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Guanidine Hydrochloride Denaturation Studies of Mutant Forms of Staphylococcal Nuclease

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Several mutant forms of staphylococcal nuclease with one or two defined amino acid substitutions have been purified, and the effects of the altered amino acid sequence on the stability of the folded conformation have been analyzed by guanidine hydrochloride denaturation. Two *nuc*- mutations, which greatly reduced the level of enzyme activity accumulated in *E coli* colonies carrying a recombinant plasmid with the mutant *nuc* gene (ie, a NUC- phenotype), both result in protein unfolding at significantly lower guanidine hydrochloride concentrations than the wild-type protein, whereas three *sup* mutations isolated on the basis of their ability to suppress partially the NUC- phenotype of the above two mutations result in unfolding at significantly higher guanidine hydrochloride concentrations. Characterization of nuclease molecules with two different amino acid substitutions, either *nuc*- + *sup* pairs or *sup* + *sup* pairs, suggests that the effect of an amino acid substitution on the stability of the native conformation, as measured by the value of $\Delta\Delta G_D$, may not be a constant, but rather a variable that is sensitive to the presence of other substitutions at distant sites in the same molecule. Surprisingly, the slopes of the log K_{app} vs guanidine hydrochloride concentration plots vary by as much as 35% among the different proteins.

Key words: protein folding, guanidine hydrochloride, staphylococcal nuclease, protein denaturation, stability mutations

Although the chemical basis for the stability of the native conformation of globular proteins in aqueous solution is understood in a qualitative sense—hydrophobic forces, hydrogen bonds, salt bridges, and van der Waals interaction all play a significant role—there is at present little quantitative information on the contributions of each of these four types of forces to the stability of any protein. If the engineering of new proteins for various scientific and technological applications is to become an exact science, it will clearly be necessary to formulate rules to predict the consequences of amino acid sequence changes on the folding equilibrium of proteins of interest. In addition, if the very formidable problem of predicting three-dimensional

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structure from amino acid sequence is to be solved in a general way, a detailed quantitative theory for calculating the strength of potential intramolecular interactions in proteins will, in all likelihood, play a key part in the overall solution.

In an effort to analyze the physical chemical determinants of protein conformational stability, a long-term study of staphylococcal nuclease has been undertaken in this laboratory. By applying the classical genetic strategy of random mutagenesis followed by isolation of mutations on the basis of phenotype, a relatively unbiased search is being carried out to identify the essential noncovalent bonds which stabilize the native conformation of this small, well-characterized protein and to elucidate the general patterns through which the information in the amino acid sequence of the polypeptide chain is integrated to determine the final solution structure.

As described elsewhere [1], a simple genetic system based on the cloned staph nuclease gene inserted into an *E coli* plasmid has been developed for isolating, mapping, and sequencing mutations that alter the amount of nuclease activity accumulated in bacterial colonies carrying this recombinant DNA plasmid. Although diminished enzyme activity is a phenotype that could reflect mutational alteration of several properties of the enzyme (substrate binding, catalytic activity, protease resistance, accessibility to transport into the periplasm, etc), it was anticipated that mutations that drastically altered folding/stability might also register a NUC- phenotype indirectly through increased proteolysis, and therefore be detected with a simple plate assay for enzyme activity [1]. To date a collection of more than 80 unique missense mutations distributed across the staph nuclease gene has been assembled [2], all of which were identified on the basis of their effects on this plate assay.

This report describes preliminary experiments that quantitate the effects of several amino acid substitutions at positions remote from the active site on the stability of staphylococcal nuclease to unfolding by guanidine hydrochloride.

MATERIALS AND METHODS

Genetic Materials

The induction, isolation, and characterization of all of the mutant alleles of the staph nuclease gene (*nuc*) used in these experiments are described in [2]. Since these mutant alleles were recovered on plasmid pFOG302, which directs *E coli* to synthesize very small amounts of incorrectly processed nuclease, the high level expression plasmid pFOG405 was constructed by replacing the upstream control region of the *nuc* gene in pFOG302, which is derived from the naturally occurring *Staphylococcus aureus* gene, with the upstream control region, ribosome binding site, initiation codon, and signal peptide of the *E coli* alkaline phosphatase gene (*pho*A; [see 3]). The product of the resulting hybrid gene is (1) regulated by levels of phosphate in the culture media; (2) transported into the periplasmic space, where the signal peptide component from the *pho*A gene is cleaved off, generating (3) mature staphylococcal nuclease protein with the correct amino terminus [Dr. John Smith and Dr. Don Oliver, personal communications]. Details of the plasmid construction and of the recombination of mutant alleles from the initial isolates on plasmid pFOG302 onto the expression plasmid pFOG405 will be published elsewhere.

