Coupling between Local Structure and Global Stability of a Protein: Mutants of Staphylococcal Nuclease†

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ABSTRACT: Staphylococcal nuclease exists in solution as a mixture of two folded (N and N′) and two unfolded (U and U*) forms. Earlier workers [Evans et al. (1989) *Biochemistry 28*, 362] have proposed that the N′/N and U/U* structural differences involve cis/trans isomerization about the Lys^{116}–Pro^{117} peptide bond with N and U cis and N′ and U* trans. The present results show that residue changes throughout the nuclease structure have large effects on the distribution of the N and N′ forms. The N′/N ratios at 313 K for nuclease H124L (N′/N = 0.07) and nuclease G79S (N′/N = 12) differ by 2 orders of magnitude. Thermodynamic parameters for equilibria linking the two folded and two unfolded substates were evaluated for seven mutants of nuclease which were found by kinetic assays to have similar enzymatic activities but by NMR spectroscopy to have a wide dispersion of thermal stabilities. Our results indicate that mutational perturbations of the N′/N equilibrium in folded nuclease (ΔG for the N ⇄ N′ reaction) are strongly coupled to changes in the stability of the N form (ΔG for the N ⇄ U reaction), but much less so to the stability of the N′ form (ΔG for the N′ ⇄ U* reaction).

A major structural difference between the two folded forms (N and N′) of the enzyme staphylococcal nuclease, as well as between the two unfolded forms (U and U*) of the protein, has been attributed to cis ⇄ trans isomerization about the Lys^{116}–Pro^{117} peptide bond: N and U cis, N′ and U* trans. Evidence for this has been educed from the following: (1) the absence of N′/N chemical shift multiplicity in nuclease P117G[1] (Evans et al., 1987) and (2) the observation of two unfolded forms (U and U*) as reported by two ^{1}H NMR peaks corresponding to the $H^{\epsilon 1}$ proton of His^{121}, a residue sequentially near Pro^{117} (Evans et al., 1987, 1989).

In this NMR study of seven mutants of the enzyme staphylococcal nuclease (Figure 1), we have found sequence-dependent effects on protein stability and on the N′/N ratio. Nucleases G79S, V23F, and L89F have higher N′/N ratios and lowered stabilities to thermal denaturation when compared to nuclease wt. Nucleases I18M and H46Y have N′/N ratios and stabilities similar to nuclease wt. Nuclease H124L has a smaller N′/N ratio and is more stable than nuclease wt.

The N′/N ratio has been found to increase with increasing temperature (Alexandrescu et al., 1989; Evans et al., 1989) or decreasing pH (Alexandrescu et al., 1989), both of which lower the stability of nuclease to denaturation. Addition of the competitive inhibitor pdTp in the presence of Ca^{2+} stabilizes nuclease to denaturation and decreases the N′/N ratio in nuclease wt (Alexandrescu et al., 1989; Evans et al., 1989) and in all mutants studied here.

Experimental Procedures

Materials. CHES, MOPS, and HEPES were obtained from Research Organics; PNPdT, salmon sperm DNA, PMSF, and EDTA were obtained from Sigma; "ultrapure" $CaCl_2$ was from Alfa; 99.8% and 99.98% ^2H_2O were from Aldrich; Tris and calf intestine alkaline phosphatase were from Boehringer Mannheim; pdTp was from Pharmacia. *Escherichia coli* strains SE6004 carrying plasmids for nuclease wt (pFOG405), V23F (A113), I18M (C210), H46Y (B204), G79S (A121), L89F (T232), and F76V+H124L (31A1) were gifts from Dr. D. Shortle (The Johns Hopkins University) (Shortle & Lin, 1985). The nuclease H124L sample was purified from a T7 promoter overexpression system (Wang et al., 1990) [plasmid-pTSN2CC, *E. coli* strain BL21-DE3 (Studier & Moffat, 1986)] provided by Dr. D. M. LeMaster (Northwestern University). Nuclease H124L obtained from the pTSN2CC plasmid has identical properties to nuclease obtained from the Z101 mutant (H124L variant) of the pFOG405 plasmid (A. T. Alexandrescu and A. P. Hinck, unpublished results).

Preparation of Protein Samples. Nucleases produced from the pFOG405 plasmid and its variants were purified by the method of Serpersu et al. (1986). Nuclease production in the pFOG405 plasmid is under the control of an alkaline phosphatase promoter and is induced by growth in low phosphate media. Starter cultures of *E. coli* containing the overexpressing nuclease gene were grown overnight in an L broth–ampicillin medium at 37 °C. The cells were induced to overproduce

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[1] Abbreviations: CHES, 2-(cyclohexylamino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; $H^{\epsilon 1}$, resonance from the histidine $C^{\epsilon 1}$ proton; NMR, nuclear magnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; pdTp, thymidine 3′,5′-bisphosphate; PMSF, phenylmethanesulfonyl fluoride; PNPdT, thymidine 5′-(*p*-nitrophenyl phosphate); pH*, pH value uncorrected for the deuterium isotope effect; ppm, parts per million; *r*, Pearson correlation coefficient; rms, root mean square; T_m, temperature for which there are equal concentrations of folded and unfolded forms (K_{TS} = 1); TSP, 3-(trimethylsilyl)-propionate-d_4; nuclease wt (wild type), enzyme with the sequence of nuclease from the Foggi strain of *Staphylococcus aureus* (Cone et al., 1971). Mutant nucleases are designated as follows: I18M, Ile^{18} replaced by Met; V23F, Val^{23} replaced by Phe; H46Y, His^{46} replaced by Tyr; G79S, Gly^{79} replaced by Ser; L89F, Leu^{89} replaced by Phe; P117G, Pro^{117} replaced by Gly; H124L, His^{124} replaced by Leu; F76V+H124L, double mutant with Phe^{76} replaced by Val and His^{124} replaced by Leu.

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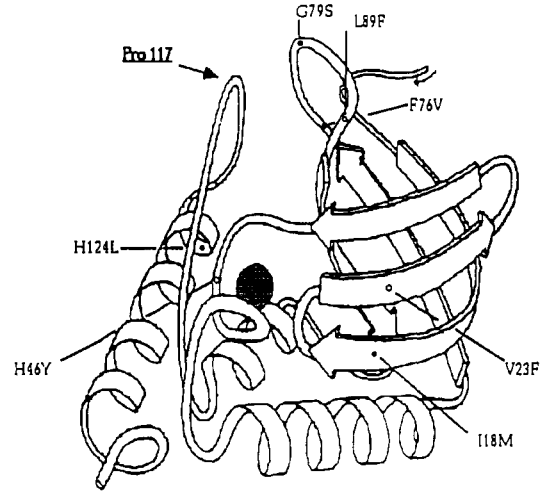


FIGURE 1: Richardson diagram of staphylococcal nuclease (Richardson, 1981) showing the locations of the mutated residues and Pro[117]. The drawing is based on the crystal structure of nuclease wt complexed with Ca^{2+} and pdTp (Cotton et al., 1979).

nuclease by transfer to a low-phosphate MOPS (50 μg/mL)–ampicillin medium (Serpersu et al., 1986). The low-phosphate (0.1 M) medium derepresses the alkaline phosphatase promoter and triggers overexpression of the nuclease gene downstream. Two hours after induction, PMSF was added to prevent the proteolytic degradation of the overproduced protein. About 150 min after addition of PMSF, the cells were centrifuged for 10 min at 5000*g* and collected as a pellet. The pellet was resuspended and stirred in cold 1 M Tris and 2.5 mM EDTA, pH 10.5. This treatment ruptures the outer cell membrane and releases the soluble contents of the periplasmic space. The crude nuclease extract was centrifuged twice for 10 min at 8000–10000*g* and the cell sap was discarded. The crude extract of nuclease was purified by the method of Serpersu et al. (1986). HEPES buffer (1 M) was added to the crude nuclease preparation to a final concentration of 10% in order to bring the pH to 9.2. The mixture was loaded on either a Bio-Rex 70 or preferably a C-25-CM-Sephadex cation-exchange column. After loading, columns were immediately washed with 2–4 column volumes of equilibration buffer (0.9 M Tris, 2.3 mM EDTA, 0.1 M HEPES, pH 9.2) and 2–4 column volumes of wash buffer (0.2 M Tris, pH 7.6) and eluted with buffer (0.5 M Tris, 1 M NaCl, pH 7.6). The presence of nuclease in the eluted fractions was detected by UV absorbance (a 1 mg/mL solution of nuclease has $A_{280} = 0.93$). Samples were dialyzed once against 50 mM phosphate and 0.1 M NaCl, pH 7; once against 10 mM phosphate and 0.1 M NaCl, pH 7, to remove Tris; and twice against deionized, distilled water. A white precipitate often formed during the last procedure which was removed by centrifugation for 10 min at 10000*g* and discarded. The protein was lyophilized and stored as a dry powder at −20 °C.

