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Histidine 121 of Staphylococcal Nuclease. Correction of the $H^{\delta 2}$ ^1H NMR Assignment and Reinterpretation of the Role This Residue Plays in Conformational Heterogeneity of the Protein

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Abstract: Heteronuclear two-dimensional NMR studies of wild-type staphylococcal nuclease containing histidine residues uniformly labeled with carbon-13 (26% isotope) have led to full analysis of the aromatic parts of the histidine ^1H and ^{13}C spin systems. The $^1H^{\delta 2}$ and $^{13}C^{\delta 2}$ resonances of His^{121} were found to be split as the result of the $N_{a1} \rightleftharpoons N_{a2}$ conformational equilibrium described previously and attributed to cis–trans isomerism about the Lys^{116}–Pro^{117} peptide bond (N_{a1}, cis; N_{a2}, trans).[3] The relative intensities of the pair of $^1H^{\delta 2}$ peaks of His^{121} showed the same dependence on ligation at the active site as do the relative intensities of the pair of $^1H^{\epsilon 1}$ peaks from the same residue. A double-mutant enzyme (nuclease G79S+H124L), which exhibited a drastically altered $[N_{a1}]$ to $[N_{a2}]$ ratio, provided additional evidence that the pair of $^1H^{\delta 2}$ peaks and the pair of $^1H^{\epsilon 1}$ peaks of His^{121} report on the same conformational equilibrium ($N_{a1} \rightleftharpoons N_{a2}$). The unusual chemical shift of the $^1H^{\delta 2}$ of His^{121} is attributed to diamagnetic shielding by the aromatic ring of Tyr^{91} as verified by ring-current calculations based on two X-ray structures for wild-type staphylococcal nuclease.[18] Since both the $^1H^{\delta 2}$ and $^{13}C^{\delta 2}$ resonances showed large chemical shift changes on going from the N_{a1} state ($^1H^{\delta 2}$ = 4.3 ppm; $^{13}C^{\delta 2}$ = 117 ppm) to the N_{a2} state ($^1H^{\delta 2}$ = 4.9 ppm; $^{13}C^{\delta 2}$ = 121 ppm), the conformational change must alter the relative orientations of the His^{121} and Tyr^{91} rings. The results correct earlier misassignment of peaks around 6.3 ppm (at pH* 7.8) to $^1H^{\delta 2}$ of His^{121} along with misinterpretation of the effect of the $N_{a1} \rightleftharpoons N_{a2}$ equilibrium on this residue.[6]

The enzyme staphylococcal nuclease provides a convenient model system for investigations of protein conformational heterogeneity in solution. Multiple forms of the protein have been detected on the basis of splitting of ^1H NMR signals from histidine residues.[1-10] Concentration-dependent spectral heterogeneity has been attributed to dimerization of the protein.[7] At lower concentrations where the enzyme is monomeric, splitting of the $^1H^{\epsilon 1}$ signals from histidines 8, 121, and 124 has been attributed to a conformational equilibrium between two states (N_{a1} and N_{a2})[11] whose difference is thought to involve isomerism about the Lys^{116}–Pro^{117} peptide bond (N_{a1}, cis; N_{a2}, trans).[3,4] The position of the $N_{a1} \rightleftharpoons N_{a2}$ equilibrium depends on the protein sequence

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and solution conditions[4,5,8]; complexation with the competitive inhibitor thymidine 3',5'-bisphosphate (pdTp), in the presence of Ca^{2+}, shifts the equilibrium in the direction of the N_{a1} form.[2-5,7,8] Histidine 46, however, appears to be unaffected by the Lys^{116}–Pro^{117} isomerism,[9] and recent evidence[10] suggests that the doubling of NMR signals from His^{46}, which was reported initially in 1970,[1] arises from isomerism about the His^{46}–Pro^{47} peptide bond; the resulting substrates are denoted N_{b1} and N_{b2}.[12]