Protein Purification

E coli strains carrying the appropriate mutant plasmid were induced for protein synthesis by growth at 37°C in complete MOPS media [4] supplemented with only

0.1 mM potassium phosphate. At the point in the growth curve where cell division stops due to phosphate starvation and synthesis of staph nuclease is induced, the protease inhibitor phenylmethylsulfonyl fluoride is added to the culture media to a final concentration of 1 mM. At the point of maximal protein accumulation (approximately 3–4 hours later), the cells are harvested by centrifugation at 4°C. The cell pellet from 500 ml of culture is resuspended in 10 ml ice-cold 1 M Tris HCl, pH 10.5/2.5 mM EDTA with gentle agitation for 20 min. After pelleting the cells, the supernatant (which is typically 0.5–1 mg/ml nuclease, usually at a level of purity greater than 50%) is mixed with 1 ml of 1 M HEPES to reduce the pH to approximately 9.2, and the mixture is passed over a small pre-equilibrated BioRex 70 column. After extensive washing with 0.2 M Tris HCl, pH 7.6, nuclease is eluted from the column with 0.5 M Tris HCl, pH 7.6/1 M NaCl and then dialyzed overnight against 2 mM PIPES, pH 6.8/50 mM NaCl. Protein concentration is determined by UV absorption at 280 nm [5] and purity (greater than 95–99%) confirmed by SDS polyacrylamide gel electrophoresis.

Guanidine Hydrochloride Denaturation Curve Analysis

To quantitate the equilibrium between the native and denatured conformations as a function of guanidine hydrochloride concentration, intrinsic fluorescence of the single tryptophan residue at residue position 140 was monitored [6]. A SPEX Fluorolog II spectrophotofluorometer was used for all measurements with the cuvette holder thermostatted at 20.0°C. The excitation wavelength was 295 nm, and intensity of emission at 325 nm was recorded. Samples of purified protein in 2.00 ml of 100 mM NaCl/25 mM potassium phosphate, pH 7.0 at final protein concentration of 40 to 50 μg/ml were weighed into a 1.0 cm × 1.0 cm path length quartz fluorescence cuvette. A 6 M solution of guanidine hydrochloride (Bethesda Research Labs, UltraPure Lot 40320) was prepared and neutralized with a small amount of Tris base to give a final pH of 7.0 on dilution to 1 M. This one solution was used for all of the experiments reported here. No correction for pH changes as a function of guanidine concentration have been made [7], although these changes become significant at high concentrations—pH at 1.5 M is 6.3.

To determine the relative fluorescence with increasing denaturant, the cuvette was removed, an aliquot of 6 M guanidine hydrochloride was added, the solution was mixed for 30 seconds via a magnetic stirring bar inside the cuvette, and the cuvette placed back in the spectrofluorometer. The approach to equilibrium was followed over time (typically requiring from 0 to 4 min at concentrations around the midpoint) and the final equilibrium value of fluorescence was recorded 5 min later. Aliquots of 6 M guanidine hydrochloride (added with calibrated Gilson Pipetman pipettors) were used that increased the final concentration of denaturant by 50 or 100 mM increments, simplifying the correction of fluorescence intensity for sample dilution and the charting of data. The apparent equilibrium constant K_{app} was calculated using the equation:

$$K_{app} = I_N - I/I - I_D \quad \text{[ref 8]} \tag{1}$$

where I is the fluorescent intensity of the sample, I_N is the extrapolated value of fluorescence for the native state (assumed to be 1.00; see Fig. 1), and I_D is the extrapolated value for the denatured state.