Enzyme Assays. The temperature for all assays was maintained at 21 °C with a thermostated circulating water bath. The assay buffer was 0.1 M CHES and 10 mM $CaCl_2$, pH 9.0. Nucleases were assayed against the PNPdT substrate by the alkaline phosphatase coupled assay method of Grissom and Markley (1989). Nuclease catalyzes the hydrolysis of PNPdT to *p*-nitrophenyl phosphate and thymidine. Alkaline phosphatase converts *p*-nitrophenyl phosphate to *p*-nitrophenol and phosphate. Concentrations of PNPdT were determined from $\epsilon_{330} = 2.4 \times 10^3\ M^{-1}\ cm^{-1}$. The rate of production of *p*-nitrophenol was determined in virgin 1-cm polystyrene cuvettes at 405 nm, where $\epsilon_{405} = 1.83 \times 10^4\ M^{-1}\ cm^{-1}$. A double-beam spectrophotometer (substrate, phosphatase, and nuclease in the sample cell; substrate and phosphatase in the reference cell) was used in order to subtract the background rate of *p*-nitrophenol production due to alkaline phosphatase. Typically, 2–5 μg of nuclease and 0.5 μg of phosphatase were used for 1-mL assays.

Nucleases were assayed against single-stranded DNA by the method of Cuatrecasas et al. (1967a). Stock solutions of salmon sperm DNA were heat-denatured by heating to 90 °C for 30 min, followed by rapid cooling in an ice bath for 15 min. Concentrations of single-stranded (heat-denatured) DNA were determined from $A_{260} = 1.08$ for a 50 μg/mL solution (Cuatrecasas et al., 1967a). The rate of hydrolysis of the DNA substrate was followed by the increase in A_{260}. Typically, 0.05 μg of nuclease was used for 1-mL assays. Cuvettes were washed extensively between assays (30 times), and a base-line trace was collected for 2–3 min after the addition of DNA to ensure that no contaminating nuclease activity remained from the previous assay. Because the exact molecular weight of the DNA substrate was not known, activity was measured in terms of ΔA_{260}/min, and V_m is reported rather than k_{cat}. V_m and K_m values were obtained by fitting hyperbolic substrate saturation curves to

$$v = VS/(K_m + S)$$

by using Cleland's HYPERO program (Cleland, 1979).

NMR Samples. Only samples that could be purified to >90% homogeneity were used. Lyophilized nuclease powder was taken up in a 99.8% ^2H_2O solution containing 0.3 M NaCl. TSP was added for use as an internal chemical shift standard. A coarse pH adjustment was made by the addition of 0.1–2-μL aliquots of 1 N ^2HCl and KO^2H. The samples were lyophilized twice from 99.8% ^2H_2O and twice from 99.98% ^2H_2O and taken up in a volume of 99.98% ^2H_2O such that the sample was 0.3 M in NaCl. Precipitate resulting from this procedure was pelleted and discarded. The pH was adjusted to its final value prior to NMR spectroscopy. The pH was chosen to optimize the separation of the His $H^{\epsilon 1}$ resonances from N, N′, and unfolded forms. To fully exchange unexchanged amide protons, samples were heated to T_m for approximately 10 min in the NMR spectrometer. Data were acquired during heating of the sample in order to follow the exchange of amide resonances. As soon as no amide resonances were observed in the His $H^{\epsilon 1}$ region, the sample was removed from the spectrometer and cooled to room temperature.

The dimeric form (N″) has been observed at high protein concentrations in nuclease wt (Alexandrescu et al., 1989) and in all of the mutants studied here with the exception of G79S. Because measurement of the distribution of the N and N′ forms was the primary motivation for the present work, nuclease concentrations were chosen low enough (<1.0 mM) so that the amount of the N″ form would be negligible. Additional considerations for this choice of protein concentration were the sharpening of His $H^{\epsilon 1}$ resonances and the lessened susceptibility to irreversible thermal denaturation of some nucleases (V23F and F76V+H124L) at low concentrations.

The following sample conditions were used for the NMR studies: wt, 0.85 mM protein, pH* 5.31; I18M, 0.7 mM protein, pH* 5.42; V23F, 0.2 mM protein, pH* 5.35; H46Y, 0.5 mM protein, pH* 5.38; G79S, 1.0 mM protein, pH* 5.28; F76V+H124L, 0.3 mM protein, pH* 5.21; L89F, 0.5 mM protein, pH* 5.38; H124L, 0.5 mM protein, pH* 5.20. All pH* values were measured at room temperature.

NMR Spectroscopy. Data were collected on Bruker AM-500 spectrometers operating at 500 MHz, except that, for nuclease wt, data were collected on a Bruker AM-600 spec-

trometer operating at 600 MHz. Free induction decays (FIDs) were digitized into 8K data point arrays. The spectral width was 12 ppm. A presaturation pulse sequence was used to suppress the residual water peak. A 90° pulse angle (typically 8–12 μs) was used, with a delay of 2.6 s between pulses. Because of the low protein concentrations used in this study, between 512 and 2048 averaged transients were required to obtain satisfactory spectra. The temperature for each experiment was checked against an external 80% ethylene glycol/DMSO standard (obtained from Bruker instruments).

Quantitation of Peak Areas. The conformational substates of nuclease are in slow exchange on the NMR time scale. In order to determine the equilibrium concentrations of the four nuclease forms, the areas of their associated His $H^{\epsilon 1}$ resonances were measured. The $H^{\epsilon 1}$ resonances of nuclease wt have been assigned by comparison to mutants lacking one of the four His residues (Alexandrescu et al., 1988). His pK_a values as well as the chemical shifts of the $H^{\epsilon 1}$ resonances in folded forms vary little between nuclease wt and the nuclease mutants used for this study; this makes it possible to identify the $H^{\epsilon 1}$ resonances in mutants by comparison to nuclease wt (Alexandrescu et al., 1988, 1989).

Areas of resonances were calculated from computer simulations of line widths and peak heights by means of the GLINFIT program (A. D. Bain, Bruker User Group software). The GLINFIT program allows the user to simulate a portion of the experimental spectrum. A difference spectrum and an rms error are computed based on the agreement of the positions, heights, and widths of resonances in the simulated and experimental spectra. The areas of the $H^{\epsilon 1}$ resonances from His^{121} were used to determine K_1 (N'/N), except for G79S where His^8 was used. The value for K_{TS} $[(U + U^*)/(N + N')]$ was determined by dividing the total areas of His $H^{\epsilon 1}$ resonances from the unfolded species ($U + U^*$) by the total areas of $H^{\epsilon 1}$ resonances from the folded species ($N + N'$). FIDs were transformed without resolution enhancement or with a line broadening of 1 Hz. In the case of nuclease G79S, it was necessary to multiply FIDs by a $\pi/10$ shifted since bell function to resolve the His^8 N and N′ resonances. To measure K_{TS} values, spectra were phased in the His $H^{\epsilon 1}$ region. The base line for the His $H^{\epsilon 1}$ region was corrected by fitting points from open regions to either side of the peaks of interest to a spline function. To measure K_1 values, spectra were rephased for the His^{121} $H^{\epsilon 1}$ resonances, and a new base-line correction was applied. Lorentzian line shapes were used to simulate spectra transformed without resolution enhancement; Gaussian line shapes were used to simulate spectra transformed with resolution enhancement. Three factors were considered in fitting each resonance: (1) the superposition of simulated and experimental spectra, (2) the minimization of features in the difference spectrum generated by the GLINFIT program, and (3) the minimization of the rms error for the fit.

Quantitation of K_1 and K_{TS} Values as a Function of Temperature. Between 17 and 21 temperature points were collected for each protein. Points near T_m were collected at 1-deg intervals. Increasing and decreasing temperatures were interleaved to check the reversibility of the transitions. The N $\rightleftarrows$ N′ and folding $\rightleftarrows$ unfolding transitions were found to be completely reversible. ΔH values were determined from plots of ln K vs $1/T$ by using the van't Hoff formalism:

$$d(\ln K)/d(1/T) = -\Delta H/R \qquad (1)$$

Only a subset of the temperature data were used in the determination of ΔH_1 values. Data could not be analyzed at low temperatures because line broadening leads to overlap of $H^{\epsilon 1}$ resonances from the N and N′ forms; accurate areas could not be determined at high temperatures, where the proteins were mostly unfolded, because peaks from N and N′ became vanishingly small. For all nucleases, plots of ln K_1 vs $1/T$ were linear throughout the entire temperature range studied.

