PROTEINS: Structure, Function, and Genetics 1:81–89 (1986)

Mutant Forms of Staphylococcal Nuclease With Altered Patterns of Guanidine Hydrochloride and Urea Denaturation

David Shortle and Alan K. Meeker
Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT **Eleven mutant forms of staphylococcal nuclease with one or more defined amino acid substitutions have been analyzed by solvent denaturation by using intrinsic fluorescence to follow the denaturation reaction. On the basis of patterns observed in the value of m—the rate of change of log K_{app} (the apparent equilibrium constant between the native and denatured states) with denaturant concentration—these proteins can be grouped into two classes. For class I mutants, the value of m with guanidine hydrochloride is less than the wild-type value and is either constant or increases slightly with increasing denaturant; the value of m with urea is also less than wild type but shows a marked increase with increasing denaturant concentration, often approaching but never exceeding the wild-type value. For class II mutants, m is constant and is greater than wild type in both denaturants, with the increase being consistently larger in guanidine hydrochloride than in urea. When double or triple mutants are constructed from members of the same mutant class, the change in m is usually the sum of the changes produced by each mutation in isolation. One plausible explanation for these altered patterns of denaturation is that chain-chain or chain-solvent interactions in the denatured state have been modified—interactions which appear to involve hydrophobic groups.**

Key words: protein stability, protein denaturation, denatured state, structural intermediates

INTRODUCTION

The native conformation of many proteins in aqueous solution under physiological conditions of temperature, salt, and pH is only marginally stable relative to the denatured state, with free energies of denaturation typically falling in the range of 5–15 kilocalories.[1] With such small stabilization free energies, it is not surprising that single amino acid substitutions often have profound effects in reducing, or occasionally increasing, the relative stability of the native state. Although plausible qualitative explanations for these effects can often be proposed, particularly in those cases where the structure of the native conformation of the wild-type protein has been determined by X-ray crystallography,[2] a general theory capable of predicting the quantitative consequences of amino acid substitutions on protein stability is not available.

One obvious approach toward developing such a quantitative theory would be to choose a simple protein, isolate a large collection of mutant forms by using the new methods of recombinant DNA and in vitro mutagenesis, and then systematically characterize how each mutant form differs from wild type in its denaturation behavior. Although virtually any protein could be chosen for such a study, the feasibility of obtaining accurate denaturation data that would be interpretable in a straightforward way is higher for monomeric proteins consisting of single folding domains that reversibly denature/renaturate at high protein concentrations. And since there are 19N possible single mutant forms of a polypeptide chain of N amino acids, a general strategy is needed for efficiently identifying those mutant forms that are most likely to be informative.

The classical studies of research groups led by Anfinsen[3] and others[4] clearly demonstrated several technical advantages of staphylococcal nuclease as a model system for the study of protein folding and stability. In an attempt to build on this earlier work, our laboratory has initiated a combined genetic and physical chemical analysis of this small protein. After isolation of the gene for *Staphylococcus aureus* and development of a plasmid-based genetic system,[5] a collection of mutant forms was identified by the conventional genetic strategy of screening large pools of randomly induced mutations with a simple plate assay for enzyme activity.[6] Preliminary characterization of a number of the mutant forms from this collection indicated that the plate assay used can easily detect mutations which produce small to very large changes in the stability of the protein, as assessed by solvent denaturation.[7,8]

In this report, data are presented that suggest amino acid substitutions in some mutant forms of staphylococcal nuclease can modify the sensitivity of this protein to solvent denaturation through their effects on the denatured state.

MATERIALS AND METHODS

A 6 M solution of guanidine hydrochloride (Bethesda Research Labs, Ultrapure lot 40320) was pre-

Received May 29, 1986; accepted June 18, 1986.

pared and neutralized with a small amount of disodium phosphate to give a final pH of 7.0 on dilution to 1 M. A 10 M solution of urea (BioRad lot 26580) was prepared in 25 mM sodium phosphate, 0.1 M sodium chloride buffer, pH 7.0, divided into 3-ml aliquots, and immediately stored at −70°C until use. Concentrations were confirmed by measuring the index of refraction.[9] A stock single solution of each denaturant was used for all experiments here described.

Correctly processed (149 amino acids long) wild-type and mutant forms of staphylococcal nuclease were prepared from a *pho*A-nuclease gene hybrid and purified by chromatography on BioRex 70 as previously described.[7,10] Protein concentration was determined by UV absorption at 280 nm (1 mg/ml = 0.93)[11] and purity (90–99%) was confirmed by SDS polyacrylamide gel electrophoresis. Mutant proteins are designated by the amino acid substitutions they contain by using the one letter code to refer first to the wild-type amino acid, followed by the residue position number, and the mutant amino acid substituted at that position.

To determine K_{app}, an apparent equilibrium constant of denaturation, intrinsic fluorescence of the single tryptophan residue at position 140 was monitored as a function of denaturant concentration on a SPEX Fluorolog II spectrophotofluorometer. For all studies, the excitation wavelength was 295 nm and the emission wavelength was 325 nm. Samples of protein in 2.00 grams of 100 mM sodium chloride/25 mM sodium phosphate, pH7.0, with a protein concentration of 40–50 micrograms/ml were maintained at 20.0°C in a 1.0 cm × 1.0 cm quartz fluorescence cuvette. To change the concentration of denaturant, the cuvette was removed, an aliquot of stock denaturant solution was added, the solution was mixed for 15 seconds via a magnetic stirring bar in the cuvette, and the cuvette was placed back in the spectrofluorometer. Readings were taken approximately 10 minutes later, a time interval previously determined to be more than sufficient for the establishment of equilibrium.