The $^1H^{\delta 2}$ signal of His^{121}, which was not observed in an earlier NMR study of the pH dependence of the aromatic spectral region[5] or in subsequent extensive assignments of wild-type nuclease (nuclease wt)[13] or nuclease H124L,[15-17] was assigned by Stanczyk et al.[6] to two or more peaks around 6.9 ppm at pH* 7.8 (uncorrected glass-electrode reading). Their approach was to selectively deuterate the aromatic rings of the phenylalanine, tyrosine, and tryptophan residues of nuclease wt so as to have an unhindered view of the $^1H^{\delta 2}$ signals of the four (protiated) histidines.[6] Since the pattern of signals at 6.9 ppm did not change upon formation of the nuclease wt–pdTp–Ca^{2+} ternary complex, these authors concluded that "the cis–trans isomerism of Pro^{117} is not the only cause for conformational heterogeneity in [staphylococcal nuclease]".[6]

We show here that the assignment strategy based on selective deuteration[6] was flawed and that the $^1H^{\delta 2}$ resonances of His^{121} at pH* 7.8 are located close to the water resonance in the aliphatic spectral region in both the N_{a1} (4.3 ppm) and N_{a2} (4.9 ppm) forms of nuclease wt. The unusual ^1H chemical shift is predicted by ring-current calculations based on X-ray structures of the nuclease wt–pdTp–Ca^{2+} ternary complex.[18]

Materials and Experimental Procedures

We first noted the unusual chemical shift of the $^1H^{\delta 2}$ signal of His^{121} in analyzing the histidine spin systems of the nuclease H124L–pdTp–Ca^{2+} ternary complex.[9] These experiments have been replicated with nuclease wt in order to compare the results with those reported earlier.[6] Sample conditions were chosen to mimic those used by Stanczyk et al.:[6] 4 mM nuclease wt, 0.3 M KCl, pH* 7.8 in ^2H_2O at 30 °C; both in the absence of Ca^{2+} and pdTp and in their presence (protein:Ca^{2+}:pdTp = 1:6:3).

Recombinant nucleases wt and G79S+H124L[19] were overproduced in *Escherichia coli* by using the T7 expression system.[20] The plasmid containing the nuclease wt sequence was constructed as described previously for the nuclease H124L plasmid,[16] with the exception that the *Nde I-Sau 3a* fragment containing the nuclease wt gene was used.[21] The G79S+H124L plasmid was constructed by subcloning the 0.7 kb *Sal I-Hind* III fragment of plasmid pTSN2CC[17] (4.8 kb) into the vector M13mp11 to create M13mp11*. Single-stranded uracil-substituted M13mp11* DNA was prepared and isolated[22] and used subsequently to generate the desired mutation by oligonucleotide site-directed mutagenesis.[22] Successful mutagenesis was confirmed by dideoxy-DNA sequencing of the M13mp11* replicative form according to the protocol described in the *Sequenase Manual* (U.S. Biochemicals, Cleveland, OH). The *Sal I-Hind* III 0.7-kb fragment was then excised and combined with the 4.1-kb *Sal I-Hind* III fragment of plasmid pTSN2CC. The fragments were ligated and transformed into competent *E. coli* strain JM103.

