Hydrogen-1 NMR Evidence for Three Interconverting Forms of Staphylococcal Nuclease: Effects of Mutations and Solution Conditions on Their Distribution†

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ABSTRACT: It has been known for several years that ^{1}H NMR spectra of the enzyme staphylococcal nuclease contain resonances due to conformational heterogeneity [Markley, J. L., Williams, M. N., & Jardetzky, O. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 645–651]. One source of conformational heterogeneity has been attributed recently to cis/trans isomeriation of the Lys^{116}–Pro^{117} peptide bond [Evans, P. A., Dobson, C. M., Kautz, R. A., Hatfull, G., & Fox, R. O. (1987) *Nature (London) 329*, 266–268]. In this paper we present evidence for three interconverting folded forms of nuclease. Forms N and N′ are monomeric; form N″ appears at higher nuclease concentrations and probably corresponds to dimerized enzyme. Saturation transfer was used to demonstrate that exchange occurs between the denatured state and N″. The effects of temperature, pH, and Ca^{2+} and nucleotide binding on NMR spectra of nuclease were examined. When the temperature is increased or the pH is lowered, form N′ is favored relative to N. Binding of a competitive inhibitor (thymidine 3′,5′-bisphosphate plus calcium ion) strongly favors one form of nuclease. ^{1}H NMR spectra of wild-type nuclease, the single-mutant nucleases L89F and H124L, and the double-mutant nuclease F76V+H124L were compared. In the unligated proteins, the equilibrium constant for the conformational equilibrium N $\rightleftarrows$ N′ is approximately 0.1 in wild-type nuclease and nuclease H124L; by contrast, this equilibrium constant is about 0.7 in nuclease L89F and 1.2 in nuclease F76V+H124L under similar conditions.

The thermodynamic hypothesis put forth by Anfinsen (1973) states that the structure of a protein at equilibrium is determined by its amino acid sequence and the experimental conditions. Current views of protein structure have been shaped largely by results from X-ray crystallography. Such structural determinations, although extremely useful, provide only a static model of the protein, and the structure often is determined only for one set of conditions.

A possibility that must be considered is that the final product of the protein folding process in solution is not a single structure, but rather two or more structures in equilibrium. In this paper we present NMR evidence for the presence of at least three folded forms of nuclease in equilibrium at high protein concentrations. The operational definition of these forms is as follows: N is the major monomeric form of the protein in uncomplexed wild-type nuclease; N′ is the minor monomeric form of uncomplexed wild-type nuclease; and N″ is a minor form of uncomplexed nuclease that is favored by high protein concentrations. We have monitored the relative distribution of these forms as a function of the amino acid sequence and solution conditions.

The crystal structure of the wild-type nuclease–Ca^{2+}–pdTp ternary complex (Cotton et al., 1979) revealed only one form of the protein in the crystal lattice with the Lys^{116}–Pro^{117} peptide bond in the cis conformation. The present work shows that one conformational form of nuclease is favored in this ternary complex in solution.

One possible source of conformational heterogeneity in solution is cis/trans isomerization of X–Pro peptide bonds. Proline residues in proteins are technically difficult to observe directly by NMR,[1] but indirect evidence for cis/trans isomerization of X–Pro peptide bonds has been obtained by observing resonances from amino acids near the X–Pro peptide bond. Such evidence has been obtained for Pro^{24} in corticotropin fragment 1–32 by observing splitting of resonances from nearby Tyr and Val residues (Toma et al., 1978) and for Pro^{114} in ribonuclease A fragment 105–124 (Stimson et al., 1982).

By using site-directed mutagenesis, Evans et al. (1987) have shown that changing Pro^{117} to Gly causes the disappearance of resonances from the N′ form of nuclease. On the basis of this result and the kinetics of the interconversion, they have attributed the N $\rightleftarrows$ N′ interconversion to a cis $\rightleftarrows$ trans isomerization about the Lys^{116}–Pro^{117} peptide bond. The present results do not provide a critical test of this hypothesis;

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[1] Abbreviations: CHES, 2-(cyclohexylamino)ethanesulfonic acid; FID, free induction decay, H_ϵ, resonance from the histidine C_ϵ proton; H_ϵ^D, H_ϵ resonance derived from a histidine in a denatured or unfolded state; NMR, nuclear magnetic resonance; pdTp, thymidine 3′,5′-bisphosphate; pH*, pH value that is not corrected for the deuterium isotope effect; PNPdT, thymidine 5′-(*p*-nitrophenyl phosphate); ppm, parts per million; rms, root mean square; SSB, shifted sine bell; TSP, 3-(trimethylsilyl)propionate-d_4; N, monomeric form of nuclease that is the major form in wild-type nuclease; N′, monomeric form of nuclease that is a minor form in wild-type nuclease [this form corresponds to form N* in the nomenclature of Evans et al. (1987)]; N″, aggregated form of nuclease; N^{TC}, nuclease conformation in the nuclease–Ca^{2+}–pdTp ternary complex. NMR resonances from the three nuclease species are identified as follows: unprimed, N form; primed, N′ form; double primed, N″ form; superscript D, denatured form. Nuclease wt (wild type) refers to the sequence of the enzyme from the Foggi strain of *Staphylococcus aureus* (Cone et al., 1971) (all of the proteins discussed in this paper were isolated from *Escherichia coli* strains coding for specific nuclease sequences). Mutants are designated as follows: nuclease H46Y, His^{46} replaced by Tyr; nuclease L89F, Leu^{89} replaced by Phe; nuclease H124L, His^{124} replaced by Leu; nuclease F76V+H124L, nuclease double mutant with Phe^{76} replaced by Val and His^{124} replaced by Leu.

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they do show, however, that mutations far away from the Lys[116]–Pro[117] site alter the N ⇄ N′ equilibrium.

Experimental Procedures

Materials. pdTp was obtained from Pharmacia and ultrapure $CaCl_2$ from Alfa. ^2H_2O, 99.8% and 99.98%, was from Aldrich. KO_2H and ^2HCl were obtained from MSD Isotopes. Thymidine 5′-(*p*-nitrophenyl phosphate) (PNPdT) NH_4^+ salt was from Sigma, CHES was from Research Organics, and calf intestine alkaline phosphatase was from Boehringer Manheim. *Escherichia coli* strain SE6004 carrying the plasmids for wild-type nuclease (pFOG405), nucleases L89F (T232), H124L (Z101), and F76V+H124L (31A1) were gifts from Dr. D. Shortle (The Johns Hopkins University). The H124L sample used for the 2D-exchange spectrum was a gift from Dr. D. M. LeMaster (Yale University). Nuclease H124L was overexpressed in *E. coli* under the control of a T7 promoter that facilitates the production of large amounts of nuclease (D. M. LeMaster, personal communication). Nuclease H124L purified from this strain has NMR and enzymatic properties (A. P. Hinck, unpublished results) identical with those of nuclease H124L expressed by *E. coli* strain SE6004 carrying the Z101 plasmid (Shortle & Lin, 1985; Alexandrescu et al., 1988).