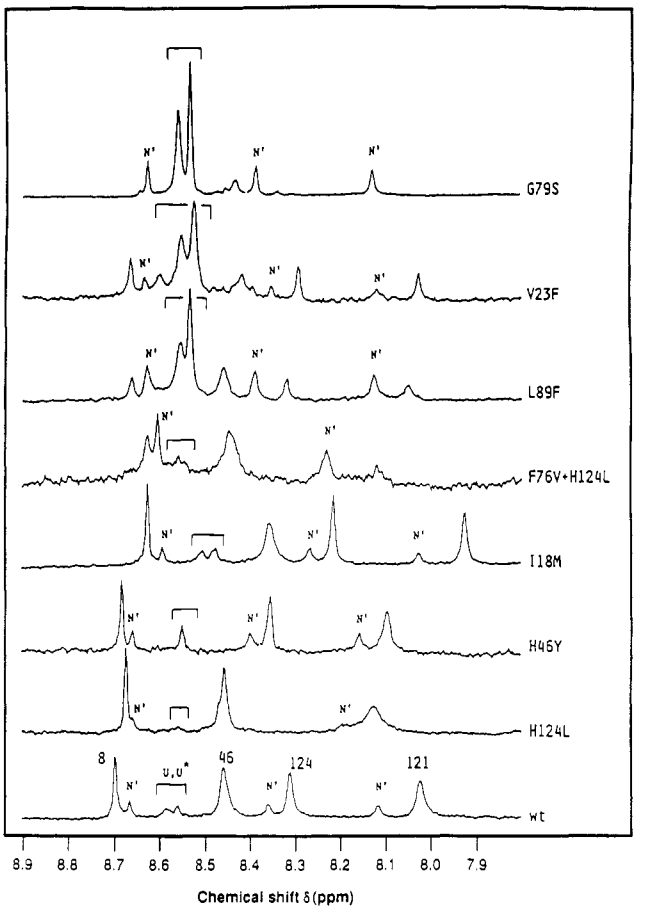


FIGURE 2: Histidine $H^{\epsilon 1}$ region from ^1H NMR spectra of nuclease wt and nucleases G79S, V23F, L89F, I18M, F76V+H124L, H46Y, and H124L a temperature of 313 K. In each spectrum, the order (from left to right) of $H^{\epsilon 1}$ resonances corresponding to the N conformational substate is as follows: His^8, His^{46}, His^{124}, His^{121}. Nuclease H124L and nuclease F76V+H124L lack His^{124}; nuclease H46Y is missing His^{46}. Positions of N′ resonances are indicated. The brackets are used to denote resonances from unfolded nuclease (U and U* forms).

Results and Discussion

Influence of Amino Acid Sequence on the Distribution of Nuclease Forms at 313 K. Effects of mutations on the conformational forms of nuclease are shown in Figure 2; all spectra were acquired at 313 K. The resonances from the N form are labeled by residue numbers (spectrum of nuclease wt); the resonances from the N′ form are indicated in each spectrum. The brackets enclose peaks arising from unfolded nuclease (U and U* forms). The N and N′ forms can be distinguished by their dependence on inhibitor (pdTp) binding. The N′ form resonances decrease in intensity and the N form resonances increase in intensity as pdTp is added (Figure 3). Note in the right panel that signals from both N and N′ shift when pdTp is added. This implies that both forms bind pdTp. The decrease of the N′/N ratio as pdTp is added indicates that the N form has a higher affinity for the inhibitor than the N′ form. By contrast with nuclease wt, a much higher concentration of pdTp is required to effect the N′ → N transition with nuclease G79S. The N′ form in nuclease wt is essentially eliminated at (nuclease wt):pdTp ratios above 1:1 (Alexandrescu et al., 1989).

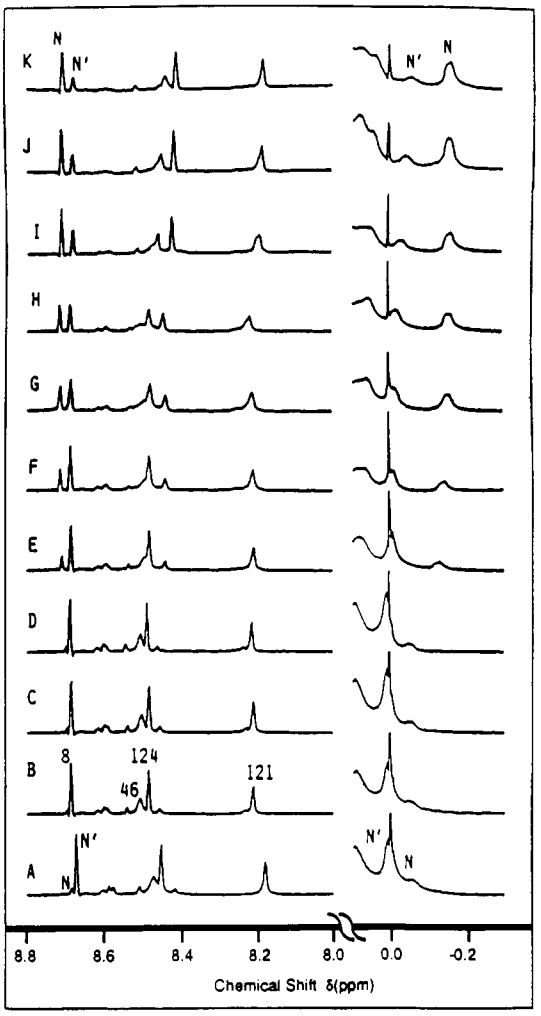
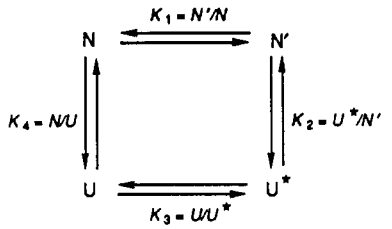


FIGURE 3: Effects of added Ca^{2+} and pdTp on the ^1H NMR (500-MHz) spectrum of nuclease G79S. Left panel, histidine $H^{\epsilon 1}$ region; right panel, resonances from C^{γ} methyl protons of Val^{74} (Wilde et al., 1988). Resonances from His^{121} in the N and N′ forms were unresolved at this pH but can be separated near pH 5.5. The sharp resonance at 0 ppm is from the chemical shift standard TSP. Conditions were as follows: 2 mM nuclease G79S, pH* 5.28, 301 K. (A) 0 Ca^{2+}, 0 pdTp. (B) 1:1 Ca^{2+}, 0 pdTp. (C) 3:1 Ca^{2+}, 0 pdTp. (D) 6:1 Ca^{2+}, 0 pdTp. (E) 6:1 Ca^{2+}, 0.5:1 pdTp. (F) 6:1 Ca^{2+}, 1:1 pdTp. (G) 6:1 Ca^{2+}, 2:1 pdTp. (H) 6:1 Ca^{2+}, 3:1 pdTp. (I) 8:1 Ca^{2+}, 4:1 pdTp. (J) 12:1 Ca^{2+}, 6:1 pdTp. (K) 20:1 Ca^{2+}, 10:1 pdTp.

The two folded (N and N′) and the two unfolded (U and U*) substates of nuclease are linked by the equilibria:



(2)

In order to determine the four equilibrium constants K_1, K_2, K_3, and K_4, three independent variables were measured: K_1, K_3, and K_{TS}.

(1) The K_1 equilibrium constant is readily measurable from the ratio of the areas of N and N′ His^{121} $H^{\epsilon 1}$ resonances.

(2) The K_3 equilibrium constant is not easily measurable [see Evans et al. (1989) and Alexandrescu (1989; Figure 3.3.1)]. Separate $H^{\epsilon 1}$ resonances from His^{121} in the U and U* forms only can be observed under a limited set of conditions. The resolution of the U and U* resonances is dependent on pH, temperature, and the nuclease variant. The intensity of the U resonance is a small fraction (4%) of that of the U* resonance; hence, accurate measurements of U/U* can be obtained only when the amount of unfolded protein is appreciable (e.g., near T_m). Owing to its low intensity, the identity of the U resonance had to be verified by magnetization-transfer experiments. Because of these considerations, K_3 was measured only for nuclease H124L at pH* 5.5 at a temperature of 326 K. A value of $K_3 = 0.044 \pm 0.004$ was used (Alexandrescu, 1989).[2]

In the treatment that follows we have used the following two assumptions: (*i*) *Unfolded nuclease was assumed to behave as a random coil with respect to cis/trans isomerization of the* Lys^{116}–Pro^{117} *peptide bond.* In a random coil, substitutions far from Pro^{117} should not affect the position of the U/U* equilibrium; thus all the mutations considered here are assumed to have the same U/U* ratio (the H124L substitution, which is seven positions away from Pro^{117}, is the mutation closest in the sequence to Pro^{117}). This assumption will be tested by future NMR studies of isotopically labeled nuclease mutants in the denatured state. (*ii*) *The cis/trans equilibrium in unfolded nuclease was assumed to be independent of temperature.* This assumption is supported by the observation that the ΔH value for cis/trans isomerization of X–Pro peptide bonds in model peptides (the temperature dependence of $K_{cis/trans}$) is very small [5 kJ/mol (Torchia, 1972)] or zero (Madison & Schellman, 1970; Maia et al., 1971, Brandts et al., 1975; Evans et al., 1989). Thus a value of $K_3 = 0.044 \pm 0.004$ was used in all of the calculations of K_1, K_2, and K_4 values that follow.

