Kinetic and Conformational Effects of Lysine Substitutions for Arginines 35 and 87 in the Active Site of Staphylococcal Nuclease†

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ABSTRACT: The high-resolution X-ray crystal structure of staphylococcal nuclease (SNase) suggests that the guanidinium groups of Arg 35 and Arg 87 participate as electrophilic catalysts in the attack of water on the substrate phosphodiester. Both arginine residues have been replaced with "conservative" lysine residues so that both the importance of these residues in catalysis and the effect of changes in electrostatic interactions on active site conformation can be assessed. The catalytic efficiencies of R35K and R87K are decreased by factors of 10^4 and 10^5 relative to wild-type SNase, with R87K showing a very significant reduction in its affinity for both DNA substrate and the competitive inhibitor thymidine 3′,5′-bisphosphate (pdTp). The thermal denaturation behavior of both mutant enzymes differs from that of wild type both in the absence and in the presence of the active site ligands Ca^{2+} and pdTp. Both the ^1H NMR chemical shifts and interresidue nuclear Overhauser effects (NOEs) of residues previously assigned to be in the hydrophobic core of SNase are altered in R35K and R87K. These observations, similar to those recently reported by our laboratories for substitutions for Glu 43 [Hibler, D. W., Stolowich, N. J., Reynolds, M. A., Gerlt, J. A., Wilde, J. A., & Bolton, P. H. (1987) *Biochemistry 26*, 6278; Wilde, J. A., Bolton, P. H., Dell'Acqua, M., Hibler, D. W., Pourmotabbed, T., & Gerlt, J. A. (1988) *Biochemistry 27*, 4127], suggest that lysine substitutions are not conservative in SNase and disrupt the conformation of the active site. Thus, the kinetic properties of R35K and R87K cannot be used to describe the roles of Arg 35 and Arg 87 in catalysis by the wild-type enzyme. Furthermore, on the basis of the effects of these conservative substitutions for Arg 35 and Arg 87, we predict that other substitutions for these residues, including neutral substitutions, are also likely to cause conformational alterations in the active site.

Although the use of site-directed mutagenesis to alter proteins and enzymes is routine in many laboratories, there is a growing realization that detailed information about the secondary and tertiary structures of a wild-type protein is required if amino acid substitutions are to be rationally selected and generated. In addition, if the functional properties of mutant proteins are to be properly interpreted, secondary and tertiary structural information is also required for each mutant protein. This need for structural information is especially acute in the application of site-directed mutagenesis to problems in mechanistic enzymology where structure and function (catalysis) can be expected to be interdependent. If amino acid substitutions introduced within the active site of an enzyme induce even small changes in conformation, partition of catalytic efficiency among the functional groups within the active site certainly cannot be easily achieved. For example, substitutions of charged functional groups are likely to perturb the ionization properties or positions of other charged functional groups, thereby thwarting attempts to quantitatively assign the importance of each charged functional group in catalysis as is discussed below.

This cautious point of view is based, in part, on our previously described studies of substitutions for Glu 43 in staphylococcal nuclease (SNase) (Hibler et al., 1987; Wilde et al., 1988) and more recent evidence acquired on dihydrofolate reductase (Adams et al., 1989; Perry et al., 1989). The three-dimensional structure of SNase was first reported at 1.5-Å resolution (Cotton et al., 1979) and recently has been independently refined to 1.65 Å (Loll & Lattman, 1989). These crystal structures, which are essentially identical in most aspects, suggest that Glu 43 is positioned to act as a general base to assist in the direct, in-line attack of a water molecule on the phosphodiester bond of a bound DNA substrate (Figure 1). These structures also reveal that the essential Ca^{2+} and the guanidinium groups of Arg 35 and Arg 87 are in close proximity to the phosphodiester bond being hydrolyzed. The positioning of these cationic groups suggests that they function as electrophilic catalysts to assist in the attack of the nucleophilic water on the substrate. It is tempting to speculate on the quantitative importance that might be assigned to both general basic (Glu 43) and electrophilic (Arg 35 and Arg 87) catalysis, about 10^{15}-fold, for the hydrolysis of DNA catalyzed by SNase.