Enzyme Assays. All assays were carried out in 100 mM CHES and 10 mM $CaCl_2$, pH 9.0, 294 K, according to the coupled assay method of Grissom and Markley (1987). Nuclease concentrations were determined from $\epsilon_{280,1\%} = 9.3$. PNPdT concentrations were determined from $\epsilon_{330} = 2.4 \times 10^3$ M^{-1} cm^{-1}. Nuclease catalyzes the hydrolysis of PNPdT to *p*-nitrophenyl phosphate and thymidine. Alkaline phosphatase converts *p*-nitrophenyl phosphate to *p*-nitrophenyl and phosphate. The rate of production of *p*-nitrophenol was determined in virgin 1-cm polystyrene cuvettes at 405 nm, where $\epsilon_{405} = 1.83 \times 10^3$ M^{-1} cm^{-1}. K_{cat} and K_m values were obtained by fitting hyperbolic substrate saturation curves to

$$v = VA/(K_m + A)$$

by using the HYPERO program of Cleland (1979).

Sample Preparation. The nuclease wild type and nuclease mutant samples used for NMR study were all >95% pure as judged by gel electrophoresis (supplementary material Figure 1). All NMR samples contained 0.3 M NaCl. All chemical shift values are reported relative to a TSP standard. Nuclease purification and NMR sample preparation was carried out as described previously (Alexandrescu et al., 1988), with the following exception. For nuclease wild type, L89F, and H124L samples at low concentrations, amide protons that were not exchanged out by lyophilization were exchanged by heating the sample at 315–320 K for 10–15 min. When used at high concentrations (above 5 mM), this procedure led to the appearance of an additional peak in the histidine region (peak X in Figure 8). This peak apparently represents irreversibly denatured (possibly aggregated) nuclease. The mutant nuclease F76V+H124L seems to be especially susceptible to this transformation. Samples of nuclease F76V+H124L were lyophilized repeatedly from ^2H_2O and kept at room temperature during NMR experiments. Production of this form during preexchange of concentrated nuclease samples (≥5 mM) can be avoided by keeping the temperature below 315 K.

Data Analysis. Free induction decays (FIDs) were digitized into 8-k data-point arrays on an ASPECT-3000 computer. For clarity, most spectra presented in this paper were multiplied by a $\pi/25$ SSB function for resolution enhancement. Integrated areas were calculated with the GLINFIT program (A. D. Bain, Bruker User Group software); unenhanced spectra were usually used, because the SSB function distorts line shapes from Lorentzian and gives larger rms errors for line fits. The base line in the histidine region was corrected by using a spline function before line fitting. The GLINFIT program allows the user to simulate a portion of the spectrum and computes an rms error based on the agreement of the positions, heights, and widths of peaks in the simulated and experimental spectra.

To determine the relative populations of forms N, N′, and N″, the H_ϵ^8 resonance was used for line fitting, since the lines from the three species are best resolved for this histidine. Areas for H_ϵ^8, $H_\epsilon^{8\prime}$, and $H_\epsilon^{8\prime\prime}$ (the resonances corresponding to the N, N′, and N″ forms, respectively) were added and normalized to give a total area of 100% for each fit. The fraction (percent) of each resonance is described as a function of experimental conditions. The rms errors for each fit are given in the figure legends.

Concentration Study. A stock solution of 0.3 M NaCl in 100% ^2H_2O was prepared from NaCl lyophilized from ^2H_2O to exchange out water of hydration. This solution was used to dilute the protein samples. Protein concentrations were determined from $\epsilon_{280,1\%} = 9.3$. The pH* of the samples was kept within ±0.1 unit of pH* 6.4. For some of the more concentrated samples (>3 mM) spectra were acquired as a function of time after dilution. No changes in the spectra were apparent for periods up to 1 h. This indicates that the N ⇄ N″ equilibrium is established rapidly relative to the time scale of acquiring NMR spectra. To maintain the same signal to noise ratio for all the spectra in the concentration series, the number of scans to be used at each concentration was calculated from

$$N_d = N_c(\mathrm{conc_c}/\mathrm{conc_d})^2$$

where $\mathrm{conc_d}$ is the concentration of the diluted sample, $\mathrm{conc_c}$ is the concentration of the most concentrated sample, and N_c is the number of scans used for the most concentrated sample. Thus, 32 scans were collected for an 8.2 mM sample, and 445 scans were collected for a 2.2 mM sample. To determine whether aggregation was reversible, a 3.5 mM sample of nuclease L89F (N″ = 37%) was diluted to 0.55 mM (N″ = 13%). Aggregation was completely reversible as monitored by the increase of $H_\epsilon^{8\prime\prime}$ resonances (see Figure 2 of the supplementary material) when samples were concentrated by ultrafiltration (using Amicon Centricon 10 microconcentrators).

Two-Dimensional Exchange Experiment for Nuclease H124L. The 2D exchange data were acquired with a NOESY pulse sequence with time proportional phase incrementation (TPPI) (Johnston et al., 1986). The sample was 6.3 mM nuclease H124L, pH* 5.74, 325 K. A τ_m of 1 s was used; 504 spectra containing 8-k data points were collected. The FIDs were multiplied by a $\pi/2$ SSB in ω_1 and a $\pi/2$ SSB in ω_2 for the plot in Figure 3A. The transformed spectrum was zero filled to a final size of 1-k data points in ω_1 and 8-k data points in ω_2; (Hz/Pt) $\omega_1 = 11.8$, (Hz/Pt) $\omega_2 = 1.5$. In order to obtain sufficient resolution to separate the three H_ϵ^8 resonances, the H_ϵ region of a complete 2D spectrum was "chopped" by using the ZOOM routine (Z. Zolnai, unpublished work) and subjected to an inverse Fourier transform. The resulting subset of time-domain data was zero filled three times in both dimensions [(Hz/Pt) $\omega_2 = 0.4$, (Hz/Pt) $\omega_1 = 7.7$] and transformed with a $\pi/2$ SSB in ω_1 and a $\pi/8$ SSB in ω_2 (Figure 3B,C). ZOOMing was necessary because the same amount of zero filling on the original data set would have produced a 64 kword × 4 kword = 256 000 kword file, which would have been