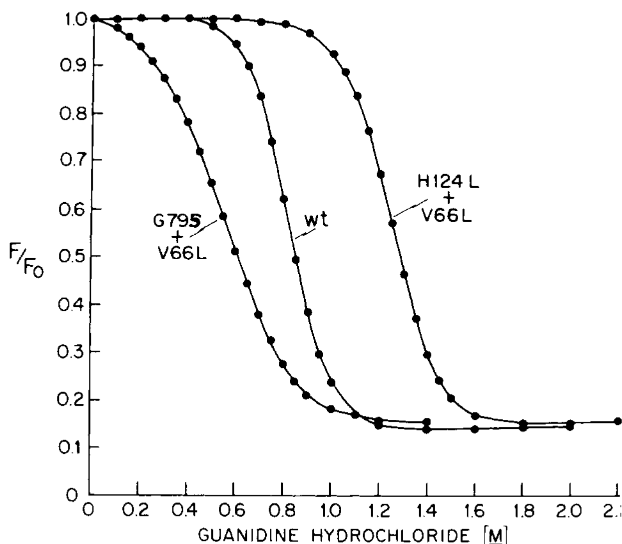

Fig. 1. Relative fluorescent intensity of tryptophan 140 in wild-type (wt) and two mutant forms of staphylococcal nucelase as a function of guanidine hydrochloride concentration. F_0 is the intrinsic fluorescence in the absence of guanidine hydrochloride on excitation at 295 nm and monitoring intensity at 325 nm. The small differences in F/F_0 at high denaturant concentrations among the three proteins is primarily a result of differences in the baseline signal from the photon counter in the absence of sample (ie, dark counts).

RESULTS AND DISCUSSION

In this report, mutant proteins are referred to by the mutant amino acid substitution(s) they contain, 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. For example, F76V designates the form of Foggi strain staphylococcal nuclease which has the wild-type phenylalanine residue at position 76 replaced with a mutant valine residue. Proteins F76V and G79S are derived from *nuc-* mutant alleles that produce very low levels of nuclease activity in recombinant *E coli* [2]. Proteins F76V + H124L, G79S + H124L, and G79S + V66L are derived from NUC+ revertants induced by mutagenesis of the *nuc-* alleles for F76V and G79S. As described in [2], the mutations encoding the substitutions H124L, H124R, and V66L are suppressor mutations (*sup*) that can partially correct the NUC- phenotype of a variety of other *nuc-* mutant alleles. Proteins H124L, H124R, V66L, H124L + V66L, and H124R + V66L are derived from the corresponding mutant alleles that were generated by recombination of the *sup* mutations, first out of the double mutant form and into a wild-type background and then with each other.

A reasonable estimate of the stability of the native conformation of a protein, ie, the Gibbs free energy of unfolding or denaturation ΔG_D, can be obtained by quantitating the effect of denaturants such as urea or guanidine hydrochloride on the unfolding equilibrium constant K_D [7–9]. Given the equation:

$$\Delta G_D = -RT \ln K_D , \quad (2)$$

the value of K_D under a given set of solution conditions—pH, temperature, ionic strength, and denaturant concentration—can be directly translated into a measure of the stability of the folded state. The value of greatest interest is $K°_D$ in the absence of denaturant, and since $K°_D$ can almost never be measured directly in the absence of denaturants or elevated temperatures, K_D (or more appropriately K_{app}) is measured over a range of denaturant concentrations and then an extrapolation to zero denaturant is carried out [8]. Although the mechanism by which denaturants alter the folding equilibrium is not known, leaving considerable doubt as to the "correct" way to carry out this extrapolation, it has been determined empirically that a linear extrapolation on a plot of log K_{app} versus denaturant concentration often yields a value of $\Delta G°_D$ that is in reasonably good agreement with values determined from thermal denaturation studies [8]. In addition, one thermodynamic formulation of the action of solvent denaturants is consistent with this linear relationship of ΔG_D to denaturant concentration [10].

A variety of physical techniques have been applied to the determination of K_{app} in the presence of denaturants. Earlier spectroscopic studies on staphylococcal nuclease by Cuatrecasas and Anfinsen [11,12] had shown that the single tryptophan residue at position 140 is quite inaccessible to solvent in the native conformation, resulting in a four- to eightfold higher quantum yield of intrinsic fluorescence on excitation at 295 nm for the native than for the denatured conformation. Although the relative fluorescence of this tryptophan as a function of denaturant only monitors the change in environment of a single residue, the values of K_{app} for acid denaturation determined by fluorescence were essentially identical to those obtained by other, more global probes of conformation such as intrinsic viscosity, circular dichroism at 222 nm, UV absorption at 280 nm, and proton NMR of the four histidine residues [11]. In light of this fact plus its greater sensitivity, precision, and ease of data collection, fluorescence was chosen over other physical techniques for the studies described in this report.