Under essentially identical conditions, the ellipticity at 222 nm (or between 300 nm and 205 nm) was determined as a function of guanidine hydrochloride concentration on a JASCO J-500C spectropolarimeter in the laboratory of Dr. Richard Armstrong, University of Maryland.

The apparent equilibrium constant K_{app} was calculated by using the equation[12]

$$K_{app} = I_n - I / I - I_d$$

where I is the fluorescence intensity (or ellipticity) of the sample, I_n is the extrapolated value of fluorescence for the native state, and I_d is the extrapolated value for the denatured state.

RESULTS

Denaturation With Guanidine Hydrochloride

As described previously,[7] denaturation with guanidine hydrochloride was monitored by the intrinsic fluorescence of the single tryptophan at position 140. For all of the proteins analyzed in this report, the fluorescence intensity as a function of denaturant concentration followed a sigmoidal curve, starting at a relative value of 1.00 for the native protein and falling to a final value of approximately 0.15. The only quantitative differences between the various proteins was the steepness of the curve and the concentration at the inflection or midpoint. Therefore, to obtain the apparent equilibrium constant K_{app}, the baseline fluorescence for the native state was extrapolated as a constant value of 1.00,[7] even for those mutant forms for which no baseline data could be collected due to the onset of denaturation at the lowest guanidine concentrations. (For the protein I18M+A69T, this value had to be corrected to 102.5 on the basis of a calculated $K_{app} = 0.03$ at zero denaturant concentration). At high denaturant concentrations, the fluorescence of all proteins converged to essentially a straight line with a small positive slope of 0.005 per M guanidine hydrochloride.

Data were analyzed by plotting the logarithm of K_{app} as a function of guanidine hydrochloride concentration and graphically fitting the data to a straight line, from which the midpoint concentration C_m and the slope m were obtained. At least two data sets, and sometimes three or four, were obtained for each protein; values of C_m and slope m consistently agreed to within 2%.

More than 40 different mutant forms of staphylococcal nuclease, each with a single amino acid substitution, were analyzed. While it was expected that C_m should vary considerably between mutants and wild type, the observation that the value of m also varied by as much as 25 percent was surprising in view of the fact that this parameter reflects the differential interaction of guanidine hydrochloride with the native and denatured states (see Discussion). Approximately 50 percent of all of the mutant proteins analyzed exhibited values of m less than 0.95 that of wild-type protein (defined as class I), and mutant proteins in which m was greater than 1.05 (class II) represented approximately 15 percent. The mutant proteins described in this report were chosen from this larger collection because (1) they exhibited some of the largest deviations from the wild-type value of m and (2) they could be easily recombined to form double and triple mutants proteins[6] in which the cumulative effect of these mutations could be determined. The positions of the substituted amino acid residues in the native conformation of the wild-type proteins are indicated in Figure 1.

As shown in Figure 2 and Table I, three single mutant forms in class I were observed to have m values between 0.78 and 0.89 that of wild-type pro-

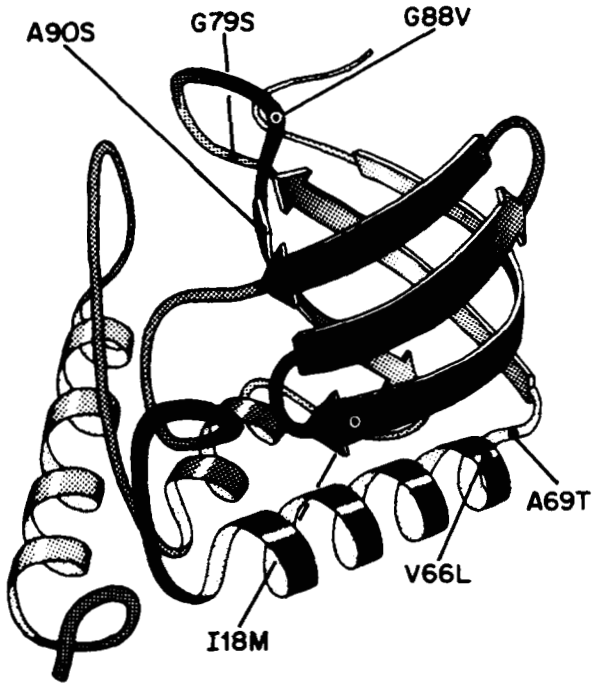

Fig. 1. Positions of the α-carbons of the amino acid residues altered in the mutant nucleases. This diagram of the three-dimensional structure of wild-type staphylococcal nuclease was drawn and copyrighted by Jane Richardson[28] and is used with her permission.

tein. Two pairs of these mutations were combined to give double mutant proteins. For nuclease V66L+G88V the observed value of m (0.57) is close to that expected if the effect of each mutation is additive (0.60), whereas in the case of nuclease V66L+G79S, m is considerably smaller than the expected additive value (0.61 vs. 0.71). For the triple mutant V66L+G79S+G88V, m is also close to the expected value (0.51 vs. 0.49). The midpoint denaturant concentrations C_m for the multiple mutant proteins can be seen in each case to have an intermediate value relative to the C_ms of the mutant forms from which it was generated, but no simple relationship between the actual value of C_m for the single and double mutant forms is apparent. Consequently, when the free energy of denaturation in buffer alone $\Delta G°_{app}$ is calculated by using the linear extrapolation of log K_{app} to zero denaturant concentration,[12] the changes in $\Delta G°_{app}$ of the multiple mutants are also not additive, as seen in Table I.