(3) The K_{TS} (two-state) equilibrium is defined as

$$K_{TS} = (U + U^*)/(N + N') \quad (3)$$

It is the ratio of unfolded (U and U^*) to folded (N and N') forms of nuclease and represents an averaged equilibrium constant for the unfolding of the N and N′ forms.[3] K_{TS} was determined rather than K_2 or K_4 because of the following considerations: (i) Separate U and U* $H^{\epsilon 1}$ resonances can be resolved only for His^{121} and only under a limited range of conditions. (ii) His $H^{\epsilon 1}$ protons are moderately labile for exchange with deuterium. A sample of nuclease dissolved in ^2H_2O will exhibit different intensities for the $H^{\epsilon 1}$ resonances from His^8, His^{46}, His^{121}, and His^{124}, depending on the history of the sample in ^2H_2O, the environment of the His residues in nuclease, and the rate of $^1H \rightarrow {}^2H$ exchange. There is no simple way to decompose the poorly resolved group of U* $H^{\epsilon 1}$ resonances into contributions from individual His residues (e.g., His^8, His^{46}, His^{124}, His^{121}), since these may contribute different intensities.

Plots of ln K_{TS} vs $1/T$ displayed an upward curvature at low temperatures for all the nucleases studied. This curvature is due to the large positive heat capacity (ΔC_p) for protein unfolding (Privalov & Khechinashvili, 1974). Because of the large heat capacity of protein unfolding, the dependence of ΔG on temperature is given by

$$\Delta G_{TS} = \Delta H^\circ_{TS} - T\Delta S^\circ_{TS} + \Delta C_p[T - T^\circ - T \ln (T/T^\circ)] \quad (4)$$

[2] Evans et al. (1989) have reported $K_3 = 0.053$ for nuclease wt at 322 K. In nuclease wt, however, the unfolded $H^{\epsilon 1}$ resonance of His^{124} overlaps the U form $H^{\epsilon 1}$ resonance of His^{121}. Since the $H^{\epsilon 1}$ resonance of His^{124} is missing in nuclease H124L, the U/U* value determined for nuclease H124L is likely to be more accurate than that for nuclease wt and is used here.

[3] If unfolding of nuclease is monitored by an experimental technique that does not distinguish between different folded and unfolded forms of the protein, the transition will appear to obey two-state thermodynamics (only one folded and one unfolded state). For a given set of conditions, the equilibrium constant for unfolding is given by K_{TS} = unfolded/folded.

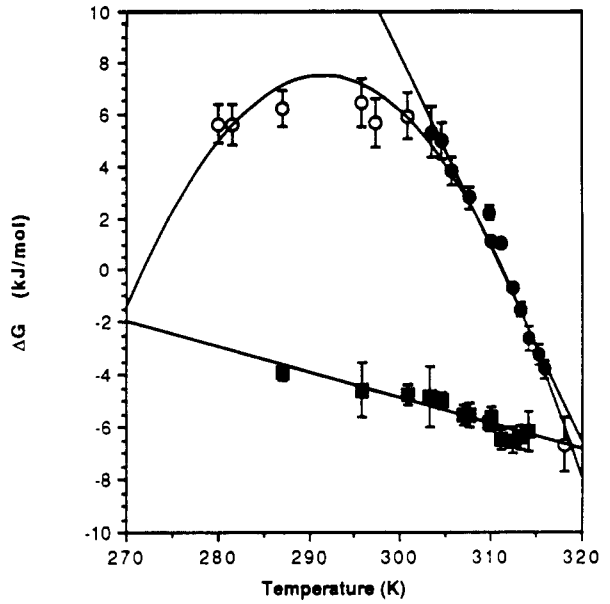


FIGURE 4: Illustration of the procedure used to determine ΔH_1 and ΔH_{TS} for nuclease G79S. (○) Complete stability curve data set fitted to $y = -1600 + 11x - (1.9 \times 10^{-2})x^2$, $r = 0.99$. (●) Limited linear portion of the stability curve data used for the determination of T_m, ΔH_{TS}, and ΔS_{TS}. Equation: $y = 230 - 0.74x$, $r = -0.99$. (■) ΔG_1 vs T data used to determine ΔH_1. Equation: $y = 24 - (9.4 \times 10^{-2})x$, $r = -0.94$. Uncertainties in the ΔG values are indicated by error bars.

ΔG_{TS} has a bell-shaped dependence on temperature: the "stability curve" (Becktel & Schellman, 1987). The second derivative of ΔG with respect to temperature (the curvature) is given by $\partial^2 \Delta G_{TS}/\partial T^2 = -\Delta C_p/T$. It is possible, in principle, to determine ΔC_p by fitting denaturation curves to second-order polynomials ($y = ax^2 + bx + c$) and obtaining solutions for $\partial^2 \Delta G_{TS}/\partial T^2 = -\Delta C_p/T$. In practice this treatment produces unacceptable errors for ΔC_p: (1) The K_{TS} values at low temperatures are small and subject to larger errors than those near T_m. (2) It is usually necessary to obtain K_{TS} values at temperatures below the freezing point of ^2H_2O to ascertain the full extent of the curvature of the stability curve.[4] Because of these considerations, only the limited linear subsets of stability curve data near T_m ($0.1 \leq K_{TS} \leq 10$) were used for the determination of ΔH_{TS} values by van't Hoff analysis (Becktel & Schellman, 1987) (see Figure 4).

If K_1 and K_3 are known, K_{TS} can be decomposed into separate K_2 and K_4 contributions:

$$\frac{1}{K_{TS}} = \frac{K_4 K_3 (1 + K_1)}{K_3 + 1} \tag{5}$$

From eq 2

$$K_1 K_2 K_3 K_4 = 1 \tag{6}$$

It follows that K_{TS} can also be expressed as

$$\frac{1}{K_{TS}} = \frac{(1/K_2)(1 + 1/K_1)}{K_3 + 1} \tag{7}$$

or

$$\frac{1}{K_{TS}} = \frac{K_4 K_3 + 1/K_2}{K_3 + 1} \tag{8}$$

[4] In practice, ΔC_p is determined by varying pH and obtaining ΔH values as a function of T_m. ΔC_p is then calculated from the slope of ΔH vs T_m (Becktel & Schellman, 1987). The measurement of ΔC_p for each mutant would have been excessively demanding of spectrometer time (since accurate data can be obtained only at low protein concentrations) and impractical at pH values where the His $H^{\epsilon 1}$ resonances are not well resolved.

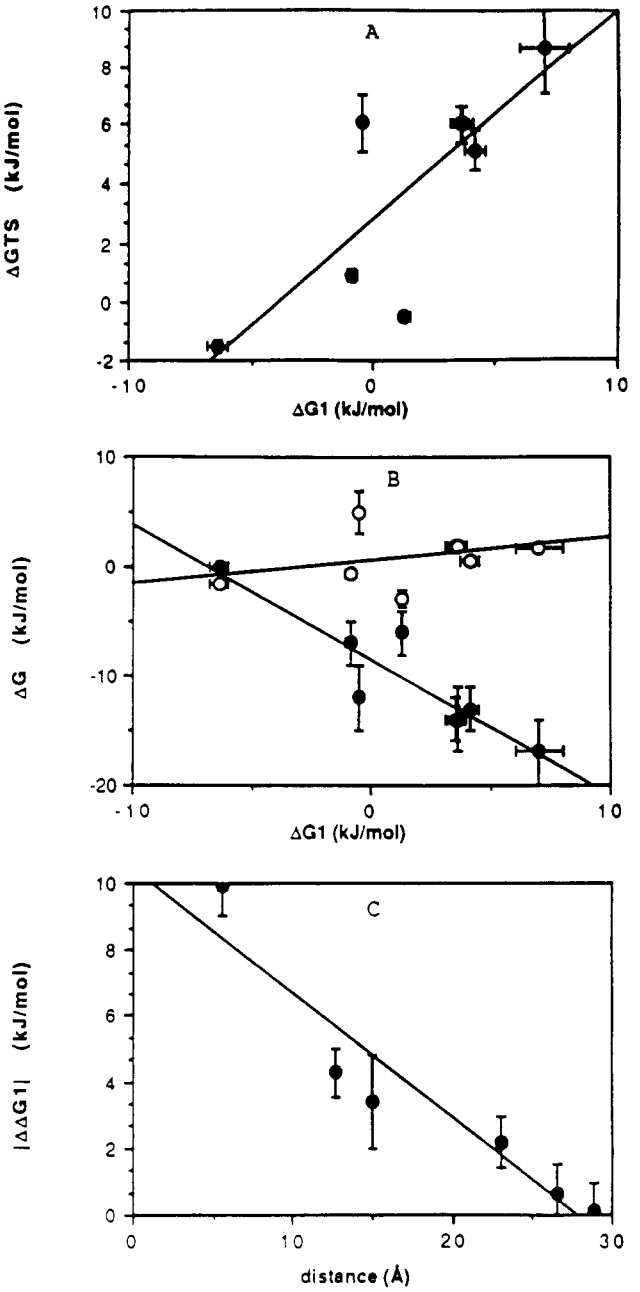


FIGURE 5: Correlation plots showing empirical relationships between various thermodynamic parameters from Table I (see eq 2). Each point on the scatter diagram represents the parameters of one of the mutants studied. Error bars represent uncertainties in ΔG values. (A) Plot of ΔG_{TS} vs ΔG_1. Equation: $y = 2.8 + 0.72x$, $r = 0.80$. (B) (○) Plot of ΔG_2 vs ΔG_1. Equation: $y = 0.48 + 0.20x$, $r = 0.34$. (●) Plot of ΔG_4 vs ΔG_1. Equation: $y = -8.6 - 1.2x$, $r = -0.91$. (C) Plot of $|\Delta\Delta G_1|$ vs C^α–C^α distance of substituted residue from Pro^{117}. Equation: $y = 10 - 0.38x$, $r = -0.95$. Nuclease F76V+H124L (a double mutant) was excluded from the analysis in (C).