The Asp, Gln, Asn, Ser, and Ala substitutions for Glu 43 were characterized by kinetic and some biophysical properties, and detailed structural studies have focused on the "conservative" Asp substitution, E45D. All five mutant proteins are similarly reduced in catalytic efficiencies by factors ranging from 1400-fold to 5000-fold (Hibler et al., 1987). Detailed investigations of solution-state and crystal structure have focused on E43D, given the labor- and time-intensive methods of both NMR spectroscopy and X-ray crystallography. The use of one- and two-dimensional ^1H NMR experiments in conjunction with isotopically labeled proteins revealed

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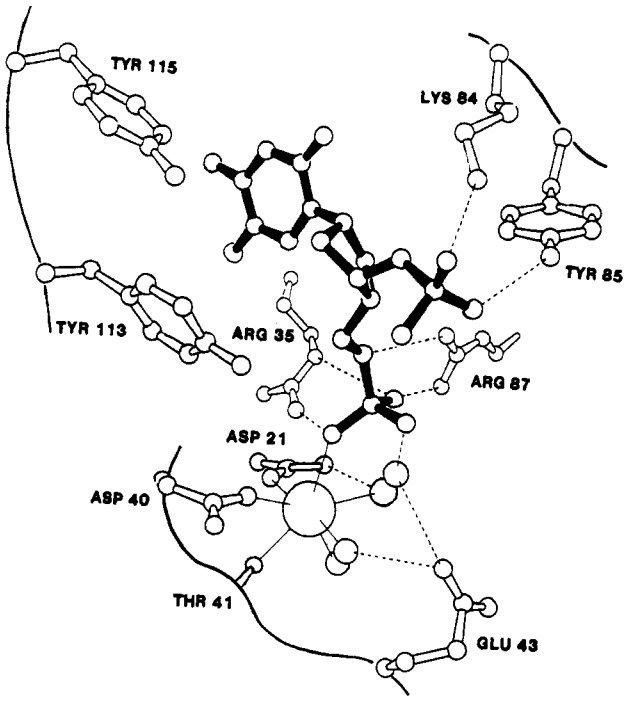

FIGURE 1: The active site of wild-type SNase (Loll & Lattman, 1989).

that a conformational change involving Leu 25, Tyr 27, Phe 34, Val 74, and Phe 76 accompanied the Glu to Asp substitution at residue 43 (Wilde et al., 1988). These residues are located in the hydrophobic core of the protein approximately 25 Å from the position of the substitution. Subsequent X-ray crystallographic studies have revealed that the coordination geometry of the essential Ca^{2+} is altered in the mutant protein (P. Loll and E. E. Lattman, personal communication). Apparently, the β-carboxylate group of Asp 43 is unable to "reach" the water coordinated to the metal ion (as observed in the structure of the wild-type protein), and as a result, the carboxylate group of Asp 43 hydrogen bonds with the amide hydrogens of Thr 44 and Lys 45 and the imidazole of His 46. Other acidic amino acid residues within the active site (Asp 19, Asp 21, and Glu 52) are also affected by this change in the position of the anionic carboxylate group of residue 43, and the positions of their side chains are altered in the structure of E43D. Large changes in geometry are not observed in the hydrophobic core in the crystalline state where interproton distances were observed to differ in solution by ^{1}H NMR spectroscopy.

In this article we report observations on the effects of "conservative" lysine substitutions for Arg 35 and Arg 87, the putative electrophilic catalysts in the hydrolysis reaction, on the structure and catalytic activity of SNase. While both lysine and arginine residues have a single cationic charge, the side chain of lysine is one bond shorter than that of arginine so the position of the charge on the side chain should differ from that in the wild-type protein. Our premise is that these subtle substitutions will allow sensitive evaluation of the importance of electrostatic effects in determining active site geometry; on the basis of our experiences with substitutions for Glu 43, if conformational alterations are observed with these conservative replacements, we would predict that other substitutions, including neutral substitutions, would cause significant conformational alterations within the active site. Kinetic analyses of the reactions catalyzed by R35K and R87K reveal that the V_{max}/K_m values for both mutant proteins are 10^4–10^5 less than that measured for wild-type enzyme. The thermal stabilities, ligand binding, and one- and two-dimensional ^{1}H NMR spectra of both mutant proteins suggest that the conformations of both mutant proteins differ from that of the wild-type protein, with the effect of the "conservative" substitution being more pronounced in R87K than in R35K. Thus, as in the case of E43D, we anticipate that quantitative analyses of the kinetic properties of these mutant enzymes and other substitutions at these positions cannot be directly used to deduce the precise roles of these residues in catalysis as has been previously suggested (Serpersu et al., 1987).