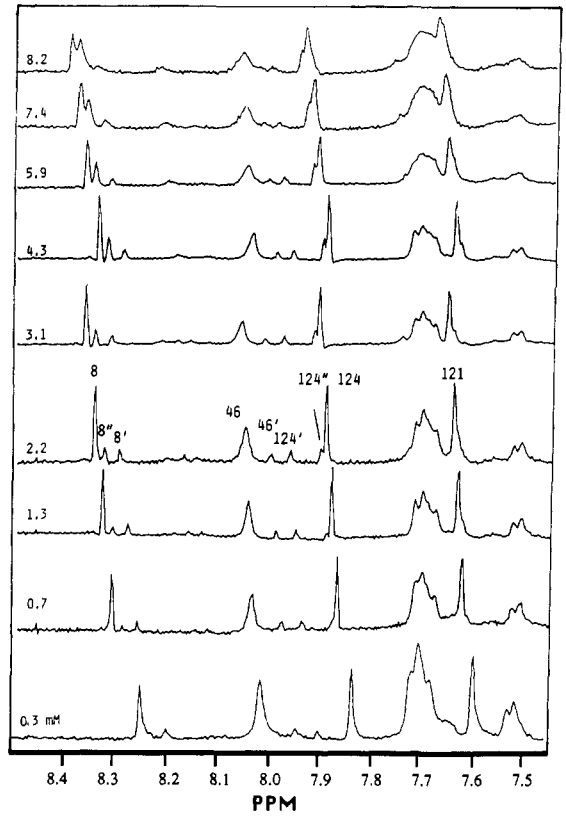

FIGURE 1: Dependence of the 500-MHz ^1H NMR spectrum of nuclease wt on the concentration of nuclease in the sample at pH* 6.4 ± 0.1. A sample that was initially 8.2 mM in nuclease wt was diluted with a solution of 0.3 M NaCl in 100% D_2O. The temperature was maintained at 310 K. The rms errors for each line fit of His^8 resonances, in percent, are indicated in parentheses (top to bottom): 8.2 mM nuclease, 32 scans (28.3) pH* 6.31; 7.4 mM nuclease, 39 scans (13.8) pH* 6.32; 5.9 mM nuclease, 62 scans (12.9) pH* 6.34; 4.3 mM nuclease, 116 scans (10.5) pH* 6.35; 3.1 mM nuclease, 224 scans (13.5) pH* 6.27; 2.2 mM nuclease, 445 scans (11.4) pH* 6.29; 1.3 mM nuclease, 1273 scans (23.7) pH* 6.31; 0.7 mM nuclease, 4391 scans (18.4) pH* 6.32; 0.3 mM nuclease, 10439 scans (20.57) pH* 6.54. Assignments of the H_ϵ peaks are noted in the figure. The changes in chemical shift of the H_ϵ resonances are due to small variations in pH between the samples. The group of resonances at 7.7 ppm does not titrate with pH.

cumbersome to process. During the course of the experiment (24 h at 325 K), about 20–30% of the sample was denatured irreversibly. While the presence of irreversibly denatured protein should not have affected the connectivities observed in Figure 3, it precluded quantitative determination of exchange rates by this method.

Ca^{2+} and pdTp Titration. Ultrapure $CaCl_2$ from a 0.94 M solution in 100% ^2H_2O and pdTp in a 0.17 M solution in 100% ^2H_2O were added to a 3.2 mM nuclease sample. The protein concentration was not decreased significantly by the small volumes added during the titrations.

Temperature Series. A 3.3 mM sample of nuclease at pH* 6.40 was used to follow changes in the intensities of the different forms as a function of temperature. Temperatures were calibrated against an 80% ethylene glycol/DMSO standard.

pH Titration. The pH of the samples was adjusted with 1 M stock solutions of KO^2H or ^2HCl. All pH values were measured at room temperature. The pH values in this paper were not corrected for the deuterium isotope effect and are therefore expressed as pH*. The volumes of KO^2H and ^2HCl added during the titrations were negligible compared to the sample volume. Determinations of sample concentrations by A_{280} before and after the titration showed that the sample concentrations did not change significantly during the titration.

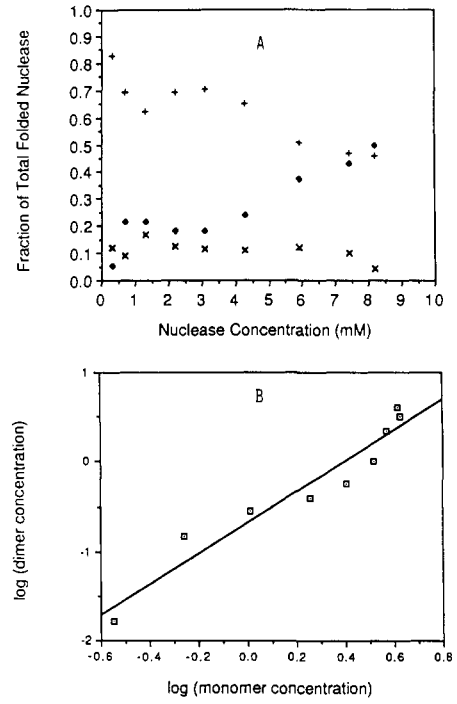

FIGURE 2: Intensities of individual histidine resonances as a function of nuclease wt concentration at pH* 6.4. (a) Plot of the integrated areas of H_ϵ resonances (Figure 1) vs nuclease concentration: (+) H_ϵ^8 (His^8 of form N); (◆) $H_\epsilon^{8''}$ (His^8 of form N″); (×) $H_\epsilon^{8'}$ (His^8 of form N′). (b) Plot of the logarithm of the dimer concentration versus the logarithm of the monomer concentration (from Figure 1).

Results and Discussion

Assignment of the H_ϵ Resonances in the Three Folded Forms. Figure 1 shows the effects of protein concentration on the spectrum of nuclease wt. The four H_ϵ resonances of nuclease have been assigned to specific histidines by comparing spectra of nuclease wt to mutants lacking one of the four histidines (Alexandrescu et al., 1988). Assignment of minor-form resonances has been based on their coordinate disappearance with a major peak when a particular histidine is replaced (Figure 3; Figure 3, supplementary material; Alexandrescu et al., 1988). The assignment of N and N′ resonances is based on their position relative to each other ($H_\epsilon^{8'}$ is upfield of H_ϵ^8; $H_\epsilon^{121'}$ and $H_\epsilon^{124'}$ are downfield from H_ϵ^{121} and H_ϵ^{124}, respectively) and their intensities (N′ resonances are smaller than N resonances in nuclease wt) in accordance with Fox et al. (1986). Resonances from the N″ form can be distinguished on the basis of their concentration dependence.