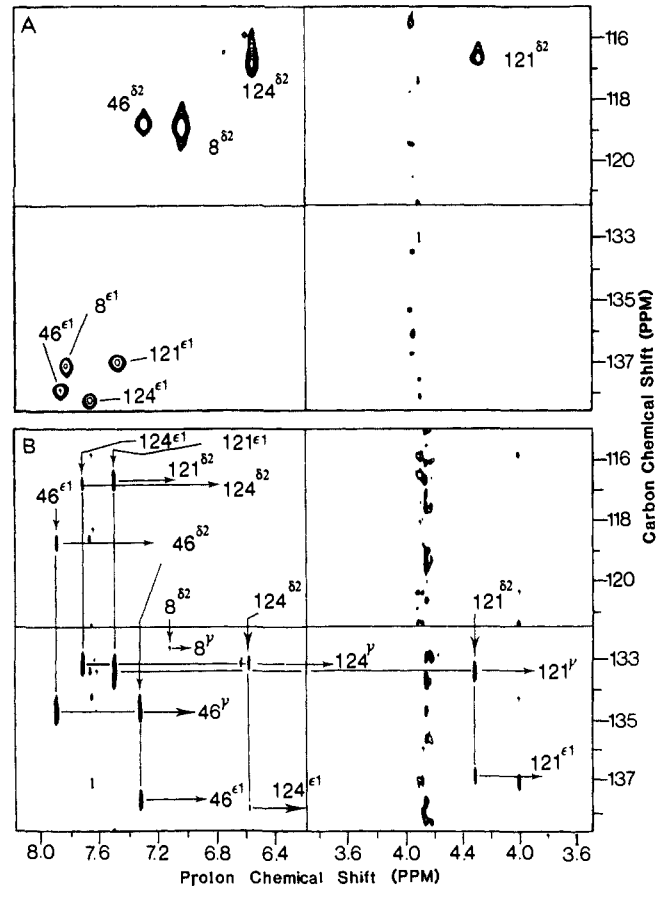

Figure 1. Heteronuclear two-dimensional NMR spectra of wild-type staphylococcal nuclease (nuclease wt)[13] containing L-[26% U-^{13}C]-histidine. The sample consisted of 4 mM protein, 12 mM pdTp, 24 mM $CaCl_2$, and 0.3 M KCl in ^2H_2O at pH* 7.8. The ^{13}C carrier frequency was placed at the center of the histidine aromatic signals (His $^{13}C^{\gamma}$, $^{13}C^{\delta 2}$, and $^{13}C^{\epsilon 1}$), and the ^{13}C spectral window was set to cover only the aromatic region. ^1H chemical shifts were referenced to internal TSP; ^{13}C chemical shifts were referenced to external dioxane whose chemical shift was assumed to be 67.8 ppm relative to the shift standard TMS. Cross-peaks assigned to the histidines are indicated by residue number and atom designator. (A) $^1H\{^{13}C\}$ SBC spectrum (11.7 T) collected at 30 °C, consisting of 202 t_1 increments with 96 averaged free induction decays (FID's) per increment. A value of $1/(2J) = 2.50$ ms was used to optimize the experiment for aromatic $^1J_{CH}$. Only positive contour levels are shown. (B) $^1H\{^{13}C\}$ MBC spectrum (14.1 T) collected at 37 °C, consisting of 176 t_1 increments with 160 FID's per increment. The period $1/(2J)$ was set to 45 ms in order to select for two- and three-bond ^1H–^{13}C couplings. This delay is shorter than the optimal value for two- and three-bond ^1H–^{13}C couplings because it was found to minimize signal loss from T_2 processes. The spectrum is presented in the mixed mode. Vertical arrows indicate ^1H chemical shifts while horizontal arrows indicate ^{13}C chemical shifts of the histidine nuclei.

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(11) The notation used here is that from ref 9. In the notation of previous publications, $N_{a1} = N$ and $N_{a2} = N'$.
(12) Since the $N_{a1} \rightleftharpoons N_{a2}$ and $N_{b1} \rightleftharpoons N_{b2}$ conformational transitions are independent, one expects to observe four conformational states for folded nuclease: N_{a1b1}, N_{a1b2}, N_{a2b1}, and N_{a2b2}.[9] To date, however, no NMR signals have been found that report simultaneously on both conformational transitions.
(13) Nuclease wt refers to recombinant nuclease produced in *E. coli* that has the same sequence as nuclease A isolated from the Foggi strain of *Staphylococcus aureus*.[14] The protein used in refs. 6 and 15 was nuclease wt with a hexapeptide N-terminal extension. The N-terminus appears to be disordered, and there is no evidence that the extension has any effect on the conformation of the rest of the protein.[15] Nuclease H124L refers to the nuclease with the same sequence as that isolated from the V8 strain of *S. aureus*. The sequence of nuclease H124L differs from that of nuclease wt by the substitution of leucine for His^{124}.
(14) Cone, J. L.; Cusumano, C. L.; Taniuchi, H.; Anfinsen, C. B. *J. Biol. Chem.* **1971**, *246*, 3103-3110. As revised by: Shortle, D. *Gene* **1982**, *22*, 181-189.
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Successful clones were picked, and the plasmid DNA was isolated and again sequenced.