Materials and Methods

The recombinant plasmid pNJS (Hibler et al., 1987), which directs the heat-inducible production of an N-terminal-modified SNase in which the heptapeptide Met-Asp-Pro-Thr-Val-Tyr-Ser is appended to the N-terminal alanine of the 149 amino acid nuclease A (Bohnert & Taniuchi, 1972), has been described. This plasmid (and derivatives thereof containing site-directed mutants in the gene for SNase) is propagated in the *Escherichia coli* strain N4830 which is auxotrophic for histidine, isoleucine, and valine and whose chromosome contains the gene for the cI857 mutation of the λ repressor. This plasmid was used instead of the secretion plasmid pONF1 (Takahara et al., 1985) used in our previous studies of substitutions for Glu 43 since biosynthetic incorporation of deuteriated amino acids for ^{1}H NMR studies is more efficiently achieved with pNJS.

Mutageneses. Both R35K and R87K were generated in M13mp19 in which a 795 bp *Bam*HI–*Sal*I restriction fragment from pNJS had been cloned; this restriction fragment contains the entire coding sequence for nuclease A and the N-terminal heptapeptide. The mutagenic primers directing the Arg 35 to Lys 35 mutation, d(CAATGACATTCA-AGCTATTATTG), and the Arg 87 to Lys 87 mutation, d-(CTGATAAATATGGAAAAGGCTTAGCG), where the positions of the mutagenic mismatches are underlined, were used in the deoxyuridine method of site-directed mutagenesis (Kunkel, 1985). Plaques containing the mutation were identified by colony hybridization followed by DNA sequence analysis. Following sequence analysis of the entire genes for both R35K and R87K, the *Bam*HI–*Sal*I restriction fragments containing the mutated genes were isolated and separately ligated with the vector *Bam*HI–*Sal*I restriction fragment of pNJS. The plasmids encoding R35K and R87K were transformed into and maintained in strain N4830.

Isolation of Mutant Nucleases. Since neither R35K nor R87K was observed to bind to the pdTp–Sepharose affinity matrix used to purify wild type and residue 43 mutants of nuclease, our previously described procedure for purifying enzymes encoded by pNJS (Hibler et al., 1987) could not be used. Instead, purification of the mutant proteins from the crude cell lysates was accomplished by chromatography on phosphocellulose as described in the literature (Fuchs et al., 1967).

Characterization of Mutant Nucleases. Kinetic, fluorescence, and one- and two-dimensional ^{1}H NMR studies were conducted as previously described (Hibler et al., 1987; Wilde et al., 1988).

Results and Discussion

Kinetic Analyses. While both wild-type nuclease and R35K could be saturated with DNA substrate at pH 9.5, the pH optimum of the wild-type enzyme, the velocity of the reaction catalyzed by R87K was observed to be a linear function of DNA concentration to 200 μg/mL, the maximum DNA concentration allowed by the spectrophotometric assay. For this reason, detailed kinetic comparisons of the pH dependence

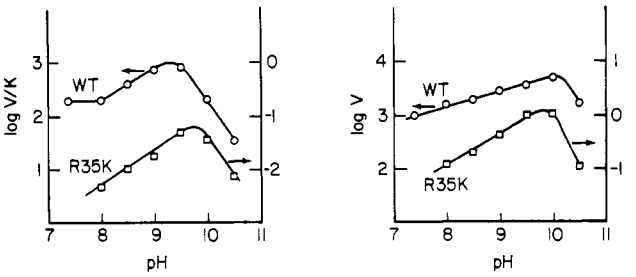

FIGURE 2: Dependence of V_{max}/K_m (left panel) and of V_{max} (right panel) on pH for both wild-type SNase and R35K. The units of V_{max} are $\Delta OD_{260nm}/(min \cdot mg)$ and of K_m are $\mu g/mL$.

of kinetic parameters on pH were not performed for this mutant enzyme. The relative velocities of the reactions catalyzed by R35K and R87K were measured at subsaturating DNA concentrations for R35K, and R87K was assessed to have a V_{max}/K_m value approximately 2-fold less than that measured for R35K.

V_{max}, K_m, V_{max}/K_m, and K_a for Ca^{2+} were measured as a function of pH for wild-type enzyme and the R35K mutant. The K_a of the wild-type enzyme for Ca^{2+}, 0.1 mM, was found to be pH independent as was the K_a of R35K for Ca^{2+}, 0.3 mM. Plots comparing V_{max} and V_{max}/K_m for these enzymes are presented in Figure 2. The observed dependencies of these kinetic parameters on pH are qualitatively similar, although the data reveal that the maximum value of V_{max}/K_m occurs approximately 0.5 pH unit higher for R35K than wild-type enzyme. Considering that the pK_a of the ϵ-amino group of Lys 35 could be approximately 2 pH units lower than the pK_a of the guanidinium group of Arg 35, the data presented in Figure 2 suggest that proton dissociation from Lys 35 is not important in the dependence of the reaction velocity on pH for this enzyme. At pH 9.5, the values for V_{max} and K_m for wild-type enzyme are 3700 $\Delta OD/(min \cdot mg)$ and 4.4 $\mu g/mL$, respectively; the same values for R35K are 1.0 $\Delta OD/(min \cdot mg)$ and 18.5 $\mu g/mL$. Based on these values, the catalytic efficiency of R35K (based on V_{max}/K_m) is 6.4×10^{-5} times that of wild-type enzyme; the catalytic efficiency of R87K is, therefore, approximately 3.2×10^{-5} times that of wild-type enzyme.