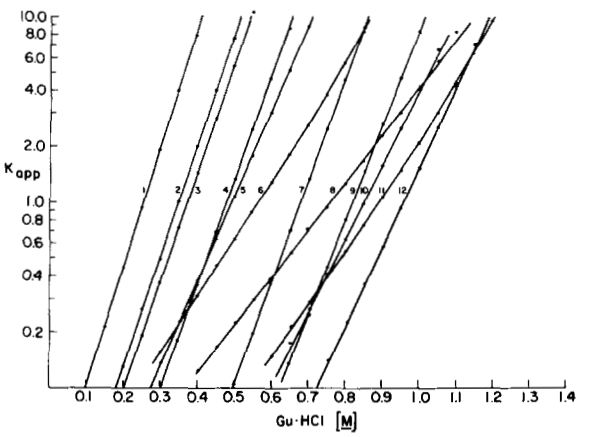

Fig. 2. A plot of the logarithm of the apparent equilibrium constant for denaturation (K_{app}) vs. concentration of guanidine hydrochloride. Numbered curves represent the following proteins: 1, I18M + A69T; 2, I18M + A90S; 3, A69T; 4, A90S; 5, G79S; 6, G79S + V66L; 7, I18M; 8, V66L + G79S + G88V; 9, wt; 10, G88V; 11, V66L + G88V; 12, V66L. See Materials and Methods for the details of the experiments.

For the three single mutants in class II, m varies from 1.07 to 1.15, and for the two double mutants, the observed m is very close to the sum of the effects of the two single mutants: m for nuclease I18M+A69T is 1.26 instead of 1.22, and for I18M+A90S 1.16 instead of 1.17. Since the changes in C_m for these two double mutants are also close to additive, the linear extrapolation of log K_{app} to zero denaturant gives a $\Delta G°_{app}$, which is quite close to the value predicted if the effects of each amino acid substitution are additive.

Denaturation With Urea

As can be seen by comparing Figure 3 with Figure 2, denaturation of this set of 12 different nucleases with urea gives results which are qualitatively similar to those obtained with guanidine hydrochloride with one major exception. Although three members of class I exhibited a slight upward curvature in plots of log K_{app} vs. guanidine hydrochloride concentration (G88V,V66L+G88V, and V66L+G79S+G88V), all six show significant upward curvature with urea as the denaturant. In each case, the observed value of m at low urea concentration is similar to the value of m for guanidine hydrochloride denaturation, and at higher concentrations m approaches, but never attains, the wild-type value. For nucleases V66L+G79S and V66L+G79S+G88V, this curvature translates into an m which increases by more than 50% over the concentration range of K_{app} from 0.1 to 10.

Since the actual value of $\Delta G°_d$ is independent of the denaturant used for its determination, agreement between $\Delta G°_{app}$ obtained with urea and with guanidine hydrochloride is expected. When the linear extrapolation to zero urea is applied to these graphs by extending the curves as straight lines with a slope equal to m for the interval K_{app} = 0.1–0.25, the estimated values of $\Delta G°_{app}$ are in reasonable agreement with those calculated from the guanidine denaturation curves for all but two of the class I mutants. This finding suggests that the rate of change of log K_{app} is a smaller value at low urea concentrations than at higher urea concentrations, rather than an artifact involving a conformational change other than denaturation. Thus the large variation in m with denatur-

Table I. Results of Denaturation Curve Analysis for Wild-Type and Mutant Nucleases

		GuHCl			Urea			GuHCl (CD)	
	Nuclease*	C_m†	m‡	ΔG°_{app}**	C_m	m***	ΔG°_{app}	C_m	m‡
	WT	0.82	1.00	5.6	2.56	1.00	6.1	0.81	0.95
Class I:	V66L	0.96	0.82	5.4	2.99	0.89 - 0.93	6.3		
	G79S	0.50	0.89	3.0	1.59	0.86 - 0.91	3.2		
	G88V	0.86	0.78	4.6	2.72	0.81 - 0.91	5.2		
	V66L+G79S	0.56	0.61	2.3	1.88	0.55 - 0.85	2.5		
	V66L+G88V	0.89	0.57	3.5	3.17	0.62 - 0.88	4.7		
	V66L+G79S+G88V	0.76	0.51	2.6	2.61	0.44 - 0.70	2.7	0.92	0.36
Class II:	I18M	0.68	1.07	5.0	2.18	1.04	5.4		
	A69T	0.37	1.15	2.9	1.22	1.11	3.2		
	A90S	0.48	1.10	3.6	1.58	1.06	4.0		
	I18M+A69T	0.26	1.26	2.2	0.91	1.08	2.3		
	I18M+A90S	0.35	1.16	2.8	1.20	1.12	3.2	0.34	1.21