Urea and guanidine hydrochloride are the two denaturants most commonly used for quantitative studies of protein stability, and both of these compounds have been demonstrated to denature reversibly wild-type staphylococcal nuclease [12]. In this preliminary study, guanidine hydrochloride denaturation curves at 20.0°C and pH 7.0 were determined for the wild-type Foggi strain nuclease and for ten mutant proteins. Figure 1 illustrates the relative intrinsic fluorescence as a function of guanidine hydrochloride for wild-type and for two double mutant proteins, one which unfolds at lower and one which unfolds at higher denaturant concentrations. In each case, the result is a single symmetrical curve that ends in a slightly inclined plateau at high denaturant concentrations representing approximately 0.15 of the initial fluorescence intensity. Since the fractional drop in fluorescence is essentially the same as that seen by Cuatrecasas et al for acid denaturation [11] and since all of the mutant proteins behave in this same manner, this curve must be a reflection of an unfolding equilibrium that changes with guanidine hydrochloride concentration.

The plots of log K_{app} versus guanidine hydrochloride concentration for the 11 proteins studied are given in Figure 2 and the numerical values of the denaturant concentration at the midpoint, Cm, plus the slopes of the best straight line fit, m, normalized to the wild-type value are listed in Table I. Several general conclusions can be immediately drawn from inspection of Figure 2: (1) The two mutant proteins F76V and G79S, which were identified on the basis of greatly reduced levels of

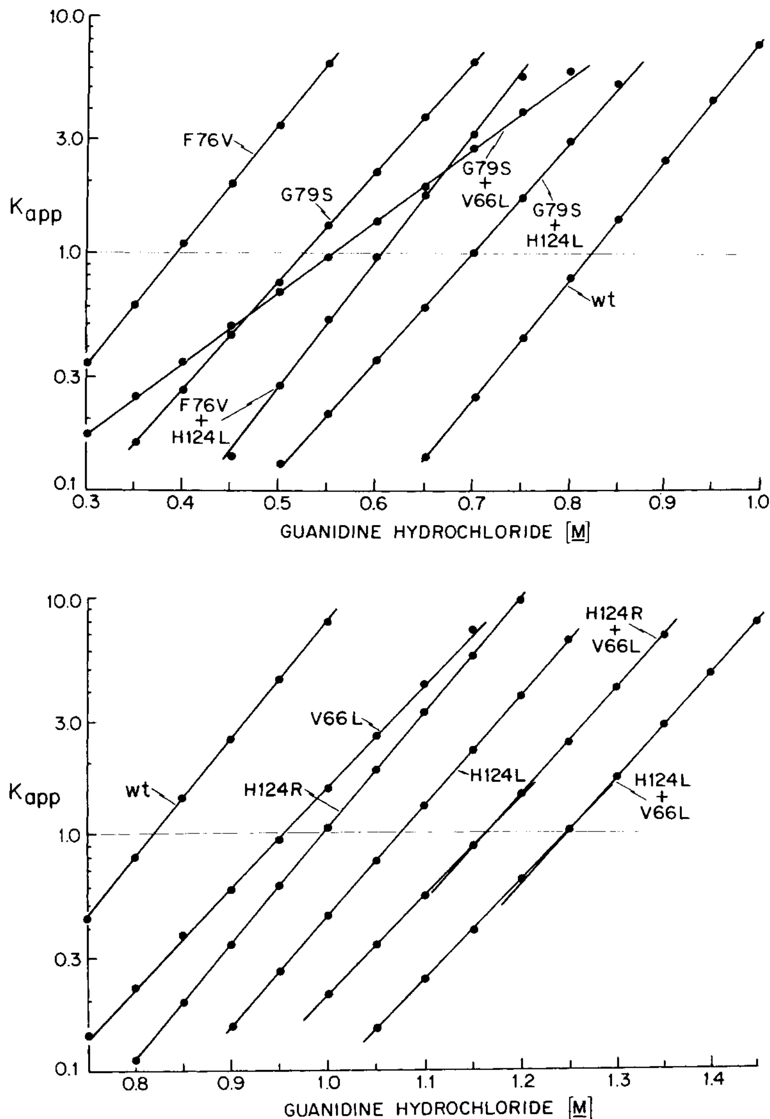

Fig. 2. Plots of the logarithm of the apparent equilibrium constant K_{app} as a function of guanidine hydrochloride concentration. See text for calculation of K_{app}. All data were collected at 20.0°C, pH 7.0 in 100 mM NaCl, 25 mM potassium phosphate buffer at a protein concentration of 40 to 50 μg/ml. No correction has been made for small changes in pH at high concentrations of guanidine hydrochloride.