Conformational and Ligand Binding Studies. The remainder of this paper presents data comparing the conformations of R35K and R87K with that of wild-type enzyme. These comparisons will allow the conclusion that the diminution in catalytic efficiency found for R35K and R87K relative to wild-type enzyme cannot be used to *quantitatively* estimate the importance of electrophilic catalysis by Arg 35 and Arg 87 in facilitating the attack of water on the phosphodiester bond of the substrate. Conformational changes are observed which presumably are primarily associated with the change in the position of the cationic charge associated with these residues.

Thermal Denaturation. The thermal denaturation of R35K, R87K, and wild-type enzyme has been monitored by the quenching of the fluorescence of Trp 140. The behavior of R35K is qualitatively similar to that observed for substitutions for Glu 43: in the absence of Ca^{2+} and pdTp, the T_m, 56.2 °C, is higher than that of wild-type enzyme, 51.9 °C; in the presence of ligands, the T_m, 58.7 °C, is less than that of wild-type enzyme, 62.4 °C. In contrast to this behavior, the T_m for R87K, 47.5 °C, is lower than that of wild-type enzyme in the absence of ligands. Since the ^1H NMR studies to be described indicate that pdTp does not bind readily to R87K, the observed T_m in the presence of ligands, 52.6 °C, is indicative of the thermal stability of the binary Ca^{2+}–R87K complex rather than the ternary pdTp–Ca^{2+}–R87K complex and, as such, cannot be compared with the values determined for R35K and wild-type SNase.

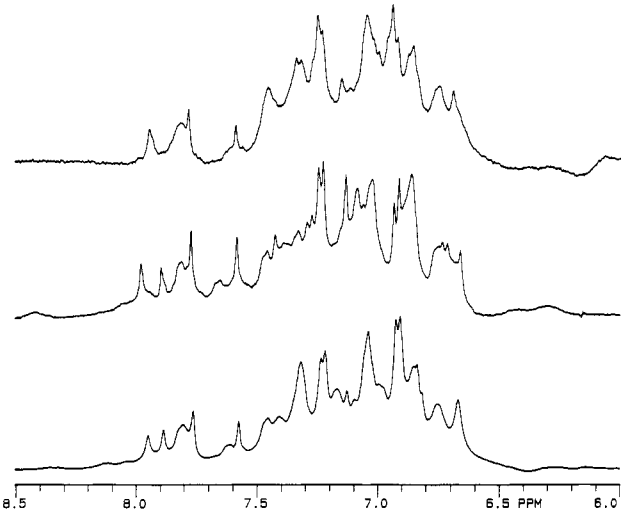

FIGURE 3: Aromatic regions of the 400-MHz ^1H NMR spectra of R35K (bottom), wild-type SNase (middle), and R87K (top) in the absence of ligands. These and all other spectra shown in the figures and in the supplementary material were obtained with 2.5 mM samples of proteins.

In analogy with our recent studies of substitutions for Glu 43, these changes in T_ms suggest that the "conservative" lysine substitutions for Arg 35 and Arg 87 perturb the conformations of the mutant enzymes. However, we note that the behavior of R87K differs from that observed for other active site mutants we have studied, including R35K, in that the substitution *decreases* the T_m in the absence of active site ligands.

^1H NMR Spectroscopy. (A) One-Dimensional Spectra of R35K, R87K, and Wild-Type SNase in the Absence of Active Site Ligands. Our previously reported studies on the effects of active site substitutions on the conformation of SNase, as evidenced by ^1H NMR spectroscopy, revealed that some substitutions alter only the conformation of the unliganded protein, other substitutions alter only the conformation of the liganded protein, and other substitutions alter the conformations of both the liganded and unliganded protein (Hibler et al., 1987). The spectrum of K48A is identical with that of wild-type SNase in the presence of ligands (with the exception of the resonances for the spatially proximal His 46), whereas the spectrum of Y85F is identical with that of wild-type SNase in the absence of ligands (with the exception of the replacement of tyrosine by phenylalanine at residue 85), and the spectra of E43D and E43S differ from that of wild type in both the presence and absence of ligands, although the differences are more pronounced in the presence of ligands.