*Mutant nucleases are designated by the amino acid substitutions they contain: the wild-type amino acid (in the one-letter code)-the residue position-the mutant amino acid.
†C_m is given in molarity (M).
‡m[d(logK_{app})/dC] is relative to the wild-type value of 5.07 log 10/M.
**ΔG°_{app} is given in kilocalories per mole.
***m is relative to the wild-type value of 1.76 $\log_{10}$/M.

ant concentration appears to reflect a significant difference between the the actions of urea and guanidine hydrochloride. The two class I mutant proteins, V66L and V66L+G88V, give values of ΔG°_{app} that are considerably larger in urea than in guanidine hydrochloride. Since these two proteins denature at the highest urea concentrations, the straight-line extrapolation to low concentrations is probably least appropriate for them, and presumably the value of m has been overestimated.

For the Class II mutants, a straight line fits the urea data as well as the guanidine hydrochloride data. Nevertheless, the magnitude of the increase in m is consistently less in urea than in guanidine by approximately 30–40%. The one exception is nuclease I18M+A69T, for which the relative increase of m in urea is much smaller than with guanidine hydrochloride (1.08 vs. 1.26). The calculated values of ΔG°_{app} are in very good agreement with those from the guanidine data, and again, as with guanidine hydrochloride as the denaturant, the two double mutants have their free energy of denaturation reduced by an amount that is close to the sum of the effects produced by the two mutations singly.

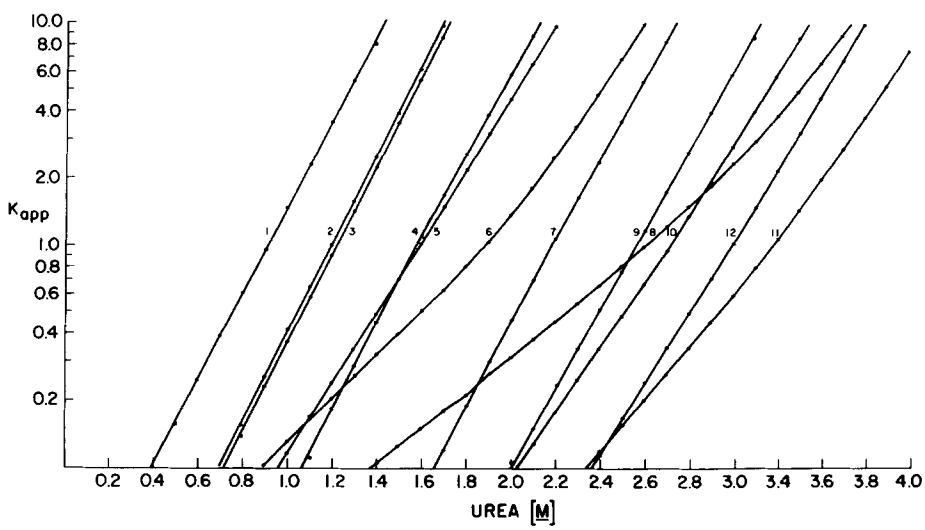

Fig. 3. A plot of the logarithm of the apparent equilibrium constant K_{app} vs. concentration of urea. See Figure 1 for the legend of curve numbers and proteins.

Circular Dichroism Studies

Studies by Anfinsen and others[13] demonstrated that, for acid denaturation of staphylococcal nuclease, the intrinsic fluorescence of the single tryptophan at 140 gives values of K_{app} which agree very well with K_{app} determined by a variety of other, more global physical methods for detecting major conformational changes. To determine if tryptophan fluorescence is also a reliable indicator of solvent denaturation, the ellipticity at 222 nm was followed as a function of guanidine hydrochloride concentration for the wt nuclease and one member from each of the two mutant classes. The change in θ_{222} for these three proteins is shown in Figure 4.

To obtain K_{app}, the θ_{222} of the native state of all three proteins was assumed to be constant at 1.0 on the basis of the behavior of the wild-type protein, and a linear extrapolation was used to calculate the θ_{222} of the denatured state in the transition region. (Since the denaturation of nuclease V66L+G79S+G88V does not appear complete at 2.0 M guanidine hydrochloride, the wild-type curve for the denatured state was used as an approximation.) The calculated value of C_m and m are listed in Table I, and for the wild-type nuclease and nuclease I18M+A90S, they are found to be essentially the same as those obtained by fluorescence at 325 nm.