Free energy values for the K_{TS}, K_1, K_2, K_3, and K_4 equilibria may be calculated from

$$\Delta G = -RT \ln K \tag{9}$$

The effects of amino acid sequence on the position of the four equilibria of eq 2 at a temperature of 313 K are summarized in Table I. We noted a weak positive empirical correlation (Figure 5A) between the position of the N′/N equilibrium (ΔG_1) and the averaged stability to thermal denaturation (ΔG_{TS}). When ΔG_{TS} was decomposed into separate ΔG_4 and ΔG_2 contributions, a strong negative correlation was observed between ΔG_1 and ΔG_4. The ΔG_2 parameter, however, was found to be nearly independent of ΔG_1 (Figure 5B).

Table I: Effects of Nuclease Sequence on Equilibria Linking Two Folded (N and N′) and Two Unfolded (U and U*) Substates of Nuclease at a Temperature of 313 K[a]

	wt	G79S	L89F	F76V+H124L	V23F	H46Y	I18M	H124L
K_{TS}	0.10 ± 0.01	1.8 ± 0.3	0.7 ± 0.2	0.10 ± 0.02	1.2 ± 0.1	0.10 ± 0.01	0.14 ± 0.02	0.037 ± 0.007
K_1	0.26 ± 0.03	11.8 ± 0.8	1.4 ± 0.3	1.2 ± 0.2	0.6 ± 0.1	0.25 ± 0.03	0.20 ± 0.02	0.07 ± 0.01
K_2	0.46 ± 0.07	1.9 ± 0.3	1.2 ± 0.4	0.17 ± 0.06	3.1 ± 0.8	0.48 ± 0.08	0.8 ± 0.1	0.5 ± 0.1
K_3	0.044 ± 0.004	0.044 ± 0.004	0.044 ± 0.004	0.044 ± 0.004	0.044 ± 0.004	0.044 ± 0.004	0.044 ± 0.004	0.044 ± 0.004
K_4	190 ± 30	1.0 ± 0.2	14 ± 4	110 ± 30	12 ± 2	200 ± 30	140 ± 30	600 ± 100
ΔG_{TS}	6.0 ± 0.6	−1.5 ± 0.2	0.9 ± 0.2	6 ± 1	−0.47 ± 0.04	6.0 ± 0.6	5.1 ± 0.7	8.6 ± 1.6
ΔG_1	3.5 ± 0.4	−6.4 ± 0.4	−0.9 ± 0.2	−0.5 ± 0.1	1.3 ± 0.2	3.6 ± 0.4	4.1 ± 0.4	7 ± 1
ΔG_2	2.0 ± 0.3	−1.6 ± 0.3	−0.5 ± 0.2	5 ± 2	−2.9 ± 0.8	1.9 ± 0.3	0.6 ± 0.1	1.8 ± 0.4
ΔG_3	8.1 ± 0.7	8.1 ± 0.7	8.1 ± 0.7	8.1 ± 0.7	8.1 ± 0.7	8.1 ± 0.7	8.1 ± 0.7	8.1 ± 0.7
ΔG_4	−14 ± 2.0	−0.08 ± 0.02	−7 ± 2	−12 ± 3	−6 ± 2	−14 ± 3	−13 ± 2	−17 ± 3
d[b]		5.5	12.7	11.5	23.0	28.9	26.5	15.0

[a] Conditions are given under Experimental Procedures. ΔG values are in units of kJ/mol; d is in units of Å. The ranges for the values reported were determined from least-squares fits of upper and lower rms error bounds on the integrals of peaks simulated with the GLINFIT program. K_1 and K_{TS} were determined experimentally; K_2 and K_4 were determined from eqs 7 and 5, respectively, by using the assumption that K_3 is independent of temperature and constant for all mutants considered here. ΔG values were calculated from $\Delta G = -RT \ln K$. [b] Distances are those between the C^α of the mutated residue and the C^α of Pro^{117}. The distance given for F76V+H124L is that between the C^α of Phe^{76} and the C^α of Pro^{117}.

These results suggest the following mechanistic hypothesis. The N → N′ transition involves the disruption of intramolecular interactions, and amino acid substitutions that promote the N → N′ transition destabilize these interactions. In the N′ state, these intramolecular interactions are removed, and denaturation by the N′ → U* pathway is independent of them (no correlation observed between ΔG_1 and ΔG_2). On the other hand, amino acid substitutions that promote the N′ → N transition stabilize intramolecular interactions that must be broken in the N → U unfolding mechanism (negative correlation observed between ΔG_1 and ΔG_4).

As to the nature of the interactions stabilizing the N form: (1) Mutations throughout nuclease affect the position of the N′/N equilibrium. (2) The position of the N′/N equilibrium correlates with the global stability of the N form. These observations suggest that the interactions stabilizing the N form are distributed and interdependent. This result may be a reflection of the high cooperativity of protein folding interactions.

Figure 5C shows that a negative correlation exists between the absolute values of $\Delta G_{1,wt} - \Delta G_{1,mutant}$ ($|\Delta\Delta G_1|$) and the distance in angstroms between the C^α of the substituted residue and the C^α of Pro^{117}. Absolute values of $\Delta\Delta G_1$ were used in this plot, because some substitutions (e.g., nuclease H124L) shift the position of the N′/N equilibrium toward the N form. Nuclease F76V+H124L was excluded from this analysis because it depends on the cumulative effects of two mutations. It should be noted, however, that while the ΔG_4 values of nuclease wt and F76V+H124L are similar, their ΔG_1 values differ by 4 kJ/mol. This suggests that the F76V substitution (11.5 Å away from Pro^{117}) has a disproportionately large effect on ΔG_1. The correlation observed in Figure 5C suggests that the position of the N′/N equilibrium is especially sensitive to mutations spatially near Pro^{117}. In particular, the substitutions that lead to the lowest ΔG_1 values (G79S, L89F, F76V) involve residues in a loop (75–91) that is adjacent to the loop (110–118) that contains Pro^{117} (Figure 1). Binding of the nuclease inhibitor pdTp, which binds roughly at the bottom of the crevice created by the two loops, strongly favors the N form (Figure 3). Evans et al. (1989) have suggested that the preference for the N conformation in the presence of pdTp could be transmitted through Tyr^{115}.[5] This mechanism could also explain the effects of the mutations in the 75–91 loop on K_1. For example, these mutations may increase the separation between the 75–91 loop and the 110–118 loop. This could increase the space accessible to Tyr^{115} and favor the trans conformation of Lys^{116}–Pro^{117}.

The correlation between $|\Delta\Delta G_1|$ and the distance from Pro^{117} might seem at odds with the conclusion that the interactions stabilizing the N form are distributed throughout the molecule. The distances spanned by these mutations, however, are quite large. For example, the distance between the C^α of Pro^{117} and the C^α of Leu^{89} is 13 Å, a significant fraction of the longest dimension of nuclease [about 35 Å (Cotton et al., 1979)]. Thus the effects of mutations more than 10 Å away on the conformation of Pro^{117} are by no means direct. Such substitutions are likely to involve significant changes in the environments of residues throughout the protein. This probably accounts for the correlation observed between ΔG_1 and ΔG_4.

Temperature Dependence of K_1 and K_{TS}. Thermodynamic values are determined most efficiently and accurately by calorimetry. A number of calorimetric studies of nuclease have been published (Griko et al., 1988; Calderon et al., 1985; Shortle et al., 1988). Griko et al. (1988) and Shortle et al. (1988) found that the ratio of calorimetric to van't Hoff enthalpy for nuclease wt is close to 1. This type of behavior is indicative of the presence of only two macroscopic states of the protein in the unfolding transition: native and denatured (Privalov & Khechinashvili, 1974). Thus the calorimetric method appears not to detect the heterogeneity of the native and denatured forms. The calorimetric trace is typically recorded at 0.5–1 K/min (Griko et al., 1988; Calderon et al., 1985; Shortle et al., 1988), while the half-times of N ⇄ N′ interconversion are on the order of 1 s (Evans et al., 1989). Thus the heat absorbed at any temperture is likely to reflect a population-weighted average of the N → N′, N → U, and N′ → U* transitions. NMR studies of nuclease wt (Evans et al., 1989; Alexandrescu et al., 1989) have shown that the protein goes from N to N′ upon heating.