Given these observations, we have used one-dimensional ^1H NMR spectroscopy to examine whether "conservative" lysine substitutions for Arg 35 and Arg 87 would alter the conformation of the unliganded and liganded states of the mutant proteins. The aromatic proton regions and the upfield-shifted proton regions of the spectra of R35K, R87K, and wild-type SNase in the absence of ligands are compared in Figures 3 and 4, respectively. Recent one- and two-dimensional spectral studies have permitted sequence-specific assignment of the resonances in the aromatic region which arise from the four histidine, three phenylalanine, seven tyrosine, and single tryptophan residues in SNase (Hibler et al., 1987; Wilde et al., 1988; Stanczyk et al., 1988; Sparks & Torchia, 1989). These studies have also allowed assignment of the resonances in the upfield-shifted proton region to specific residues that

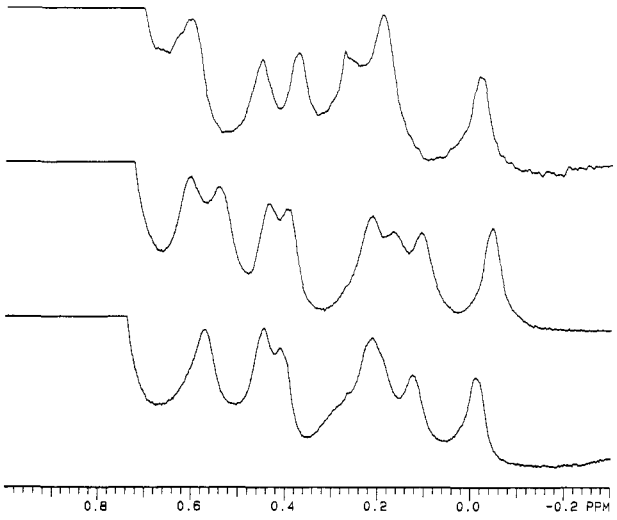

FIGURE 4: Upfield-shifted proton regions of the 400-MHz ^{1}H NMR spectra of R35K (bottom), wild-type SNase (middle), and R87K (top) in the absence of ligands.

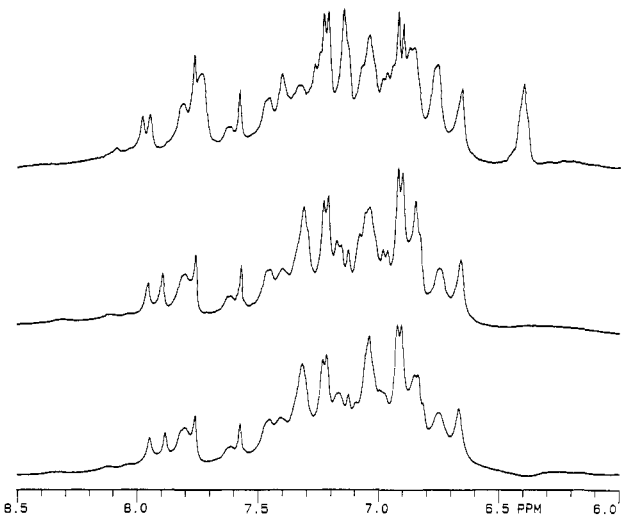

FIGURE 5: Aromatic region of the 400-MHz ^{1}H NMR spectrum of R35K in the absence of Ca^{2+} (bottom) and in the presence of 1 (middle) and 2 (top) equiv of Ca^{2+}.

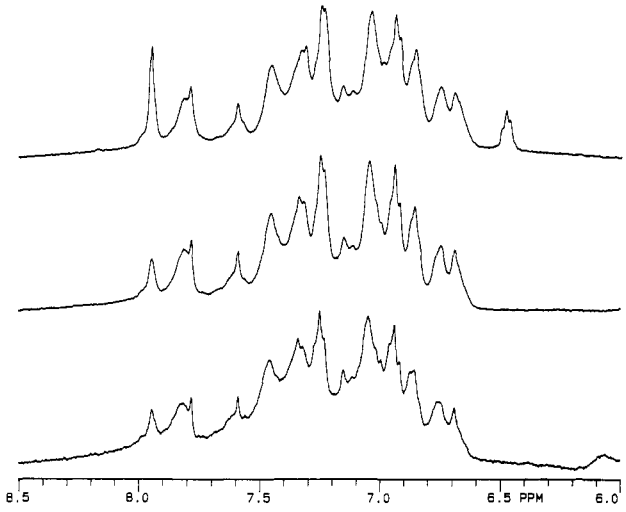

FIGURE 6: Aromatic region of the 400-MHz ^{1}H NMR spectrum of R87K in the absence of Ca^{2+} (bottom) and in the presence of 1 (middle) and 2 (top) equiv of Ca^{2+}.