Characterization of the N″ Form of Nuclease. Figure 1 shows that the intensities of resonances from the N″ form decrease relative to those of the N and N′ forms as the concentration of nuclease wt is lowered. This indicates that the N″ form is a soluble dimer or higher order aggregate of nuclease. The data rule out the possibility of N″ being a covalently modified form of nuclease since N and N″ are interconvertible. Figure 2A is a plot of the normalized fractional integrals of the H_ϵ^8 resonances in the various forms as a function of concentration. The data were obtained from the spectra in Figure 1.

The aggregation of nuclease may be described by the equation

$$nA \rightleftarrows A_n$$

with an association constant

$$K_a = [A_n]/[A]^n$$

which also can be expressed as

$$\log ([A_n]) = n \log ([A]) + \log (K_a)$$

where [A] is the concentration of monomer [(protein concentration) $(f_N + f_{N'})$ = (protein concentration) $(1 - f_{N''})$] and $[A_n]$ is the concentration of aggregate [(protein concentration)$(f_{N''})$].

A plot of log ($[A_n]$) versus log ([A]) (see Figure 2B) gives $n = 1.7$ and $K_a = 0.21$ mM^{-1} for nuclease wt at 310 K and pH* 6.4. Thus, the stoichiometry of the N″ form is most likely a dimer.

Surprisingly, the line widths of H_ϵ^8 and $H_\epsilon^{8''}$ are similar (as measured by GLINFIT). This is unexpected since the rotational correlation time of the dimer should be larger than that of the monomer, and resonances from the dimer should be broader than those from the monomer. His^8 is in a surface-exposed flexible region of the molecule. The residue may have enough conformational freedom to give rise to a sharp H_ϵ resonances even in the dimer. Corroborative evidence for this hypothesis is the presence of unexpectedly sharp histidine resonances in the NMR spectrum of the pyruvate dehydrogenase multienzyme complex when a histidine is replaced for Glu^{291} in a flexible segment of the E2p chain of PDH (Texter et al., 1988). The molecular weight of the PDH complex is approximately 4 million.

2D Exchange Spectrum of Nuclease H124L. Figure 3A shows a phase-sensitive two-dimensional exchange spectrum of nuclease H124L (nuclease H124L is missing His^{124}; thus, peaks H_ϵ^{124}, $H_\epsilon^{124'}$, and $H_\epsilon^{124''}$ are absent[2]). The cross-peaks in the figure demonstrate exchange between nuclease forms and confirm the earlier cross-assignments. The advantages of this type of experiment over one-dimensional magnetization-transfer experiments are that the problem of decoupler spillover is avoided and multiple connectivities between exchanging spin systems can be observed in a single experiment, provided that the mixing time is optimized with respect to the exchange rates (Figure 4 of the supplementary material shows a 1D steady-state NOE experiment with wild-type nuclease that indicates magnetization transfer between H_ϵ^D and $H_\epsilon^{8''}$). Figure 3B shows a ZOOMed portion of the spectrum (as described under Experimental Procedures). For His^8, we observed three connectivities, which are tentatively assigned to $H_\epsilon^D \rightleftarrows H_\epsilon^8$, $H_\epsilon^D \rightleftarrows H_\epsilon^{8'}$, and $H_\epsilon^D \rightleftarrows H_\epsilon^{8''}$ exchange as indicated in the figure. The expected H_ϵ^8–$H_\epsilon^{8'}$ and H_ϵ^8–$H_\epsilon^{8''}$ cross-peaks were too close to the diagonal to be resolved.[3]

Effect of Ca^{2+} and pdTp on the Conformation of Nuclease. Nuclease requires a Ca^{2+} ion for activity, and pdTp is a tightly binding inhibitor of nuclease. The 1.5-Å resolution crystal structure of nuclease was solved with Ca^{2+} and pdTp bound (Cotton et al., 1979) and shows only one form of nuclease in the crystal lattice. When Ca^{2+} ion and pdTp are added to a solution of wild-type nuclease, the N′ and N″ resonances disappear. Eventually only one resonance is observed for each histidine (Figure 4). A simple interpretation is that the N form of nuclease is favored over the N′ and N″ forms in the presence of Ca^{2+} and pdTp. This interpretation is not strictly correct, since the conformation of complexed nuclease appears to be different from any of the conformations of uncomplexed nuclease. To account for this, we refer to this new conformation as N^{TC}. From the chemical shifts of H_ϵ^8 and H_ϵ^{121} it would appear that the conformation of the ternary complex