Nucleases wt and G79S+H124L enriched specifically with L-[26% U-^{13}C]histidine were produced in *E. coli* strain AW608thr^{+} (histidine auxotroph), which had been transformed with both the T7 overproducing plasmid (Amp^{R}) described previously and plasmid pGP1-2 (Kan^{R}).[23] Plasmid pGP1-2 contains the gene for T7 polymerase under control of the heat-sensitive λ_{PL} promoter;[23] production of T7 polymerase is initiated by shifting the growth temperature from 30° to 42 °C. Nucleases were produced by inoculating 1 L of M9 medium[24] (supplemented with glucose to yield 10 g/L, L-[26% U-^{13}C]histidine to 30 mg/L, ampicillin to 50 μg/mL, and kanamycin to 50 μg/mL) with 5 mL of an overnight growth of the culture on LB/kanamycin/ampicillin. The 1-L cultures were grown with shaking at 30 °C until OD_{600} reached 1.0. Production of nuclease was initiated by shifting the temperature from 30 to 42 °C. The cultures were grown an additional 3 h, at which point the cells were harvested and the nuclease purified as described previously.[17] The L-[26% U-^{13}C]histidine was isolated from a protein hydrolyzate of *Anabaena* 7120 (a photosynthetic cyanobacterium) grown on [26% ^{13}C]CO_2 as its sole carbon source.[25]

Two-dimensional, ^{1}H-detected ^{13}C spectroscopy was used to analyze the combined ^{1}H and ^{13}C spin systems of the histidines.[26] Information about sample conditions and spectrometer settings is given in the figure captions.

The ring-current contribution to the chemical shift of the His^{121} ^{1}H$^{\delta 2}$ was calculated by using the FORTRAN program RCUR4 adapted from Perkins FORTRAN program for calculating Johnson-Bovey ring-current shifts.[27] The MOLEDT routine of the INSIGHT package (Biosym Technologies, Inc.) was used to add hydrogens to the heavy atoms of the X-ray structures[18] obtained from the Protein Data Bank.[28]

Results and Discussion

In the ^{1}H{^{13}C} SBC spectrum of nuclease wt, four strong ^{1}H$^{\epsilon 1}$–^{13}C$^{\epsilon 1}$ cross-peaks and four ^{1}H$^{\delta 2}$–^{13}C$^{\delta 2}$ cross-peaks were observed, both in the presence of active-site ligands (Figure 1A) and in their absence (data not shown). The ^{1}H chemical shifts of seven of these cross-peaks agreed closely with previous assignments for this protein at pH* 7.8.[5] The eighth peak, which has an unusual ^{1}H chemical shift (4.3 ppm), was assigned by difference to the ^{1}H$^{\delta 2}$ of His^{121}. Alexandrescu et al.,[5] who assigned seven of the eight histidine peaks by mutagenesis, did not locate the 4.3 ppm resonance that lies outside the aromatic spectral region.