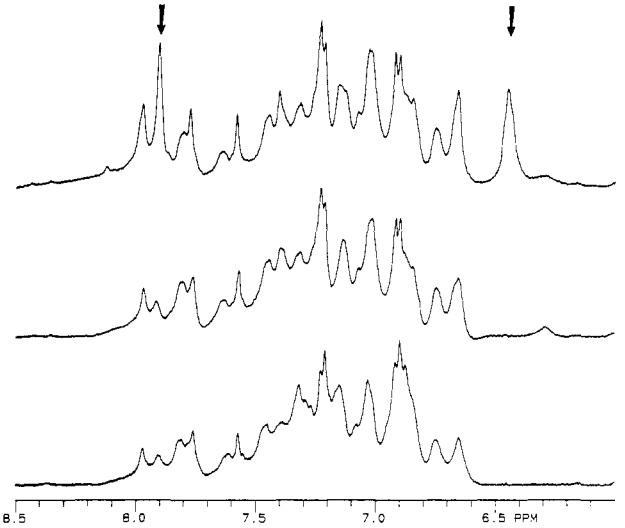

FIGURE 7: Aromatic region of the 400-MHz ^{1}H NMR spectrum of the binary complex of wild-type SNase with 2 equiv of Ca^{2+} in the absence of pdTp (bottom) and in the presence of 1 equiv (middle) and 5 equiv (top) of pdTp.

are spatially adjacent to aromatic residues and, therefore, are upfield shifted due to ring current effects. These upfield-shifted resonances are located either within the hydrophobic core of SNase (Leu 25 and Val 74 are in close proximity to Tyr 27, Phe 34, and Phe 76) or on the surface of SNase (Lys 133 and Ile 139 are in close proximity to Tyr 54 and Trp 140). Both regions are at least 15 Å removed from the essential Ca^{2+} in the active site.

The aromatic and upfield-shifted proton regions of R35K differ from those of wild type although they more closely resemble wild type than the same spectral regions of R87K. Thus, in the unliganded state, both active site substitutions appear to alter the conformation of the protein. Interpretation of the spectral differences between the mutant proteins and wild-type SNase in the presence of ligands requires additional information about the affinities of R35K and R87K for Ca^{2+} and pdTp.

(B) Titration of R35K, R87K, and Wild-Type SNase with Ca^{2+} and pdTp. Since Arg 35 and Arg 87 have both been postulated to interact electrostatically with phosphoryl oxygens of the 5′-phosphate group of pdTp, it is important to establish whether the affinities of the R35K and R87K mutant proteins for pdTp are sufficiently great that ternary complexes of proteins with Ca^{2+} and pdTp can be formed at the millimolar concentrations of proteins and ligands used in ^{1}H NMR experiments. Accordingly, we have investigated the formation of binary Ca^{2+} complexes and ternary pdTp–Ca^{2+} complexes.

Titration of wild-type SNase with Ca^{2+} induces several subtle changes in the aromatic and upfield-shifted proton regions of the spectrum (spectra in the supplementary material). The addition of more than 1 equiv of metal ion induces relatively small additional changes in the proton chemical shifts. Similar results were obtained for the addition of Ca^{2+} to R35K (Figure 5) and to R87K (Figure 6).

Titration of the binary complex of wild-type SNase and Ca^{2+} with pdTp (Figure 7) showed that the addition of 1 equiv of pdTp induces changes in both the aromatic and upfield-shifted proton regions of the spectrum and that additional pdTp does not produce further changes in the chemical shifts of protons associated with the protein. However, small changes in the chemical shifts of the H_6 and $H_{1'}$ protons of bound and free pdTp are observed when more than one pdTp per SNase is added. The changes in chemical shifts for protein resonances observed upon binding of pdTp are consistent with a conformational change induced by binding of pdTp. High-resolution X-ray data demonstrating a conformational change upon pdTp binding have recently been obtained (P. Loll, E. E. Lattman,