For nuclease V66L+G79S+G88V, a large discrepancy appears in the C_m and m obtained with the two physical probes of protein denaturation. As can be seen in Figure 4, the θ_{222} begins changing at low guanidine concentrations and continues across a very broad transition zone without reaching the wild-type value by 2.0 M guanidine hydrochloride. When compared to the comparable fluorescence curve, the θ_{222} curve is not as steep and is shifted to higher denaturant concentrations. Thus, while the two physical methods give the same denaturation profile for wild-type nuclease and nuclease I18M+A90S, for nuclease V66L+G79S+G88V tryptophan fluorescence and circular dichroism do not change coordinately on denaturation with guanidine hydrochloride, a phenomenon observed with several naturally occurring proteins and usually explained as consequence of stable intermediate states.[14,15,16]

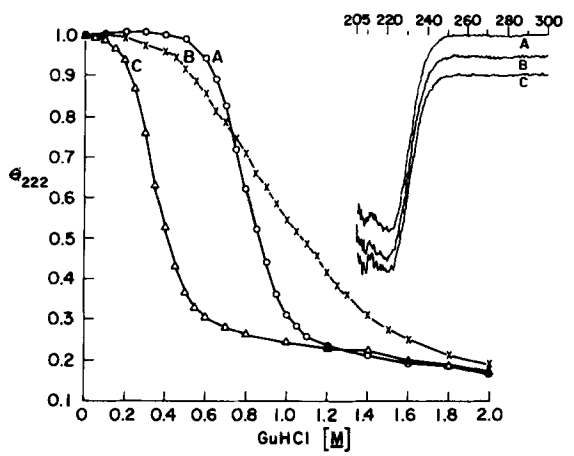

Fig. 4. A plot of the ellipticity at 222 nm as a function of guanidine hydrochloride concentration. Curve A is the wild-type nuclease; curve B is the class I mutant protein V66L+G79S+G88V; curve C is class II mutant protein I18M+A90S. Inset: Circular dichroism spectra in the absence of denaturant for the same three proteins. The x axis is in nanometers. For wild type, the peak at 221 nm corresponds to a molar ellipticity of $[\theta] = 17 \times 10^3$ deg·cm^2 per decimole.

The ellipticity in the wavelength range from 205 to 300 nm was also measured for each of these three proteins in the absence of denaturant (Fig. 4), and no significant differences were found. Spectra taken of each protein at its midpoint concentration of guanidine hydrochloride and at 1.4 M guanidine hydrochloride (data not shown) revealed only that nuclease V66L+G79S+G88V has a larger-than-wild-type ellipticity at all wavelengths below 235 nm at these denaturant concentrations.

DISCUSSION AND CONCLUSIONS

With all equilibrium techniques for monitoring protein denaturation as a function of solvent composition, the primary data consist of a sigmoidal curve representing a changing physical parameter. In order to extract apparent equilibrium constants describing the fractional ratio of molecules in the denatured and native state at a given denaturant concentration, three assumptions must be introduced. First of all, the denaturation reaction is presumed to be adequately described by two states, the native N and the denatured D. In addition, the values of the physical parameter for both the N and the D states in the transition region must be estimated, usually by assuming that the behavior of these species outside the transition region can be used to correctly extrapolate across the transition region.

When the solvent denaturation of staphylococcal nuclease and several mutant forms was monitored by tryptophan fluorescence and values of K_{app} were calculated from the data and from the most reasonable extrapolations of the fluorescence of native and denatured states, the observation was made that single amino acid substitutions can alter not only C_m, the concentration of denaturant at which $K_{app} = 1$, but also m, the rate of change of log K_{app} with denaturant concentration. Some mutant proteins, designated class I mutants, exhibited values of m with guanidine hydrochloride that were significantly less than wild-type nuclease.[7] All of these proteins also displayed reduced values of m with urea as the denaturant, but in this case, m varied significantly with the urea concentration. When the amino acid substitutions present in different class I mutants were combined into a single protein, the observed value of m was reduced by an amount roughly equal to the sum of

the reductions caused by each amino acid change individually. Other mutant proteins, designated class II, yielded constant values of m that were larger than wild type with both guanidine hydrochloride and urea, with the increase always being larger with guanidine hydrochloride. Again, the effects of two class II mutations on m in a double mutant protein were approximately additive.

Although the precise chemical mechanism(s) by which solvent denaturants such as urea and guanidine hydrochloride exert their destabilizing action on the native state of proteins remains uncertain, a number of quantitative models have been put forward to describe the dependence of the free energy of denaturation on denaturant concentration. Common to all of these models is the premise that denaturants alter the equilibrium between N and D through a preferential interaction with the denatured state. Where the various models differ is in the physical chemical description of this interaction. In the general case

$$\Delta G_d(C) = \Delta G^\circ_d - f(C)^*(X_d - X_n)$$

where $\Delta G_d(C)$ is the free energy of denaturation at a specific denaturant concentration C, ΔG°_d is the free energy of denaturation in the absence of denaturant, f(C) is the functional dependence of the free energy change on denaturant concentration C, and $(X_d - X_n)$ is the difference in the value of an interaction parameter X for the denatured and native states of the particular protein. In the formulation of solvent denaturation in general thermodynamic terms proposed by John Schellman,[17]

$$\Delta G_d(C) = \Delta G^\circ_d - k^*(A_d - A_n)^*C$$

with k representing a constant (that includes the universal gas constant, temperature, and free energy per unit protein-denaturant interaction) and A the solvent-accessible surface area. Since no other model of solvent denaturation actually predicts the linear dependence of $\Delta G_d(C)$ on denaturant concentration (i.e., m is a constant), the Schellman model is chosen for the discussion that follows. The basic argument, however, would be similar if the binding site model[18] or the Tanford model[18] had been chosen.