A more rigorous assignment of the unusual histidine ^{1}H–^{13}C cross-peak results from comparing the ^{1}H{^{13}C}SBC spectrum (Figure 1A) with the ^{1}H{^{13}C}MBC spectrum of the same sample (Figure 1B). Correspondence between the His^{121} ^{1}H$^{\epsilon 1}$ signal assigned previously[5] and the unusual cross-peak was established both by the three-bond correlation[26] between ^{13}C$^{\delta 2}$ and ^{1}H$^{\epsilon 1}$ and by the three-bond correlation between ^{13}C$^{\epsilon 1}$ and ^{1}H$^{\delta 2}$. Futhermore, the ^{1}H$^{\delta 2}$ and ^{1}H$^{\epsilon 1}$ peaks assigned to His^{121} each showed correlations to the same ^{13}C$^{\gamma}$, respectively, by two- and three-bond coupling. The connectivities form a steplike pattern that links ^{13}C$^{\delta 2}$–^{1}H$^{\epsilon 1}$ and ^{13}C$^{\epsilon 1}$–^{1}H$^{\delta 2}$ in a redundant fashion.[26] These are outlined in Figure 1B for each of the histidine residues in nuclease wt.[29]

A double-mutant enzyme, nuclease G79S+H124L,[19] was used to investigate the influence of the $N_{a1} \rightleftharpoons N_{a2}$ conformational transition on the spin system of His^{121}. Whereas $[N_{a1}]/[N_{a2}] \cong 20$ for unligated nuclease wt,[2,7] the ratio for unligated nuclease G79S+H124L is about 0.15 (Figure 2, bottom trace). Upon addition of pdTp in the presence of $CaCl_2$, the $[N_{a1}]$ to $[N_{a2}]$ ratio increases as shown by the relative intensities of the 1D ^{1}H NMR signals from the ^{1}H$^{\epsilon 1}$ of His^{8} and His^{121} corresponding to the N_{a1} and N_{a2} forms (Figure 2).

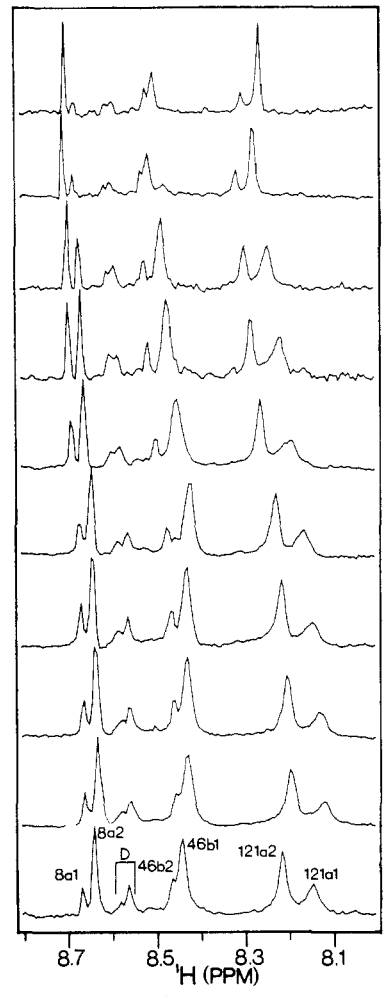

Figure 2. One-dimensional, ^{13}C-decoupled ^{1}H spectra of nuclease G79S+H124L[19] acquired under increasing concentrations of $CaCl_2$ and pdTp. Only the histidine ^{1}H$^{\epsilon 1}$ region is shown. The sample was enriched with L-[26% U-^{13}C]histidine. Each spectrum was the average of 512 FID's acquired with ^{13}C WALTZ-16 decoupling during the acquisition time. The initial 0.405-mL sample consisted of 2.08 mM nuclease G79S+H124L and 0.3 M KCl in $^{2}H_2O$ at pH* 5.26. Aliquots of 400 mM $CaCL_2$ and 91.3 mM pdTp were added such that the following molar ratios of protein:$CaCl_2$:pdTp (bottom to top) were attained: 1:0:0, 1:1:0, 1:3:0, 1:6:0, 1:12:0, 1:12:0.3, 1:12:0.6, 1:12:1.2, 1:12:2.4, 1:12:10.