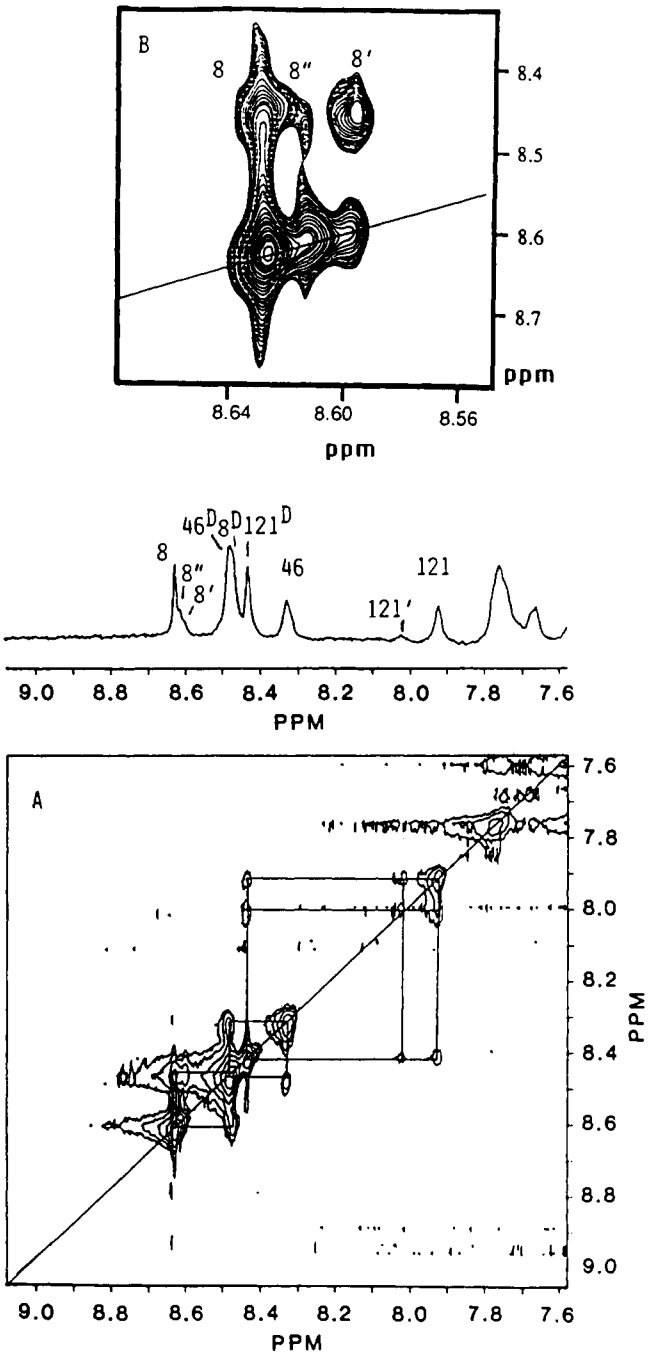

FIGURE 3: Two-dimensional-exchange experiment (^{1}H NMR at 500 MHz) for nuclease H124L. Conditions: 6.3 mM nuclease H124L, 0.3 M NaCl, pH* 5.74, 325 K. FIDs were multiplied by a $\pi/2$ SSB in both dimensions to obtain the plot in (a) and by $\pi/2$ SSB in ω_1 and $\pi/8$ SSB in ω_2 for the plot in (b). (a) Exchange pathways for His^{121}, His^{46}, and His^8. Connectivities between H_ϵ^{121}–$H_\epsilon^{121'}$, H_ϵ^{121}-denatured H_ϵ^{121}, $H_\epsilon^{121'}$-denatured H_ϵ^{121}, and H_ϵ^{46}-denatured H_ϵ^{46} are drawn. For His^8 only the H_ϵ^8-denatured connectivity is drawn (see below). (b) Expansion of the region around the H_ϵ^8 resonances. Connectivities tentatively assigned to H_ϵ^8-denatured, $H_\epsilon^{8''}$-denatured H_ϵ^8, and $H_\epsilon^{8'}$-denatured are shown (see text). The 2D spectrum was phased in the H_ϵ^8 region.

[2] The H_ϵ^{46} and $H_\delta^{46'}$ resonances overlap at this pH* (see Figure 6).

[3] Recent evidence shows that two unfolded forms of nuclease exist in solution (U and U*) and that these forms have distinct chemical shifts for His^{121} (Evans et al., 1987, 1988). Only one unfolded resonance U^{*121} was observed in our 2D exchange spectrum of nuclease of H124L (Figure 3). The lower intensity U^{121}–H_ϵ^{121} cross-peak may be missing from our 2D exchange spectrum because of insufficient S/N or overlap of the U^{121} and U^{*121} resonances at pH* 5.74. In a 1D experiment carried out under conditions identical with those of Evans et al. (1988) (except that a temperature of 327 K was used for nuclease H124L instead of 321 K for wild type, since H124L has a higher T_m than wild type), magnetization transfer was observed to both U^{121} and U^{*121} when H_ϵ^{121} was saturated. Our results confirm the observation of two denatured states in wild-type nuclease (Evans et al., 1988) for nuclease H124L.

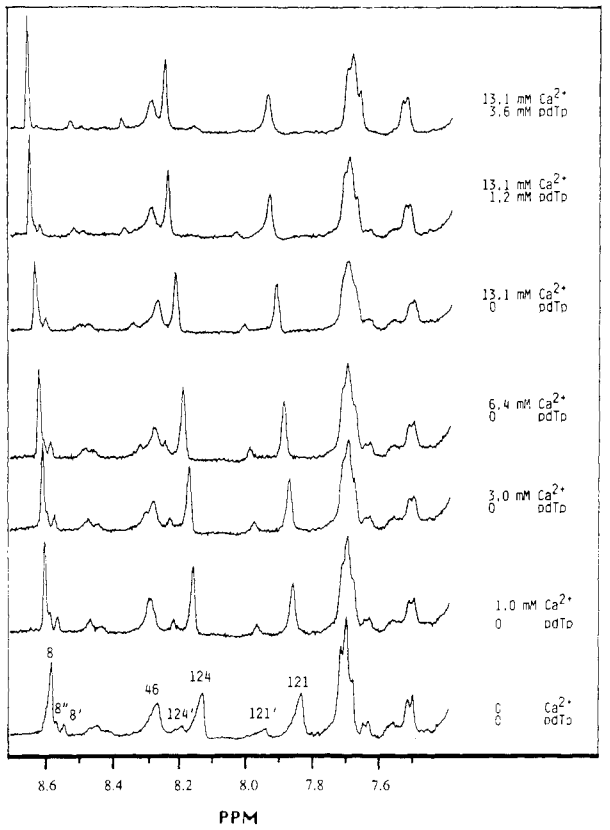

FIGURE 4: Dependence of the H_ϵ region of the 500-MHz ^1H NMR spectrum of nuclease wt on the concentration of added Ca^{2+} and pdTp. Aliquots of a 0.94 M $CaCl_2$ and 0.17 M stock solution of pdTp were added to a 3.2 mM nuclease sample, pH* 5.92, 313 K. Each spectrum was the average of 256 scans. FIDs were multiplied by a $\pi/25$ SSB function prior to transformation.

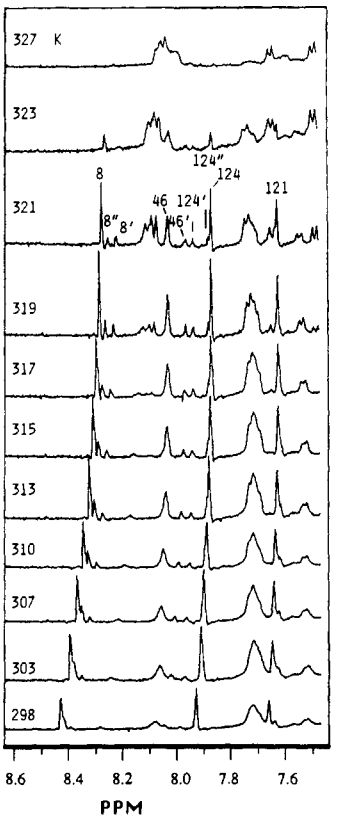

FIGURE 5: Temperature dependence of the 500-MHz ^1H NMR spectrum of 3.3 mM nuclease wt. The pH* at room temperature was 6.40. Each spectrum is the average of 128 transients. FIDs were multiplied by a $\pi/25$ SSB function prior to transformation.

(N^{TC}) is more like the N form than the N′ form.

The uncomplexed forms N, N′, and N″ exchange slowly on the NMR time scale defined by the relative positions of the histidine peaks (i.e., they give rise to separate resonances). The N^{TC} form, however, is in fast exchange with uncomplexed nuclease on the NMR time scale of the same histidine peak. No new resonances are observed when Ca^{2+} and pdTp are added. The chemical shifts of the histidine resonances at substoichiometric pdTp concentrations represent the population weighted average of the complexed forms of nuclease and uncomplexed forms of nuclease that are able to bind ligands. Since the $H_\epsilon^{8'}$ and $H_\epsilon^{121'}$ peaks shift as pdTp is added, this indicates that the N′ form can bind inhibitor. The fact that its intensity decreases as the pdTp concentration increases suggests that the N′ form has a lower affinity for pdTp than the N form.