enzyme activity in *E coli* strains carrying the corresponding mutant alleles and grown at 37°C, both unfold at significantly lower guanidine hydrochloride concentrations than does the wild-type protein. (2) Two of the three double mutant proteins (F76V + H124L and G79S + H124L), which were identified as moderate-activity pseudorevertants of the above two mutants, both unfold at higher concentrations than the corresponding parent protein. Since the rank order of enzyme activity scored in *E coli* colonies carrying these mutant alleles is the same as the order of Cm for purified

TABLE I. Guanidine Hydrochloride Denaturation Parameters for Mutant Forms of Staphylococcal Nuclease at 20°C. and pH 7.0

Protein	Cm [M]	Slope m[a]	Apparent $\Delta G_D^{\circ b}$ Observed m	Apparent $\Delta G_D^{\circ b}$ Wild-type m
wt (Foggi strain)	0.82	1.00	+5.5	+5.5
	0.82	1.02	+5.6	+5.5
	0.82[c]	0.98	+5.4	+5.5
F76V	0.39	1.00	+2.6	+2.6
F76V + H124L	0.60	1.05	+4.2	+4.0
G79S	0.53	0.92	+3.2	+3.5
G79S + H124L	0.70	0.91	+4.3	+4.7
G79S + V66L	0.55	0.61	+2.2	+3.7
	0.56	0.62	+2.3	+3.7
H124L	1.08	0.93	+6.7	+7.2
	1.08	0.95	+6.9	+7.2
H124R	1.00	0.97	+6.5	+6.7
V66L	0.96	0.84	+5.4	+6.4
H124L + V66L	1.25	0.87	+7.2	+8.3
	1.25	0.86	+7.2	+8.3
H124R + V66L	1.17	0.87	+6.8	+7.8

[a]Values have been normalized relative to m for the wild-type protein, which is 4.98 $\log_{10}$/mole.
[b]Free energy is expressed in Kcal/mole.
[c]Buffer was 25 mM sodium cacodylate, pH6.8 + 100 mM NaCl.

proteins—F76V < G79S < F76V + H124L < G79S + H124L—there appears to be a rough correlation between protein stability at 20°C and nuclease activity accumulated in bacteria grown at 37°C. (3) However the third "pseudorevertant" protein G79S + V66L has a value of Cm only slightly higher, and a slope that is 35% lower, than the parent mutant protein G79S. Linear extrapolation of the plot for this protein to zero denaturant gives an estimate of stability that is considerably lower than that of G79S. This result suggests that either the suppression effect caused by V66L is not mediated through enhanced stability or the stability at 20°C does not accurately reflect stability at 37°C. (4) All three *sup* amino acid substitutions, when present singly in an otherwise wild-type protein, result in unfolding at significantly higher concentrations of guanidine hydrochloride. (5) When the *sup* amino acid substitution V66L is combined with either of the other two *sup* substitutions—H124L or H124R—the double mutant protein is clearly more stable than H124L or H124R alone, a result that is in marked contrast to the combination of V66L with G79S. (6) Comparison of the plots for F76V alone and with H124L, G79S alone and with H124L, V66L alone and with H124L, and finally wild-type vs H124L suggest that the amino acid substitution of leucine for histidine at position 124 may add an increment of stability that is roughly constant in all four "background" molecules.

This last point can be submitted to a quantitative test by determining the change in ΔG°_D (ie, $\Delta\Delta G^\circ_D$) caused by addition of the H124L substitution to each of the four molecules. If the change in free energy is a constant, then:

$$\Delta G^\circ_{1+H124L} = \Delta G^\circ_1 + \Delta\Delta G^\circ_{H124L} , \quad (3)$$

where the subscript 1 indicates the mutant amino acid substitution present in the background molecule. Examination of the data in Table I, however, indicates that

there is at least one potential problem in applying the linear extrapolation to obtain K_{app} in the absence of denaturant. The values of slope m appear to be significantly different from the wild-type value for several single and double mutant proteins, a result that is not anticipated by any of the theoretical models for the denaturing action of guanidine hydrochloride [13] and that makes it problematic as to whether the observed value of m should be used to extrapolate to zero denaturant or, as argued by Cupo and Pace [13], the wild-type value in all cases. Obviously, the value of $\Delta G°_{app}$ depends heavily on the chosen value for m.