If the two-state approximation is assumed to adequately describe the denaturation behavior of the class I mutant proteins, then the smaller m they exhibit must, by the Schellman model, reflect a smaller value of $(A_d - A_n)$, the only adjustable parameter of the above equation. Several aspects of the data suggest that a reduction in A_d for these proteins is more likely than an increase in A_n. (1) The increase in m with increasing urea concentration is more plausibly accounted for by proposing that an initially small surface area of the denatured state A_d gets larger as the solvent becomes more denaturing rather than by proposing that an initially large surface area of the native state gets smaller. (2) The CD spectrum for the Class I mutant with the smallest m is, in the absence of denaturant, indistinguishable from the wild-type protein. (3) The CD data for this same protein clearly reveals "structure" at GuHCl concentrations at which the protein appears fully denatured by the criteria of loss of tryptophan fluorescence.

Alternatively, the anomalous patterns of denaturation for the class I mutants could be explained by a breakdown of the two-state approximation because of the appearance of a third species I intermediate in structure between N and D. As shown diagrammatically in Figure 5A, if the surface area of I is less than D and if tryptophan fluorescence does not distinguish between I and D, then fluorescence would only monitor the equilibrium beween N and I, and for this

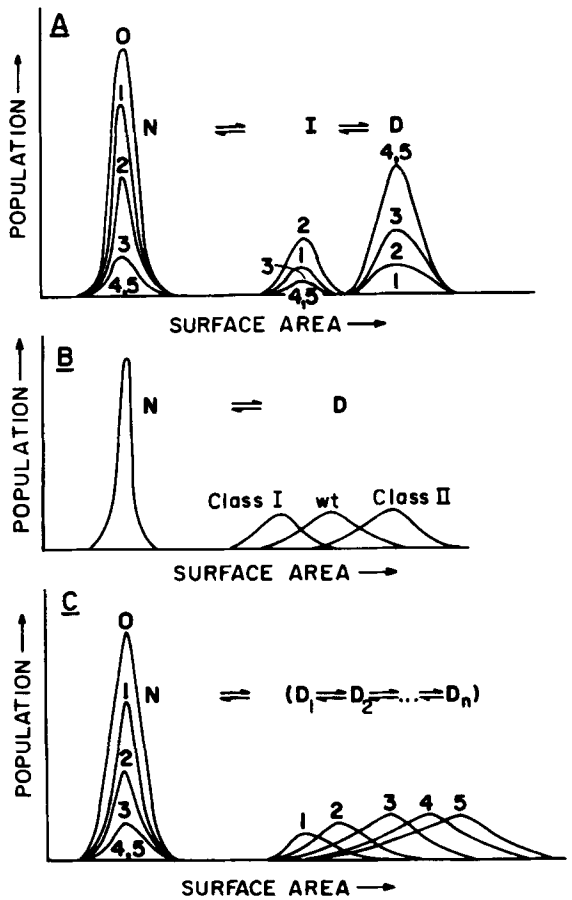

Fig. 5. Hypothetical population distribution of different microstates of staphylococcal nuclease as a function of their solvent accessible surface areas. Numbered curves represent populations at increasing denaturant concentrations. 5A) A three-distribution model consisting of N (native), I (structural intermediate), and D (denatured) macrostates. 5B) A two-distribution model in which the mean value of surface area for D can increase or decrease with changes in the amino acid sequence (class II and class I mutants, respectively). 5C) A two-distribution model in which D shifts to higher mean surface area with increasing denaturant concentrations.

reaction m would be proportional to $(A_i - A_n)$. The noncoincidence of the denaturation curves obtained with tryptophan fluorescence and circular dichroism would then be a consequence of the latter method's ability to detect equilibria between both N and I (which dominates at low denaturant concentration) and I and D (which is only complete at high denaturant concentrations).

If the two-state approximation is assumed to describe the denaturation behavior of the class II mutant proteins, then their larger-than-wild-type value of m could be explained by a proportional increase in $(A_d - A_n)$. Again, the most plausible means by which this value could increase is through an increase in A_d rather than a decrease in A_n. Although the magnitude of $(A_d - A_n)$ has not been experimentally determined for any protein, Lee and Richards[19] have estimated that for a protein of 15,000 daltons, A_d could be approximately three times the value of A_n, so that $(A_d - A_n)$ would equal to 2 A_n. If all of the 26 percent increase in $(A_d - A_n)$ for the class II mutant protein I18M+A69T were accounted for by change in the native state, A_n for this protein would have to be 48 percent the wild-type value. Given the spherical shape and dense packing of the X-ray diffraction model of staph nuclease and the lack of detectable changes in the CD spectrum of this protein in the absence of denaturant, this seems an unlikely possibility.

For this class of mutant proteins, the stabilization of structural intermediates is not a reasonable alternative explanation because the increase in $(A_d - A_n)$ implies that the state into which the native state is converted on denaturation is likely to have a larger value of A than does the wild-type denatured state. If this state effectively exposes more of its surface area (amino acid residues) to solvent, it makes no sense to call it an intermediate since it is no longer intermediate in structure between N and D but rather is a "more completely" denatured state.