The temperature dependence of the N, N′, and unfolded resonances for the nucleases discussed above is illustrated in Figures 6 and 7. The temperature dependence of K_2 and K_4 (Table II) was determined by van't Hoff analysis using K_2 and K_4 values calculated from eqs 7 and 5. Only the limited subset of temperature data where both K_1 and K_{TS} values could be determined and K_{TS} was linear with temperature were used to determine ΔH_2 and ΔH_4.

As the temperature is increased, the amount of unfolded nuclease, and consequently K_{TS}, increases. K_2 (U^*/N') increases with temperature, and K_4 (N/U) decreases with temperature. Because unfolding transitions have a large ΔC_p value (Brandts, 1964; Shiao et al., 1971; Privalov & Khechinashvili,

[5] Their argument was based on the observation (Tucker et al., 1979) that Tyr^{115} partially occupies the pdTp binding pocket in uncomplexed nuclease.

Table II: Thermodynamic Parameters for Equilibria Linking the Folded (N and N′) and Unfolded (U and U*) Substates of Nuclease[a]

	wt	G79S	L89F	F76V+H124L	V23F	H46Y	I18M	H124L
T_m	320.8 ± 0.0	311.3 ± 0.1	313.3 ± 0.1	320.0 ± 0.1	310.6 ± 0.0	320.2 ± 0.1	318.7 ± 0.1	329.2 ± 0.1
ΔH_{TS}	260 ± 30	230 ± 30	180 ± 30	220 ± 40	120 ± 20	290 ± 40	250 ± 30	360 ± 50
ΔS_{TS}	0.81 ± 0.08	0.7 ± 0.1	0.58 ± 0.09	0.7 ± 0.1	0.39 ± 0.06	0.91 ± 0.14	0.78 ± 0.09	1.1 ± 0.2
ΔH_1	54 ± 2	22 ± 1	28 ± 7	40 ± 10	50 ± 10	60 ± 10	45 ± 5	80 ± 20
ΔS_1	0.16 ± 0.01	0.091 ± 0.005	0.09 ± 0.02	0.13 ± 0.03	0.16 ± 0.03	0.18 ± 0.03	0.13 ± 0.02	0.23 ± 0.07
ΔH_2	210 ± 60	200 ± 50	140 ± 40	180 ± 90	60 ± 30	210 ± 70	220 ± 80	240 ± 80
ΔH_3	0	0	0	0	0	0	0	0
ΔS_3	−0.026 ± 0.002	−0.026 ± 0.002	−0.026 ± 0.002	−0.026 ± 0.002	−0.026 ± 0.002	−0.026 ± 0.002	−0.026 ± 0.002	−0.026 ± 0.002
ΔH_4	−280 ± 30	−230 ± 50	−180 ± 30	−240 ± 30	−130 ± 20	−280 ± 60	−250 ± 40	−370 ± 60

[a] The ΔH and ΔS values are in units of kJ/mol; T_m is in units of K. The ΔH_{TS} and ΔS_{TS} values are given at T_M. The ΔS_1 values were calculated from $\Delta G_1 = \Delta H_1 - T\Delta S_1$, for $T = 313$ K. The ΔS_3 values were calculated from $\Delta G_1 = \Delta H_1 - T\Delta S_1$, for $T = 313$ K, by using $\Delta G_3 = 8.1 \pm 0.7$ kJ/mol and $\Delta H_3 = 0$ kJ/mol. The ΔH_1 and ΔH_{TS} values were determined experimentally; ΔH_3 was assumed to be zero (Brandts et al., 1975); ΔH_2 and ΔH_4 values were determined from plots of ln K_2 (calculated from eq 7) and ln K_4 (calculated from eq 5) vs $1/T$. (See text for details.)

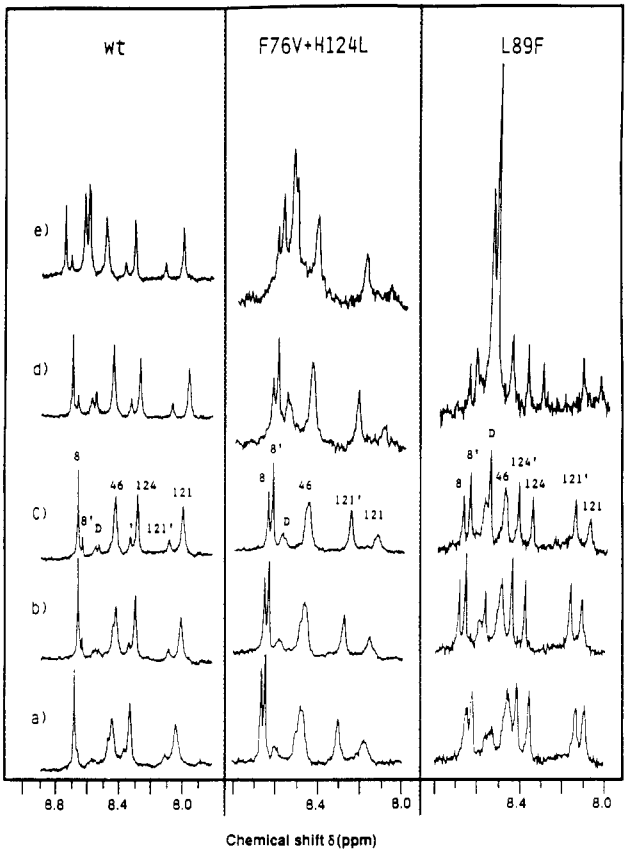


FIGURE 6: Selected points from the temperature titrations of nucleases wt, L89F, and F7lV+H124L. Parameters for each spectrum are reported as follows: temperature, K_{TS}, K_1. Nuclease wt: (a) 301 K, 0.08 ± 0.01, 0.12 ± 0.02; (b) 305 K, 0.043 ± 0.005, 0.18 ± 0.01; (c) 310 K, 0.045 ± 0.006, 0.17 ± 0.02; (d) 315 K, 0.16 ± 0.02, 0.26 ± 0.02; (e) 319 K, 0.53 ± 0.08, 0.42 ± 0.07. Nuclease F76V+H124L: (a) 301 K, 0.10 ± 0.01, 0.9 ± 0.1; (b) 308 K, 0.16 ± 0.03, 1.2 ± 0.2; (c) 310 K, 0.11 ± 0.02, 1.2 ± 0.2; (d) 315 K, 0.32 ± 0.04, 1.7 ± 0.4; (e) 320 K, 0.8 ± 0.1, 2.6 ± 1.0. Nuclease L89F: (a) 301 K, 0.11 ± 0.02, 0.9 ± 0.2; (b) 305 K, 0.20 ± 0.03, 1.0 ± 0.1; (c) 310 K, 0.40 ± 0.08, 1.5 ± 0.3; (d) 315 K, 1.4 ± 0.2, 1.3 ± 0.3.

1974), ΔH_{TS} and ΔS_{TS} are temperature dependent and the temperature dependence of ΔG_{TS} is given by eq 4 rather than by $\Delta G = \Delta H - T\Delta S$ (Becktel & Schellman, 1987). Similarly, since both N → U and N′ → U* are unfolding transitions, ΔH_2 and ΔH_4 are expected to be strongly dependent on temperature. In the absence of ΔC_p data, ΔH and ΔS for the unfolding transitions can only be compared directly for nucleases that share similar T_m values (e.g., nucleases G79S, V23F, and L89F; and nucleases I18M, H46Y, F76V+H124L, and wt).

The value of K_1 was found to increase linearly with temperature for all nucleases considered here (Figures 6 and 7).