Temperature Effects on Nuclease Species. The effects of increasing temperature on the distribution of nuclease species are shown in Figure 5. Between 315 and 323 K, resonances due to N′ increase about 2-fold, while those due to N″ decreases about 2-fold relative to the total (N + N′ + N″). The relative intensity of the major resonance is unchanged within experimental error.

pH Titration of the Nuclease Species. The pH titration was carried out at 315 K in order to achieve the maximum resolution of $H_\epsilon^{8''}$ and $H_\epsilon^{8'}$ resonances. It can be seen in the spectra of Figure 6 that the $H_\epsilon^{8'}$ resonance increases relative to H_ϵ^{8} and $H_\epsilon^{8''}$ as the pH is lowered. The H_ϵ^{124} and H_ϵ^{121} resonances of the N″ form were not well resolved from those of the N form in this experiment. H_ϵ^{124}, therefore, may be treated as the sum of $H_\epsilon^{124} + H_\epsilon^{124''}$ resonances, as can the

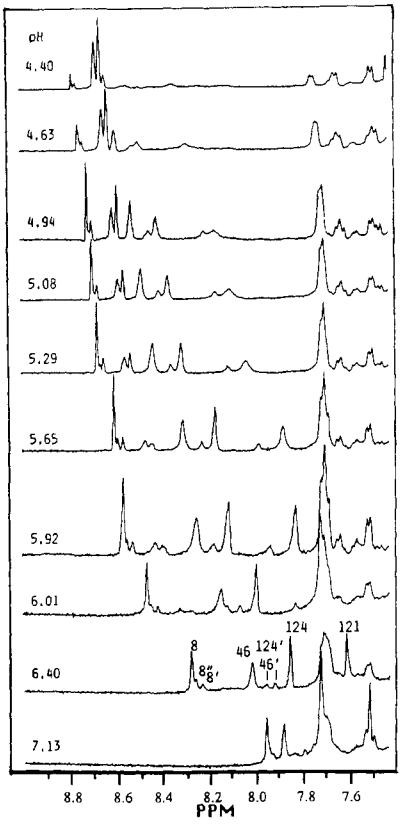

FIGURE 6: Effect of pH on the histidine region of the 500-MHz ^1H NMR spectrum of nuclease wt. Conditions: 3.2 mM nuclease wt in ^2H_2O containing 0.3 M NaCl; 315 K. Each spectrum resulted from 256 scans; FIDs were multiplied by a $\pi/25$ SSB function prior to transformation. All pH* values were determined at room temperature and not corrected for changes in pH* with temperature.

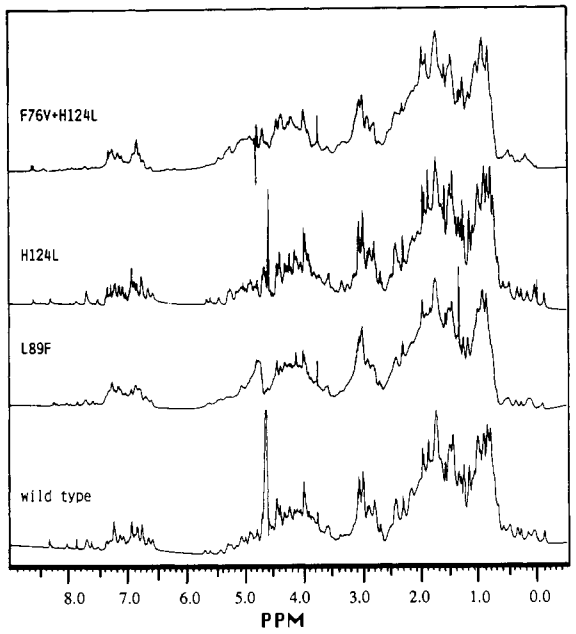

FIGURE 7: ^{1}H NMR spectra (500 MHz) of wild-type nuclease and three mutants (bottom to top): nuclease wt (3.3 mM, pH* 6.40, 310 K, 128 scans); nuclease L89F (3.3 mM, pH* 6.41, 310 K, 128 scans); nuclease H124L (1.0 mM, pH* 5.54, 315 K, 948 scans); nuclease F76V+H124L (1.0 mM, pH* 5.53, 298 K, 948 scans). All spectra were transformed without resolution enhancement.

H_ϵ^{121} resonance. The $H_\epsilon^{121\prime}$ and $H_\epsilon^{124\prime}$ resonances, which were well resolved from the corresponding resonances of the N form, also increase with decreasing pH. There is about a 4-fold increase in the integrated areas of N′ resonances relative to the total area of folded resonances (N + N′ + N″) in this pH range. The area of the resonance due to $H_\epsilon^{8\prime\prime}$ remained constant during the pH titration.

We conclude from the data presented in this section that the N′ conformation becomes relatively more favorable at low pH. Below pH* 4 at room temperature, the H_ϵ^8 and $H_\epsilon^{8\prime}$ resonances approach equal intensity [data for wild type not shown; see Figure 8 of Alexandrescu et al. (1988) for H46Y]. This is well below the pK_a* of any of the four histidines of nuclease (Alexandrescu et al., 1988), which eliminates them as possible candidates in a mechanism for the observed changes in K_{eq} = [N′]/[N] with pH. Fox et al. (1986) have reported that N and N′ peaks from a given histidine have similar pH titration curves. We confirm this and find in addition that the titration curves of the histidines in form N″ do not differ very much from their titration curves in forms N or N′. Thus, the conformational equilibria and dimerization reactions have only minor effects on the histidine pK_a values.

Nuclease Mutants. As part of our investigation of the effects of mutations on nuclease structure, we have examined several nuclease mutants by NMR. The sequence of the nuclease from the Foggi strain of *Staphylococcus aureus* is taken as the wild type. Nuclease H124L is produced in nature by the V8 strain of *S. aureus*. It has been shown that the nuclease H124L mutation acts as a "global suppressor" (Shortle & Lin, 1985); i.e., nucleases carrying the H124L mutation can alleviate the deleterious effects on protein stability of mutations at other sites. A majority of the mutants we have investigated have distributions of the N and N′ forms similar to that of the wild-type nuclease (for example, nucleases H8R, I18M, V23F, H46Y, and Y85F). Two of the mutants, however, nuclease L89F and nuclease F76V+H124L, exhibit a dramatic increase in the intensity of the N′ and N″ form resonances relative to the N form.[4] Nuclease F76V also shows increased minor forms, but the protein is difficult to obtain since it is susceptible to proteolysis during purification. The double-mutant F76V+H124L is more stable and can be purified to >95% purity if purification is carried out quickly (≤12–24 h from the time the protein is isolated as a crude extract to the time it is eluted from a Bio-Rex 70 column). It is especially important to load the protein onto the column as rapidly as possible.