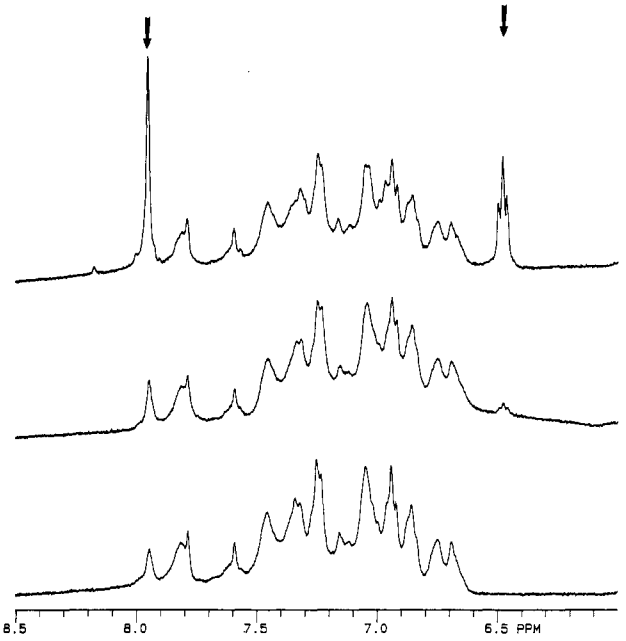

FIGURE 8: Aromatic region of the 400-MHz ^1H NMR spectrum of the binary complex of R87K with 2 equiv of Ca^{2+} in the absence of pdTp (bottom) and in the presence of 1 equiv (middle) and 5 equiv (top) of pdTp.

and R. O. Fox, personal communications).

Titration of the binary complex of R35K and Ca^{2+} with pdTp yielded similar results (spectra in the supplementary material) with the addition of 1 equiv of pdTp, inducing changes in both regions of the spectrum analogous to those observed for the wild-type protein. In contrast to the wild-type SNase, the binding of pdTp to R35K is in fast rather than slow exchange on the basis of the resonance associated with the $H_{1'}$ proton of pdTp.

Titration of the binary complex of R87K and Ca^{2+} with pdTp did not produce changes in the chemical shifts of protons associated with the protein (Figure 8). The chemical shifts of the H_6 and $H_{1'}$ protons of pdTp are nearly identical in the presence and absence of the R87K–Ca^{2+} binary complex, and the line widths of the resolved protons of pdTp are also unaffected by the presence of the binary complex. Since the titrations were performed with millimolar concentrations of the binary complexes and pdTp, the binding constant of pdTp to R87K is extremely weak compared to the binding constant of pdTp to wild-type SNase, approximately 0.2 μM under the conditions of our NMR studies. This latter value was quantitated by measuring the quenching of tyrosine fluorescence as pdTp is bound at micromolar concentrations of enzyme and pdTp. When similar determinations are performed on R35K, weak binding is observed although a value for the binding constant cannot be reliably extracted from the data; when R87K is studied, no binding can be detected by fluorescence measurements.

The extremely low affinity of R87K for pdTp, but not of R35K, is consistent with a large conformational change within the active site in R87K relative to R35K and wild type. Alternatively, binding of the 5′-phosphoryl oxygens of pdTp in wild-type protein involves Arg 87 but not Arg 35. We hesitate to specify the existence of specific interactions between the Arg 35 and Arg 87 given the distinct possibility that the conformations of the active sites of these proteins differ from that of wild-type SNase.

Having established that R35K does bind both Ca^{2+} and pdTp, it is possible to compare the one-dimensional spectrum of this mutant with that of wild-type SNase in the presence of both active site ligands. In the presence of ligands (spectra in the supplementary material), the aromatic and upfield-shifted proton regions of R35K differ from those of wild-type SNase although many features are in common with the wild type. For example, the three methyl resonances at approximately 0.2 ppm that have been assigned to one methyl group of Leu 25 and both methyl groups of Ile 139 are present in both spectra. Also, the chemical shifts of the resonances previously assigned to Val 74 (0.43 and −0.03 ppm in the spectrum of wild-type SNase) occur at different chemical shifts in R35K. Some of the spectral differences between the aromatic regions of R35K and wild type can be explained by the different spectral properties of the H_6 and $H_{1'}$ protons of pdTp, but several differences which are associated with protein resonances can be discerned. The arrows in the spectra mark the positions of the resonances for the bound and free pdTp ligand. However, the changes in chemical shifts which are noted for both the aromatic protons and the upfield-shifted methyl groups support our conclusion that the lysine substitution for Arg 35 alters the environments of the residues we have previously identified to be affected by the aspartate substitution for Glu 43. A number of these residues (Leu 25, Val 74, Tyr 27, Phe 34, and Phe 76) form part of the hydrophobic core of wild-type SNase. In the case of substitutions for Glu 43, the detection of small conformational differences in the hydrophobic core required transmission of significant conformational changes in the active site across a distance of approximately 25 Å. In the case of R35K, residue 35 is adjacent in primary sequence to Phe 34, and a change in the position of the positive charge at the end of the side chain for residue 35 (an arginine side chain is one bond length longer than a lysine side chain) could directly alter the position of the aromatic ring of Phe 34, thereby causing some of the changes in chemical shifts that have been observed.