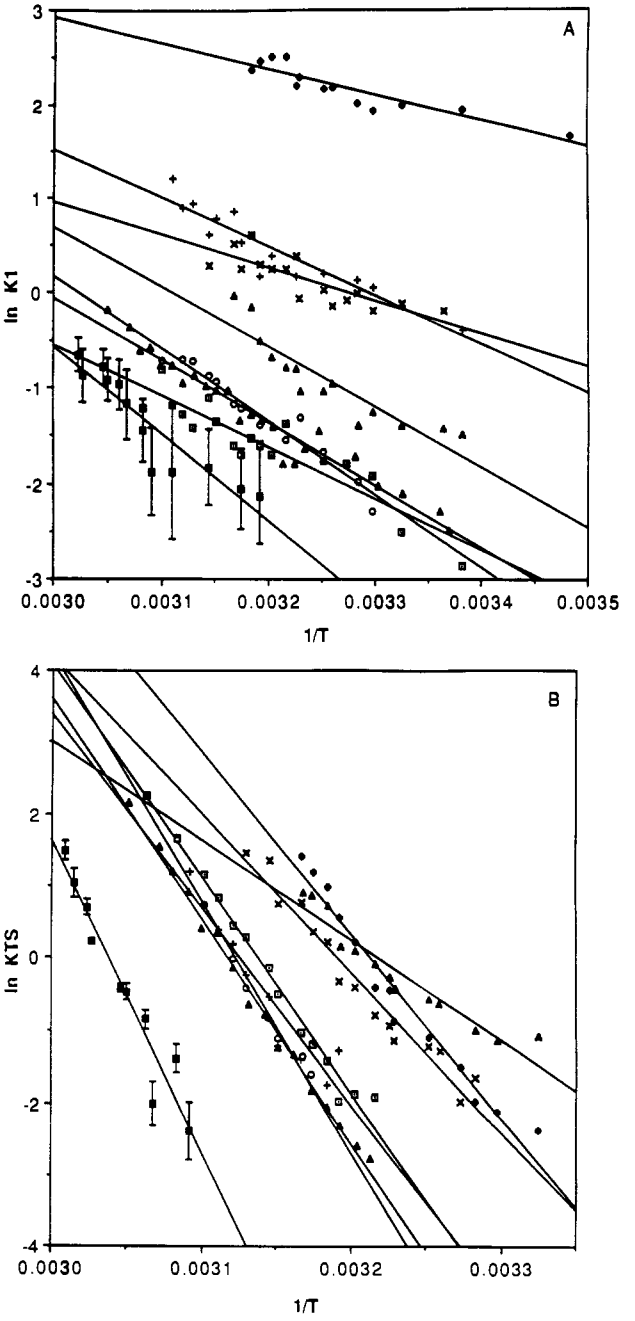


FIGURE 7: Thermodynamic (van't Hoff) plots for (A) the K_1 equilibrium and (B) the K_{TS} equilibrium. Symbols: (△) wt; (□) I18M; (▲) V23F; (○) H46Y; (♦) G79S; (×) L89F; (+) F76V+H124L; (■) H124L. Error bars are shown only for H124L; errors for the other mutants were of comparable magnitude. Thermodynamic parameters derived from these data and their associated uncertainties are given in Tables II and III.

Table III: Enthalpic and Entropic Contributions to the Stabilization of the Cis Folded Form (N) at a Temperature of 313 K[a]

	wt	G79S	L89F	F76V+H124L	V23F	H46Y	I18M	H124L
$\Delta\Delta G_{conf}$	12 ± 1	1.7 ± 1.1	7.2 ± 0.9	7.6 ± 0.8	9.4 ± 0.9	12 ± 1	12 ± 1	15 ± 2
$\Delta\Delta H_{conf}$	54 ± 2	22 ± 1	28 ± 7	40 ± 10	50 ± 10	60 ± 10	45 ± 5	80 ± 20
$\Delta\Delta S_{conf}$	0.14 ± 0.010	0.066 ± 0.008	0.07 ± 0.03	0.11 ± 0.04	0.14 ± 0.04	0.17 ± 0.05	0.10 ± 0.02	0.20 ± 0.05

[a] $\Delta\Delta G_{conf} = \Delta G_1 + \Delta G_3$; $\Delta\Delta H_{conf} = \Delta H_1 + \Delta H_3$; $\Delta\Delta S_{conf} = \Delta S_1 + \Delta S_3$. Thermodynamic parameters were calculated from K_1 and K_3 equilibria rather than from K_2 and K_4. The errors for K_2 and K_4 values are large because of the propagation of errors from the separately measured quantities in eqs 5 and 7. The errors for K_1 values are smaller, by comparison, because they were measured directly.

Table IV: Kinetic Parameters for the Enzymatic Activities of Nuclease Mutants

	wt	I18M	V23F	H46Y	G79S	L89F	H124L	F76V+-H124L
				Hydrolysis of PNPdT[a]				
K_m (mM)	4.4 ± 0.8	8.8 ± 2.2	7.4 ± 1.0	4.4 ± 0.6	14 ± 4	10 ± 3	5.6 ± 1.5	2.9 ± 0.7
k_{cat} (min^{-1})	2.1 ± 0.1	2.3 ± 0.2	18.± 0.1	0.59 ± 0.02	2.0 ± 0.3	0.020 ± 0.003	0.84 ± 0.09	1.02 ± 0.07
k_{cat}/K_m (M^{-1} min^{-1})	480 ± 80	260 ± 70	240 ± 40	130 ± 20	140 ± 40	2.0 ± 0.7	150 ± 40	350 ± 90
wt/mutant (k_{cat}/K_m)		1.8 ± 0.6	2.0 ± 0.6	3.7 ± 1.0	3.4 ± 1.2	240 ± 100	3.2 ± 1.1	1.4 ± 0.4
				Hydrolysis of Single-Stranded DNA[b]				
K_m (μg)	6.0 ± 2.6	9.0 ± 3.3	5.2 ± 2.0	4.0 ± 2.2	5.8 ± 3.1	30 ± 10	9.0 ± 3.3	3.2 ± 2.0
V_m [ΔA/(mg·min^{-1})]	2200 ± 300	3000 ± 400	2300 ± 300	1200 ± 200	2000 ± 400	1200 ± 200	3000 ± 1000	1900 ± 300
V_m/K_m	370 ± 120	330 ± 80	440 ± 120	300 ± 120	340 ± 190	40 ± 6	330 ± 80	590 ± 300
wt/mutant (V_m/K_m)		1.1 ± 0.4	0.84 ± 0.36	1.2 ± 0.62	1.1 ± 0.7	9.2 ± 3.3	1.1 ± 0.4	0.63 ± 0.38

[a] Substrate: thymidine 5′-(*p*-nitrophenyl phosphate) (PNPDT) in an alkaline phosphatase coupled assay (Grissom & Markley, 1989). Values for the PNPdT activities of wt, F76V+H124L, L89F, and H124L were reported previously (Alexandrescu et al., 1989). [b] Substrate: heat-denatured single-stranded salmon sperm DNA (Cuatrecases et al., 1967a).

Thus, as has been found for nuclease wt (Evans et al., 1989), ΔH_1 (the enthalpy change for the N → N′ reaction) is positive for all nucleases studied (Table II). This indicates that the N → N′ transition involves the net breakage of noncovalent interactions, perhaps those that constrain the Lys^{116}–Pro^{117} peptide bond to the cis conformation. The cis Pro^{117} peptide conformation (form N) is thus favored over N′ because of its lower enthalpy. Also, as found for nuclease wt (Evans et al., 1989), ΔS_1 (the entropy change associated with the N → N′ transition) is positive for all nucleases studied (Table II). This indicates that the N′ form has greater motional freedom (more disorder) than the N form. The trans Pro^{117} peptide conformation (form N′) is thus favored over N because of its higher entropy. The observed differences in ΔG_1 (Table I), as a function of nuclease sequence, result from the subtle interplay of the entropic and enthalpic forces that contribute to the N → N′ reaction. For nuclease wt at 313 K, ΔH_1 (which favors the N form) is sufficiently large to compensate for ΔS_1 (which favors the N′ form), and the N form predominates. For nuclease G79S, the ΔH_1 contribution is too small to compensate for the favorable ΔS_1 contribution, and the majority of nuclease molecules exist in the N′ form.

Evans et al. (1989) have analyzed the differences in the cis/trans ratio of folded and unfolded nuclease wt, in terms of the conformational stabilization of the cis Pro^{117} afforded by the folded conformation of the protein. For example, the quantity $\Delta\Delta H_{conf}$ is the difference in the enthalpy for cis → trans isomerization in the folded (N, N′) and unfolded (U, U*) states. From eq 2 it also follows that $\Delta\Delta H_{conf}$ is also the difference between the enthalpies for denaturation of the N and N′ forms:

$$\Delta\Delta H_{conf} = \Delta H_1 + \Delta H_3 = -(\Delta H_2 + \Delta H_4) = \Delta H_{N\rightarrow N'} - \Delta H_{U\rightarrow U^*} = -(\Delta H_{N'\rightarrow U^*} - \Delta H_{N\rightarrow U}) \quad (10)$$

Similar relationships may be derived for $\Delta\Delta S_{conf}$ and $\Delta\Delta G_{conf}$ (Table III).

The N ⇄ N′ equilibrium involves a relatively minor structural rearrangement when compared to the complete loss of structure in an unfolding transition. In contrast to K_{TS}, K_1 shows a linear dependence on temperature over the entire temperature range studied (typically 20–30 K). This behavior suggests that the ΔC_p for the N → N′ transition is negligible. If our assumption that $\Delta H_3 = 0$ is correct (Brandts et al., 1975), it follows that $\Delta\Delta H_{conf}$ is independent of temperature. Thus while ΔH_2 and ΔH_4 are both likely to have a strong temperature dependence, their sum, $\Delta\Delta H_{conf}$, should be temperature independent. In principle, it should be possible to attribute the differences in $\Delta\Delta H_{conf}$ to effects of mutations on either the N or N′ forms (or both) by comparing the ΔH_2 and ΔH_4 values for different nucleases. In practice, (1) the errors of ΔH_2 and ΔH_4 are unacceptably large for this type of comparison (Table II), and (2) ΔH_2 and ΔH_4 are likely to be strongly temperature dependent and cannot be compared for mutants with different T_m values.