The results shown in Figure 2 were used to determine conditions under which $[N_{a1}]/[N_{a2}] \cong 1$ for nuclease G79S+H124L. ^{1}H{^{13}C} SBC (Figure 3A) and ^{1}H{^{13}C} MBC (Figure 3B) spectra were recorded under these inhibitor conditions and at a temperature and pH* similar to those used for the nuclease wt sample (30 °C, pH* 7.8). The ^{1}H{^{13}C}SBC spectrum shows single strong ^{1}H$^{\epsilon 1}$–^{13}C$^{\epsilon 1}$ and ^{1}H$^{\delta 2}$–^{13}C$^{\delta 2}$ correlations from His^{8} and His^{46}, but a broadened ^{1}H$^{\epsilon 1}$–^{13}C$^{\epsilon 1}$ peak with unresolved splitting and a pair of well-resolved ^{1}H$^{\delta 2}$–^{13}C$^{\delta 2}$ peaks from His^{121}. The doubling arises from the two conformational states N_{a1} and N_{a2}. The assignments given (Figure 3A) are consistent with changes in the intensities of these peaks in ^{1}H{^{13}C}SBC spectra (data not shown) upon addition of pdTp and $CaCl_2$ (by definition, N_{a1} is the form that predominates in the presence of saturating pdTp and $CaCl_2$).[7] These results, along with multiple-bond correlation (^{1}H{^{13}C} MBC) data (Figure 3B), which provided ^{1}H$^{\epsilon 1}$–^{13}C$^{\delta 2}$ and ^{1}H$^{\delta 2}$–^{13}C$^{\epsilon 1}$ three-bond correlation cross-peaks, served to identify the combined ^{1}H–^{13}C spin system of each histidine ring of the partially complexed nuclease mutant.

X-ray structures[18] of the nuclease wt–pdTp–Ca^{2+} ternary complex (N_{a1} form) show that the ^{1}H$^{\delta 2}$ of His^{121} is situated above

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(29) The ^{13}C$^{\gamma}$–^{1}H$^{\delta 2}$ (two-bond) cross-peak from His^{8} is weaker than those seen for the other histidine residues, although it clearly is visible in Figure 1B. We observed none of the expected three-bond correlations between the ring protons (^{1}H$^{\epsilon 1}$ and ^{1}H$^{\delta 2}$) and carbons (^{13}C$^{\gamma}$, ^{13}C$^{\delta 2}$, ^{13}C$^{\epsilon 1}$) of His^{8}.

