of nuclease (Privalov et al., 1989) was measured using a vibrational densimeter (Anton Paar, Austria) and found to be 0.68 $cm^3\ g^{-1}$ for wild-type nuclease at pH 3.0 and 20 °C. No significant effect of pH on the partial specific volume was observed. Calculated heat capacities of the unfolded protein were determined as described by Makhatadze and Privalov (1990).

Acknowledgments

We thank Drs. David Shortle and Alan Meeker for the gift of strains overproducing staphylococcal nuclease and Dr. Mike Rodgers for per-

forming analytical ultracentrifugation. We also thank Drs. Yuri Griko and George Makhatadze for helpful discussions and advice. This work was supported by NIH grant GM 48036 and NSF grant MCB 9118687.

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