Therefore, the simplest way of explaining the behaviors of the class II mutant proteins is likely to be within the framework of the two-state approximation. As emphasized by Lumry et al.,[20] the N and D macrostates should each be represented as a distribution of microstates. These distributions need not be symmetric and are expected to change in midpoint and shape with the change of any variable that effects the free energies of the different microstates (temperature, solvent, amino acid sequence, etc.). For the reasons presented above, the changes in m resulting from certain amino acid substitutions can be presumed to be a consequence of changes in the denatured state, more specifically in the solvent-accessible surface area exposed in the denatured state.

As shown diagrammatically in Figure 5B, if the distribution of D microstates is plotted as a function of solvent-accessible surface area A, class II mutants represent a shift of the D distribution to greater values of A than the wild-type distribution. The class I proteins could derive their properties either from the appearance of structural intermediates (Fig. 5A), or alternatively by the same argument used for the class II mutants, through a shift of the D state to smaller average values of A_d (Fig. 5B). In other words, instead of discreet intermediates which represent separate (resolvable) distributions of microstates, the denatured state of these mutant proteins may consist of a distribution of microstates "intermediate" between the wild-type N and D states. From Figure 5B, the experimentally determined value of m would be proportional to the distance between the N and D peaks (i.e., $A_d - A_n$).

On increasing the denaturant concentration, the distribution of D states is expected to shift in the direction of increasing solvent accessible surface area, since microstates with larger values of A are stabilized to a greater degree than those with smaller values of A (Fig. 5C). If the distribution of N states is shifted to a lesser extent, $(A_d - A_n)$ and therefore m should increase with increasing denaturant concentration. This is in fact what is observed with all six of the class I mutants in urea. Variation in m with guanidine hydrochloride is only detectable in three of these mutants and is much less pronounced than in urea. However, for wild-type nuclease and the five class II mutant proteins, there is no evidence for a concentration dependence of m in either denaturant.

The predicted shift in the distribution of D states to higher average surface area with increasing denaturant concentration may be responsible for noncoincidence of the guanidine denaturation curves of protein V66L+G79S+G88V as monitored by tryptophan fluorescence and circular dichroism. The argument in this case is very similar to the one described above that invokes a structural intermediate I, with the important difference that no energy barrier is presumed to exist between the I and D states. In other words, the D states at lower denaturant concentration have the same fluorescence but greater θ_{222} than the D states at higher denaturant concentration. Consequently, the θ_{222} for the D state is incorrrectly predicted on the basis of a linear extrapolation from outside the transition region, giving rise to an apparent denaturation curve that is less steep and shifted to higher denaturant concentrations than the actual curve.

From the hypothesis that amino acid substitutions in class I and II mutants are altering the average surface area exposed in the denatured state, it follows that (1) significant interactions must occur in the denatured state of staphylococcal nuclease that prevent the chain from attaining a true statistical random-coil state under moderately denaturing conditions and that (2) these interactions can be significantly perturbed by single amino acid substitutions. The first conclusion is not surprising in view of the numerous examples of data in the literature which

suggest the denatured state of proteins under moderately denaturing conditions is more compact than a statistical random coil and can undergo physical changes with increasingly denaturing conditions.[21,22] As shown by Tanford and colleagues,[23] when solution conditions are strongly denaturing (e.g., 6 M guanidine hydrochloride), the hydrodynamic properties of the denatured state of most proteins converge toward those of a true statistical random coil.

The tentative conclusion that single amino acid substitutions can modify interactions in the denatured state may seem more surprising. As to the nature of these interactions, one possibility is that, because staphylococcal nuclease denatures at such low urea and guanidine hydrochloride concentrations, elements of its native secondary structure persist in the denatured state. In this case, the putative interactions that give rise to the difference values of m could simply be a subset of the interactions that occur in the native state. A number of models of protein folding, such as the diffusion-collision model,[24] imply transient formation of secondary structural elements in the denatured state.

Alternatively, denatured state interactions could consist of chain-chain and chain-solvent interactions different from those of the native state. The amino acid substitutions found among the class II mutants are all replacements of a buried hydrophobic amino acid with a polar amino acid (ile-met, ala-thr, ala-ser), whereas two of the three class I mutations replace a buried amino acid with a more hydrophobic one (val-leu and gly-val). This apparent correlation between local changes in hydrophobicity and changes in solvent accessible surface exposed in the denatured state raises the possibility that solvophobic interactions may be responsible for major deviations of the denatured state of proteins away from a random coil, a well-recognized phenomenon in the physical chemistry of synthetic polymers.[25]

Further insight into the nature of these putative interactions may be gained by the characterization of the properties of a much larger collection of double mutant proteins. For the class II proteins I18M+A69T and I18M+A90S, the additive changes in $\Delta G°_{app}$ and in $(A_d - A_n)$ suggest the interactions altered by each of the pairs of amino acid substitutions may be independent of each other. Since for the class I protein V66L+G79S the quantitative changes in these same parameters exhibit rather clear non-additive behavior, the argument can be made[26,27] that these two amino acids substitutions alter a set of interdependent interactions, a surprising conclusion in view of the 25-angstrom separation between these two-residue positions in the native state.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Richard Armstrong for his valuable assistance in the operation of his JA500C spectropolarimeter, and Martin Karplus for helpful discussions. This work was supported by NIH grant GM34171.