Influence of Amino Acid Sequence on the Enzymatic Activity of Nuclease. No correlation was observed between N′/N ratios (Table I) and steady-state kinetic parameters (Table IV). Of the mutations discussed in this report, only L89F affects activity. The loss of activity for L89F appears to be unrelated to the high proportion of the N′ form in this mutant. An examination of the crystal structure of the wild-type nuclease ternary complex shows that a C^δ methyl group of Leu^{89} is almost in van der Waals contact with the plane of the inhibitor pyrimidine ring. The substitution of a bulky phenylalanine is likely to interfere with substrate binding.[6] The 5-fold difference in K_m values with DNA, compared to the smaller 2-fold difference in K_m with PNPdT, may reflect poorer binding to L89F of the dA, dG, and dC moieties of the heterogeneous DNA substrate. The 2-fold decrease in V_{max} with DNA compared with the 100-fold decrease in k_{cat} with PNPdT could be due to the "stickiness" of the DNA substrate. Once DNA is bound to nuclease L89F, catalysis may be processive and mask differences between nuclease L89F and nuclease wt. Favorable contributions from an extended DNA binding site (Cuatrecasas et al., 1967b; Kraut, 1988) could

[6] A recent report by Corey and Shultz (1989) on the L89C mutant of nuclease showed that reaction of Cys^{89} with Hg^{2+} or Cu^{2+} leads to reduced catalysis, presumably as the result of steric hindrance.

also reduce deleterious effects of the L89F substitution at the active site.

Although large differences in the activities of these mutants have been reported (Shortle & Lin, 1985) based on the TBD–DNA agar plate assay (Lachica et al., 1971), this assay depends on both DNAse activity and the mobility of the protein through the agar gel. Proteolysis of nuclease by contaminating enzymes in the agar gel also may be a factor in the observed differences.

Mutations that cause large changes in the N′/N ratio produce no systematic changes in enzymatic activity (Table IV). This result can be interpreted in two alternative ways: (1) the N form, which is favored when pdTp binds, is the active form, and substrate binding, like pdTp binding, induces the transition from the inactive (or less active) N′ form to the active N form; (2) both the N and N′ forms have equivalent activity. Conclusion 1 appears unlikely on kinetic and thermodynamic grounds. Evans et al. (1989) obtained a rate constant of 1.0 s^{-1} for the N′ → N conversion of nuclease wt at 322 K. At the temperature of the nuclease assays (294 K), the rate of interconversion should be considerably slower. By comparison, Serpersu et al. (1987) estimated k_{cat} = 95 s^{-1} for the hydrolysis of DNA by nuclease wt at 297 K. Thus, the N′ → N conversion probably is too slow to show up in the initial rate of the reaction. If substrate binding altered the cis/trans ratio at Pro^{117} and converted a less active enzyme into a more active enzyme on the time scale of the initial rate measurement, one would expect to see a nonlinear reaction rate. This was not observed with the mutant nucleases. Furthermore, a very high concentration of pdTp (which binds much more tightly than any substrate) was required to bring about the conversion of N′ to N with nuclease G79S. Since $K_{1,\text{nuclease wt}}/K_{1,\text{nuclease G79S}} = 45$, a corresponding value would be expected for $K_{m,\text{nuclease G79S}}/K_{m,\text{nuclease wt}}$. However, the latter ratio is only 3.5 ± 1.5 (Table IV). The only result that seems at odds with interpretation 2 is the fact that the N form has a higher affinity for pdTp than the N′ form (Figure 3). To accommodate this result, one must assume that pdTp binds in a manner that is different from substrates and that this mode of binding is responsible for the differential affinity for N and N′. One important difference between pdTp and substrates is charge: pdTp has two additional negative charges when compared to a comparable unit of DNA substrate and three additional charges when compared to the PNPdT substrate.

If interpretation 2 is correct, then both N and N′ are folded enzyme species with equivalent enzymatic activity. It is interesting in this regard that nuclease P117G, which presumably does not accommodate a cis peptide bond, also retains full activity (Evans et al., 1987). It will be interesting to evaluate other properties of staphylococcal nuclease in terms of the N and N′ conformational substates: pre-steady-state kinetics, metal binding affinity, catalysis of RNA hydrolysis, stability to proteolysis, and protein structure and dynamics.

Conclusion

Although its physiological importance is uncertain, the presence of N ⇄ N′ conformational heterogeneity in nuclease has important implications for our understanding of the rules that govern protein folding. In the N form, Pro^{117} is located in a type VI reverse turn (Evans et al., 1989). A trans X–Pro peptide bond cannot be accommodated in this type of turn (Huber & Steigemann, 1974). The fact that changes in residues as distant in the sequence as Gly^{79} change the conformation of Pro^{117} from mostly cis to mostly trans illustrates the complexity of the amino acid code. The local turn conformation is coupled to the tertiary structure of the protein. It cannot be predicted simply by examining the sequence of the residues encompassing the turn (Kabsch & Sander, 1984).

The presence of multiple interconverting folded conformations of nuclease, and the effects of mutations on the distribution of these forms, illustrates that the amino acid code is both a structural and a dynamic code. The protein folding problem is thus not simply a matter of predicting protein structure from amino acid sequence; the flexibility inherent to the structure also must be taken into account. Nuclease wt can be considered to exist primarily in the N form, whereas nuclease G79S exists primarily in the N′ form. Nucleases F76V+H124L and L89F, however, have equal amounts of both forms. Because of the presence of conformational heterogeneity in nuclease, the determination of pure characteristics for the N or N′ forms (e.g., amide proton exchange rates) will be complicated by averaging whenever interconversion between substates is fast relative to the time scale of the measurement.

An important advantage of NMR spectroscopy is that proteins can be studied both in solution and in the solid state. It remains to be seen whether the N ⇄ N′ conformational heterogeneity is present only in solution, or if it also exists in the crystalline state. Solid-state NMR of isotopically enriched nuclease L89F or nuclease F76V+H124L may help to shed light on this critical question.

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Pulcherosine, a Novel Tyrosine-Derived, Trivalent Cross-Linking Amino Acid from the Fertilization Envelope of Sea Urchin Embryo†

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ABSTRACT: The normally hardened and aminotriazole-induced soft fertilization envelopes (FEs) of the sea urchin *Hemicentrotus pulcherrimus* and two other species were isolated and investigated for component proteins and cross-linking amino acids. From the acid hydrolysate of the hard FE of *H. pulcherrimus*, we isolated by reversed-phase high-performance liquid chromatography a novel fluorescent compound as well as dityrosine and trityrosine, the major tyrosine-derived cross-linking amino acids. These three compounds were also isolated from the reaction products of the tyrosine/horseradish peroxidase/H_2O_2 system. The structure of the novel compound, designated "pulcherosine", was determined to be 5-[4″-(2-carboxy-2-aminoethyl)phenoxy]-3,3′-dityrosine. With respect to the position of diphenyl ether bond between the tyrosine and dityrosine moieties, it is an isomer of isotrityrosine found in *Ascaris* cuticle collagen [Fujimoto et al. (1981) *Biochem. Biophys. Res. Commun. 99*, 637–643]. Isotrityrosine was not found in either of the above systems as a major component. The contents of tyrosine, dityrosine, trityrosine, and pulcherosine in the hard FE of *H. pulcherrimus* were estimated as 255, 5.5, 2.1, and 1.3 residues, respectively, per 10 000 total amino acid residues, while in the soft FE, those of tyrosine and dityrosine were 305 and 0.25 residues, respectively, and trityrosine and pulcherosine were only traces. The molar ratio of dityrosine, trityrosine, and pulcherosine in the hard FE was 100:38:24, while that for tyrosine/horseradish peroxidase/H_2O_2 reaction products was 100:3:8, respectively.

One of the conspicuous processes in the early development of sea urchin is the elevation of fertilization envelope[1] (FE)[2] observed immediately after fertilization and its subsequent hardening. Within a minute postinsemination, the vitelline layer (VL), which is slightly modified from the state in the unfertilized eggs, elevates and associates, in a Ca^{2+}-dependent manner, with several proteins and enzyme(s) secreted from the cortical granules to make a soft precursor of FE (SFE). In 5–10 min it is converted to hard FE (HFE) by intermolecularly cross-linking the tyrosine residues in some of the assembled proteins and the VL-derived scaffold, forming dityrosine (DT), trityrosine (TT), and higher homologues by the action of ovoperoxidase, which is one of the components of SFE (Foerder & Shapiro, 1977; Hall, 1978; Somers & Shapiro,

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[1] Historically this investment has been called "fertilization membrane", although it is not a common biomembrane consisting of lipid bilayer and proteins, but rather a huge complex of glycoproteins and enzyme(s).

[2] Abbreviations: FE, fertilization envelope; HFE, hard fertilization envelope; SFE, soft fertilization envelope; VL, vitelline layer; DT, dityrosine; TT, trityrosine; PC, pulcherosine; IDT, isodityrosine; ITT, isotrityrosine; HRPO, horseradish peroxidase; HPLC, high-performance liquid chromatography; FAB, fast atom bombardment; MS, mass spectrometry; ATA, 3-amino-1,2,4-triazole; BA, benzamidine (hydrochloride); MFSW, Millipore-filtered seawater; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 2-ME, 2-mercaptoethanol; res, residue(s); TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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