Table I: Distribution of Nuclease Forms in Wild Type and Mutants[a]

		N (%)	N′ (%)	N″ (%)	rms error (%)
(1) wild type	3.3 mM	66	6	28	10.6
(2) L89F	3.3 mM	35	24	41	13.9
(3) L89F	1.4 mM	39	30	31	12.8
(4) L89F	0.10 mM	60	39	0	18.3
(5) H124L	7.7 mM	71	7	22	19.3
(6) H124L	1.0 mM	91	7	2	14.6
(7) H124L+F76V	7.7 mM	28	37	35	10.1
(8) H124L+F76V	1.0 mM	39	40	22	11.8
(9) H124L+F76V	0.23 mM	44	56	50	19.4

[a] From the spectra shown in Figure 8. Fractions of the three species were determined from line fitting of the H_ϵ^8 resonances. Spectra 1–3 were transformed without resolution enhancement. For (4) the FID was multiplied by an exponential function with a line broadening of 2 Hz prior to transformation. A $\pi/25$ SSB was used for (5) and (6). For (7–9), spectra were zero filled from 8-k to 64-k and multiplied by a $\pi/25$ SSB function. Peaks in (1–6) were simulated by using Lorentzian line shapes; (7–9) were simulated by Gaussian line shapes.

A comparison of the spectra of wild-type and mutant nucleases is presented in Figures 7 and 8. Differences are manifested in the spectra of nucleases L89F and F76V+H124L relative to nuclease wt and nuclease H124L, whose spectra are similar. The chemical shifts of high-field methyl resonances (−0.5 to 0.8 ppm), particularly the most upfield shifted resonance which has been assigned to methyl protons of Val[74] (Gerlt, 1987; Wang et al., 1988), and some of the C_α resonances downfield of the residual HDO peak (5.0–6.0 ppm) are altered by these mutations. Some of these changes represent shifts in the positions of resonances in the N form and cannot be explained simply by increased contributions from N′ or N″ resonances.

Figure 8 shows expansions of the histidine H_ϵ regions from the spectra of these proteins.[5,6] Both the N″ and N′ forms are more populated in nuclease L89F relative to wild type and in nuclease F76V+H124L relative to nuclease H124L, but in both cases the increase in the N′ form is larger than that in the N″ form. These data are summarized in Table I. Another significant feature is that for a given histidine the separation between the resonances from each form is different in all the proteins (see Figure 8). Not only do the L89F and

[4] The assignment of N and N′ resonances in nucleases H124L, L89F, and F76V+H124L is based on their relative positions in the spectra in reference to the spectrum of nuclease wt and on the observation that increasing temperature or lowering pH causes increases in the N′/N intensity ratio in each of these mutants as it does in nuclease wild type.

[5] The sharp histidine resonances of nuclease may be unique in their sensitivity to the conformational heterogeneity. For less-resolved or broader resonances, the minor forms may be manifested only as line broadening. For example, the three H_ϵ^8 resonances have similar line widths in all mutants (Figure 8), while the overall spectra of L89F and F76V+H124L appear broader than those of wild-type nuclease and nuclease H124L (Figure 7).

[6] J. Gerlt (1988) and co-workers have found that the C_δ proton of His[121] is split by an additional conformational heterogeneity that they have attributed to two alternative hydrogen-bonding positions for the imidazole group of that histidine. This result may explain why H_ϵ^{121}–H_δ^{121} cross-peaks were not observed in COSY spectra of nuclease (Wang et al., 1988). It also suggests that additional minor forms may be present that are not resolved on the basis of the C_ϵ proton signals.