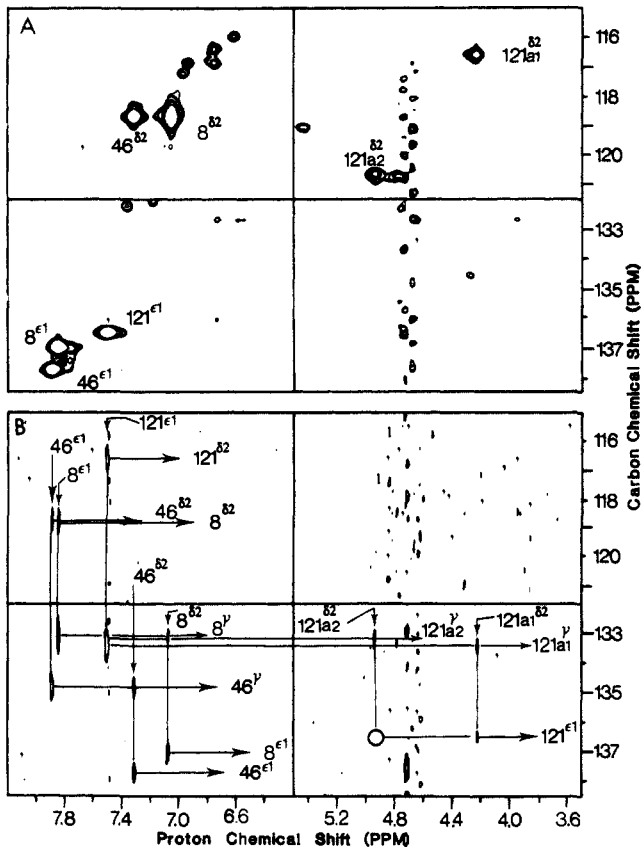

Figure 3. Two-dimensional, proton-detected ^{13}C NMR spectra of nuclease G79S+H124L[19] containing L-[26% U-^{13}C]histidine. The sample contained 4 mM protein, 4.8 mM pdTp, 16 mM $CaCl_2$, and 0.3 M KCl in ^2H_2O at pH* 7.8. Other conditions were as described in Figure 1. Cross-peaks assigned to the histidines are indicated by residue number and atom designator. (A) $^1H\{^{13}C\}$ SBC (11.7 T) spectrum recorded with 238 t_1 increments and 128 averaged FID's per increment. The delay $1/(2J)$ was set to 2.5 ms to optimize for aromatic $^1J_{CH}$. Additional spectral parameters were set as described in Figure 1. Only positive contours are shown. (B) $^1H\{^{13}C\}$ MBC (14.1 T) spectrum recorded with 174 t_1 increments and 160 averaged FID's per increment. The delay $1/(2J)$ was set to 70 ms to optimize for two- and three-bond $^1H-^{13}C$ couplings. Additional spectral parameters were adjusted as in Figure 1. The spectrum is presented in the mixed mode. Vertical arrows indicate ^1H chemical shifts while horizontal arrows indicate ^{13}C chemical shifts of the histidine nuclei. The circle indicates a cross-peak that is visible at lower contour levels.

and to the middle of the aromatic ring of Tyr[91]. The calculated upfield shift for $^1H^{\delta 2}$ was 3.08 ppm, which is close to the observed upfield shift of 2.8 ppm from the expected random-coil chemical shift of 7.1 ppm.[30] Since both the $^1H^{\delta 2}$ and $^{13}C^{\delta 2}$ resonances show large chemical shift changes at pH* 7.8 on going from the N_{a1} conformational form ($^1H^{\delta 2}$ = 4.3 ppm; $^{13}C^{\delta 2}$ = 117 ppm) to the N_{a2} form ($^1H^{\delta 2}$ = 4.9 ppm; $^{13}C^{\delta 2}$ = 121 ppm), the conformational change must alter the relative orientation of His[121] and Tyr[91] rings. The chemical shift of $^1H^{\delta 2}$ of His[121] moves closer to the random-coil value on going from the N_{a1} form to the N_{a2} form but remains strongly shielded. By contrast, the chemical shift of $^{13}C^{\delta 2}$ of His[121] goes from a shielded environment in N_{a1} to a deshielded environment in N_{a2}. The results suggest that the angle θ between the His[121] and Tyr[91] rings has a value of $54.7° < \theta \leq 90°$ in N_{a1} but $\theta < 54.7°$ in N_{a2} (54.7° is the "magic angle" at which the diamagnetic anisotropy is zero). This hypothesis can be tested by an examination of $^1H-^1H$ NOE values.

The above results demonstrate that His[121] does not provide an exception to the pattern of $N_{a1} \rightleftharpoons N_{a2}$ structural heterogeneity reported previously.[2-4,7,9] The peaks around 6.9 ppm observed by Stanczyk et al.[6] probably arose from incompletely deuterated aromatic residues. Ironically, the main conclusion of Stanczyk et al.,[6] namely that staphylococcal nuclease has conformational heterogeneity that is not resolved upon formation of the ternary complex, has been established rigorously by studies of His[46] (Figure 2 and refs 9 and 10). These newer results show that native, monomeric, staphylococcal nuclease does exhibit two classes of conformational heterogeneity but that His[121] does not report on both as had been claimed.[6]

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Registry No. L-His, 71-00-1; L-tyr, 60-18-4; staphylococcal nuclease, 9013-53-0.

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