In Table I, both approaches have been used to obtain $\Delta G°_{app}$. When the observed m is used to obtain $\Delta G°_{app}$, $\Delta\Delta G°_{H124L}$ ranges from a low of +1.1 kcal/mole with G79S to a high of +1.8 kcal/mole with V66L. When the wild-type value of m is used for all extrapolation, the range is from +1.2 kcal/mole with G79S to +1.9 kcal/mole with V66L. Based on the fairly high reproducibility of the midpoints and slopes for a given protein from experiment to experiment, some of which are given in Table I plus many others that are not shown, it would appear that this range of values is larger than experimental error. However, if the effect of the V66L substitution is similarly examined in the background of wild-type, G79S, H124L, and H124R molecules, a far greater range of values is obtained. When observed m is used to obtain $\Delta G°_{app}$, $\Delta\Delta G°_{V66L}$ ranges from −1.0 kcal/mole for G79S to +0.4kcal/mole for H124L, and when the wild-type value of m is used for the extrapolation, $\Delta\Delta G°_{V66L}$ calculates out to be +0.2 kcal/mole for G79S and +1.1 kcal/mole for both H124L and H124R. For this particular amino acid substitution, the change in free energy of denaturation by guanidine hydrochloride appears to be extensively modified by single amino acid substitutions elsewhere in the polypeptide chain.

CONCLUSIONS

There are several ways of viewing the preliminary stability data presented in this paper. One perspective, which is favored by this author, is that the linear extrapolation to zero denaturant concentration should not be applied to all of the mutant forms of staph nuclease because some of the amino acid substitutions involved are significantly altering the proteins' interaction with guanidine hydrochloride. Clearly, the large changes in m values for the plots of log K_{app} versus guanidine hydrochloride concentration could reasonably be interpreted as a reflection of such altered interactions [9,10]. If so, continued study of these and other mutant forms of staphylococcal nuclease may shed some light on the mechanism of denaturation by compounds like guanidine hydrochloride and urea.

A second possibility is that the transition being monitored by tryptophan fluorescence involves a second reaction in addition to unfolding of the polypeptide chain. For example, a local "breathing" reaction involving residues surrounding tryptophan 140 could provide another mechanism for fluorescence quenching. Any mutant protein in which such a second reaction was altered disproportionately relative to the unfolding reaction could not be accurately modeled by the two-state approximation. Similarly, the two-state approximation might be inappropriate if some of the amino acid substitutions are causing significant stabilization of folding intermediates [13]. However, there is no clear evidence for the breakdown of two-state behavior with these mutant forms of staph nuclease. In fact, the remarkable linearity of lnKapp with respect to guanidine hydrochloride concentrations for all proteins except H124L

+ V66L and H124R + V66L suggests that, if present, any second reaction must change in parallel with the unfolding reaction as a function of guanidine hydrochloride concentration.

Perhaps the most interesting possibility is that the calculated values of $\Delta G°_{app}$ are essentially correct and that the changes in the free energy of denaturation $\Delta\Delta G°_D$ caused by individual amino acid substitutions are not strictly additive when multiple substitutions are combined in a single nuclease molecule. Instead, energetically important interactions can take place between residues that are a considerable distance apart in the native conformation and thereby change the value of $\Delta\Delta G°_D$ caused by an amino acid substitution. (In this respect, it is intriguing that residues G79S and V66L are on essentially opposite sides of the native conformation, with several beta-strands intervening between them.) In this case, a conformational change can be invoked as the physical pathway through which the interaction occurs [see references 14 and 15 for a thorough discussion of this argument]. If in fact this is the correct conclusion to be drawn from these data, then future studies to quantitate the ΔH and ΔS of unfolding by thermal denaturation and microcalorimetry should confirm the values of free energy change presented in Table I. Perhaps as more thermodynamic data are collected on these and related mutant forms of staphylococcal nuclease, it will be possible to document the presence of these putative conformational changes and to determine if they represent changes in the structure of the native state or of the denatured state.

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