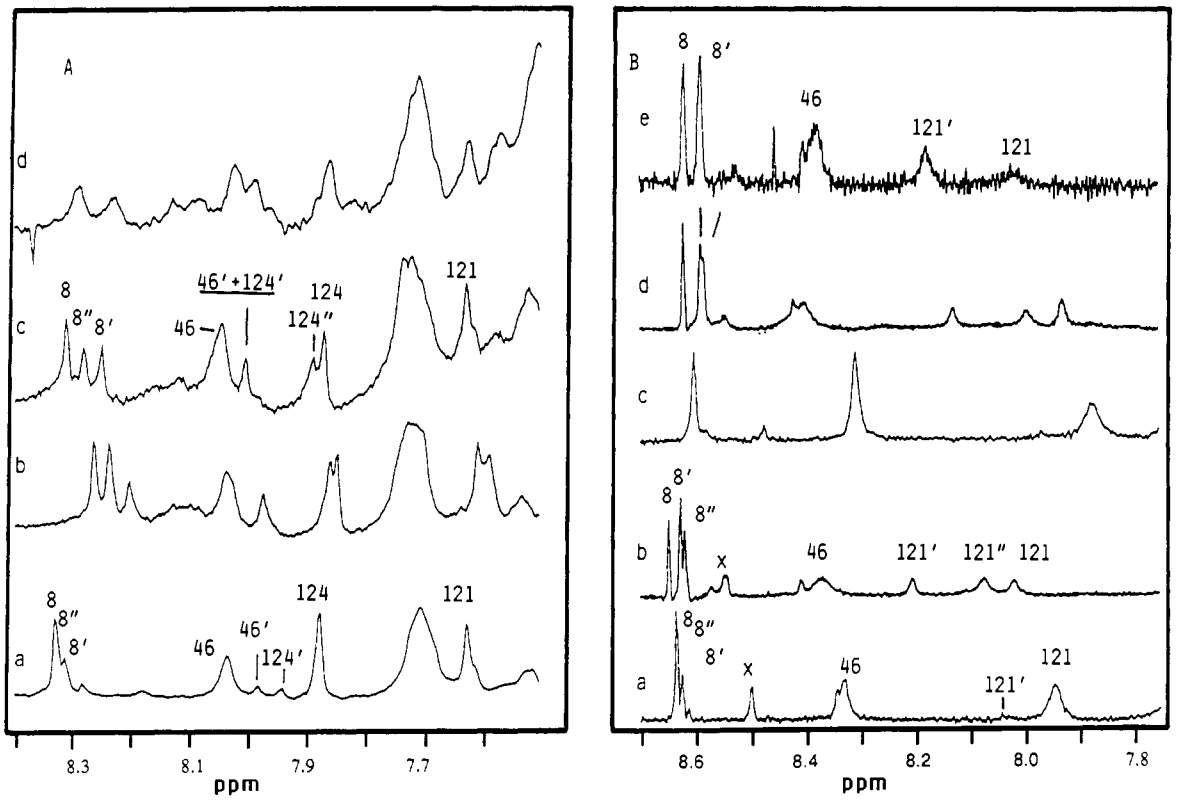

FIGURE 8: Sequence effects on the distribution of conformational states of staphylococcal nuclease as revealed by 500-MHz ^{1}H NMR spectra of the histidine region of the proteins. (A) Comparison of nuclease wt and nuclease L89F: (a) nuclease wt (as in Figure 7); (b) nuclease L89F (as in Figure 7); (c) nuclease L89F (1.4 mM, pH* 6.40, 310 K, 512 scans); (d) nuclease L89F (0.1 mM, pH* 6.44, 310 K, 15800 scans; the spectrum was multiplied by an exponential function with a line broadening of 2 Hz prior to transformation). (B) Comparison of nuclease H124L and nuclease F76V+H124L: (a) nuclease H124L (7.7 mM, pH* 5.53, 315 K, 16 scans); (b) nuclease F76V+H124L (7.7 mM, pH* 5.52, 298 K, 64 scans); (c) nuclease H124L (as in Figure 7); (d) nuclease F76V+H124L (as in Figure 7); (e) nuclease F76V+H124L (0.23 mM, pH* 5.5, 305 K). All spectra of nuclease H124L were transformed with a $\pi/25$ SSB; all spectra of nuclease F76V+H124L were zero filled from 8-k data points to 64-k data points and transformed with a $\pi/25$ SSB.

Table II: Kinetic Parameters for the Hydrolysis of Thymidine 5′-(*p*-Nitrophenyl phosphate) (PNPdT) Catalyzed by Wild-Type and Mutant Staphylococcal Nuclease

nuclease	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	$(k_{cat}/K_m)_{wt}/(k_{cat}/K_m)_{mutant}$
wt	4.4 ± 0.8	2.1 ± 0.1	480 ± 80	1
L89F	10 ± 3.0	0.020 ± 0.003	2.0 ± 0.7	240 ± 100
H124L	5.6 ± 1.5	0.84 ± 0.09	150 ± 40	3.2 ± 1.1
F76V, H124L	2.9 ± 0.7	1.0 ± 0.07	350 ± 90	1.4 ± 0.4

F76V+H124L mutations alter the distribution among the slowly interconverting forms, but they influence the local environments of the histidines within these forms. Addition of Ca^{2+} ion and pdTp favors one form in each of these mutants. The H_ϵ resonances of this form have chemical shifts similar to those of the N form.

Shortle (1986) reported the following C_m values (where C_m is the guanidine hydrochloride concentration at the midpoint of denaturation) for these variants: nuclease H124L, 1.08 M > wild type, 0.82 M > F76V+H124L, 0.60 M. We have determined a C_m value of 0.32 M for nuclease L89F. The kinetic parameters of the mutants discussed in this paper are shown in Table II. Only nuclease L89F has reduced activity.[7] The fact that nuclease L89F retains considerable activity is surprising since we expected the mutation to interfere with substrate binding at the active site on the basis of the crystal structure (Cotton et al., 1979).

Conclusion

In this paper we have presented evidence for the presence of at least three slowly interconverting folded forms of staphylococcal nuclease (forms N, N′, and N″). N is the major monomeric folded form of nuclease wt, N′ is a minor monomeric form in nuclease wt, and N″ is probably a dimeric form of nuclease. We have shown that the equilibrium populations of these three forms of nuclease can be changed by altering the protein concentration or sequence or by changing solution conditions such as temperature or pH. It is unclear whether the conformational heterogeneity in nuclease has any physiological significance. However, since nuclease has been used as a model protein for the study of protein structure and folding, it is important to fully understand the range of possible nuclease conformational states.

The major form of folded nuclease (N) is believed to contain a cis peptide bond between residues Lys^{116} and Pro^{117} on the basis of the presence of a cis peptide bond at this position in the structure of the nuclease–Ca^{2+}–pdTp ternary complex. Evans and co-workers (Evans et al., 1987) have suggested that N′ represents a folded form of nuclease with a trans Lys^{116}–Pro^{117} peptide bond. In the denatured random coil state, X–Pro peptide bonds are thought to be presented in a ratio of approximately 20% cis to 80% trans (Creighton, 1984). If the cis/trans isomerization of the Lys^{116}–Pro^{117} is assumed to be the difference between the N and N′ structures, then the 90% cis/10% trans ratio observed for this peptide bond requires about 2 kcal/M of stabilization energy from other

[7] With single-strand DNA as a substrate, nuclease L89F shows only a 9-fold reduction in turnover relative to wild type. A more detailed discussion of the enzymatic activities of L89F and other mutants will be presented in a subsequent paper.

regions of the protein. Minor reductions in the stability of the overall nuclease structure, through amino acid substitutions,[8] titration of ionizable groups, or heating, would be expected to allow the Lys^{116}–Pro^{117} bond to take the more stable trans configuration. This is what we have observed.

Two-dimensional NMR experiments are typically carried out by using 10–15 mM protein concentrations. For molecules the size of nuclease this corresponds to protein solutions with 75–83% water. Protein crystals used for X-ray crystallography typically contain 30–70% water (Matthews, 1985). Therefore, protein concentrations used for NMR spectroscopy frequently approach those in the crystalline state, which by definition is an ordered protein aggregate. If a protein is to be studied by this method, it is important to verify whether the high concentrations normally used for two-dimensional NMR lead to aggregation.[9]

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Supplementary Material Available

Figures showing (1) the SDS–PAGE gel of the wild-type nuclease sample used for Figure 1, (2) reversibility of aggregation for nuclease L89F, (3) assignment of $H_\epsilon^{46\prime}$, and (4) a 1D NOE experiment showing magnetization transfer between denatured H_ϵ^{8} and $H_\epsilon^{8\prime\prime}$ (6 pages). Ordering information is given on any current masthead page.

Registry No. PNPdT, 2304-08-7; staphylococcal nuclease, 9013-53-0; calcium, 7440-70-2; thymidine 3′,5′-bisphosphate, 2863-04-9; histidine, 71-00-1.

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[8] Leu^{89} and Phe^{76} are each about 10 Å away from Pro^{117}.

[9] For 2D NMR experiments used in the sequential assignment of the nuclease–Ca^{2+}–pdTp ternary complex (Wang et al., 1988), it has been possible to work with concentrations of nuclease H124L as high as 5 mM. Under these conditions only the N^{TC} form is present.