REFERENCES

1. Pfeil, W. The problem of protein stability. Mol. Cell. Biochem. 40:3–28, 1981.
2. Perutz, M.F. What the abnormal hemoglobins teach us about the folding problem. In: "Protein Folding." Jaenicke, R. (ed.). Amsterdam: Elsevier/North Holland Biomedical Press. 1980:317–322.
3. Anfinsen, C.B. Principles that govern the folding of protein chains. Science 181:223–230, 1973.
4. Tucker, P.W., Hazen, E.E., Cotton, F.A. Staphylococcal nuclease reviewed: a prototypic study in contemporary enzymology. IV. The nuclease as a model for protein folding. Mol. Cell. Biochem. 23:131–141, 1979.
5. Shortle, D. A genetic system for analysis of staphylococcal nuclease. Gene 22:181–189, 1983.
6. Shortle, D., Lin, B. Genetic analysis of staphylococcal nuclease: identification of three intragenic "global" suppressors of nuclease-minus mutations. Genetics 110:539–555, 1985.
7. Shortle, D. Guanidine hydrochloride denaturation studies of mutant forms of staphylococcal nuclease. J. Cell. Biochem. 30:281–289, 1986.
8. Shortle, D. Stability mutants of staphylococcal nuclease: a correlation between nuclease activity in an agar gel assay and stability to guanidine hydrochloride denaturation. In: "Protein Engineering." Inouye, M., Sarma, R., (eds.). New York: Academic Press, Inc. 1986, in press.
9. Nozaki, Y. The preparation of guanidine hydrochloride. Methods Enzymol. 26:43–50, 1972.
10. Serpersu, E.H., Shortle, D., Mildvan, A.S. Kinetic and magnetic resonance studies of effects of genetic substitution of a CA2+-liganding amino acid in staphylococcal nuclease. Biochemistry 25:68–77, 1986.
11. Fuchs, S. Cuatrecasas, P., Anfinsen, C.B. An improved method for the purification of staphylococcal nuclease. J. Biol. Chem. 242:4768–4770, 1967.
12. Pace, C.N. The stability of globular proteins. Crit. Rev. Biochem. 3:1–43, 1975.
13. Anfinsen, C.B., Schechter, A.N., Taniuchi, H. Some aspects of the structure of staphylococcal nuclease. Part II. Studies in solution. In: "Structure and function of proteins at the three-dimensional level." Cold Spring Harbor Symposia on Quantitative Biology. VolXXXVI. Cold Spring Harbor, New York: The Cold Spring Harbor Laboratory, 1972:249–255.
14. Wong, K.P, Tanford, C. Denaturation of bovine carbonic anhydrase b by guanidine hydrochloride. J. Biol. Chem. 248:8519–8523, 1973.
15. Kuwajima, K., Nitta, K., Yoneyama, M., Sugai, S. Three-state denaturation of alpha-lactalbumin by guanidine hydrochloride. J. Mol. Biol. 106:359–373, 1976.
16. Desmadril, M., Yon, J.M. Existence of intermediates in the refolding of T4 lysozyme at pH7.4. Biochem. Biophys. Res. Commun. 101:563–569, 1981.
17. Schellman, J.A. Solvent denaturation. Biopolymers 17:1305–1322, 1978.
18. Tanford, C. Protein denaturation. Part C. Theoretical models for the mechanism of denaturation. Adv. Protein Chem. 24:1–95, 1970.
19. Lee, B., Richards, F.M. The interpretation of protein structures: estimation of static accessibility. J. Mol. Biol. 55:379–400, 1971.
20. Lumry, R., Biltonen, R., Brandts, J.F. Validity of the "Two-State" hypothesis for conformational transitions of proteins. Biopolymers 4:917–944, 1966.
21. Aune, K.C., Salahuddin, A., Zarlengo, M.H., Tanford, C. Evidence for residual structure in acid- and heat-denatured proteins. J. Biol. Chem. 242:4486–4489, 1967.
22. Tsong, T.Y. The trp-59 fluorescence of ferricytochrome c as a sensitive measure of the overall protein conformation. J. Biol. Chem. 249:1988–1990, 1974.
23. Tanford, C. Kawahara, K., Lapange, S. Proteins as random coils. I. Intrinsic viscosities and sedimentation coefficients in concentrated guanidine hydrochloride. J. Am. Chem.

Soc. 89:729–736, 1967.
24. Karplus, M., Weaver, D.L. Protein-folding dynamics. Nature 260:404–406, 1976.
25. Flory, P.J. Chapter XII. In: "Principles of Polymer Chemistry." Ithaca, New York. Cornell University Press. 1953.
26. Ackers, G.K., Smith, F.R. Effects of site-specific amino acid modification on protein interactions and biological function. Annu. Rev. Biochem. 54:597–629, 1985.
27. Carter, P.J., Winter, G., Wilkinson, A.J., Fersht, A.R. The use of double mutants to detect structural changes in the active site of the tyrosyl-tRNA synthetase (Bacillus stearothermophilus). Cell 38:835–840, 1984.
28. Richardson, J.S. The anatomy and taxonomy of protein structure. Adv. Protein Chem. 34:167–339, 1981.