(C) Detection of the Conformational Changes in R35K by Two-Dimensional NOE Spectroscopy. Given the observation that R87K does not bind pdTp and that its thermal melting behavior differs significantly from that of the other active site mutants we have studied, our further discussion of conformational changes will focus on R35K and on E43D for which high-resolution structural information is now available. The one-dimensional spectral data presented in the previous section reveal that the same resolved upfield methyl groups that were affected by substitutions for Glu 43 are also affected by R35K. X-ray crystallographic studies now support our conclusions, based upon qualitative analysis of NMR data (Hibler et al., 1987; Wilde et al., 1988), that substitutions for Glu 43 induce changes within the active site that are likely to be mechanistically significant. Such qualitative analysis is, therefore, likely to be sufficient for assessing whether significantly different conformations occur within the active sites of R35K and R87K relative to wild type.

Two-dimensional NOE correlation spectra of SNase are particularly well resolved in the section involving the aromatic protons and upfield-shifted methyl groups. Our previous studies have assigned all of the correlations involving Leu 25, Val 74, Tyr 27, Phe 34, and Phe 76 in this spectral region (Wilde et al., 1988). These assignments have recently been independently confirmed and greatly expanded in elegant work (Sparks & Torchia, 1989). The spectra in Figure 9 are slices for the *pro-R* Val 74 methyl group (−0.03 ppm in wild-type SNase) taken from two-dimensional NOE correlation spectra for R35K, E43D, and wild-type SNase in the presence of active site ligands. The correlations that are present have been assigned as follows: 7.8 ppm, F76 ortho; 7.5 ppm Y27 meta;

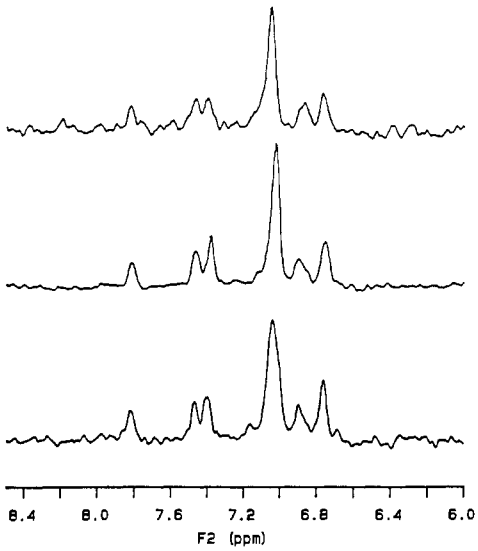

FIGURE 9: Slices from two-dimensional nuclear Overhauser effect correlation spectra that show the correlations between the *pro-R* methyl group of Val 74 (−0.03 ppm) and aromatic protons for protiated E43D (bottom), wild-type SNase (middle), and R35K (top). The intensities of the diagonal peaks for the *pro-R* methyl group of Val 74 and the intensities of the entire spectra were equivalently used to normalize the intensities of the slices extracted from the different spectra; this procedure was also used to normalize the slices shown in Figure 10 and in Figures 9–12 of the supplementary material.

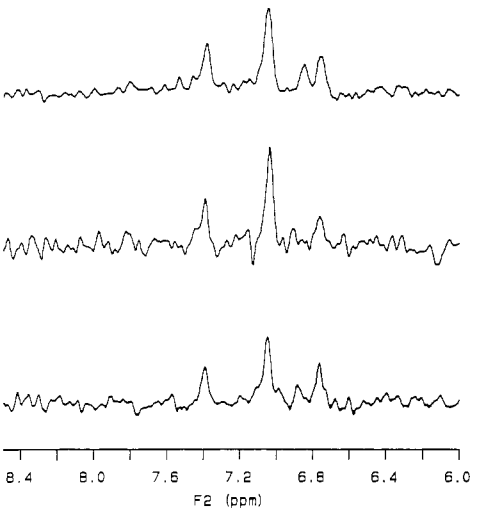

FIGURE 10: Slices from two-dimensional nuclear Overhauser effect correlation spectra that show the correlations between the *pro-R* methyl group of Val 74 (−0.03 ppm) and aromatic protons for samples of E43D (bottom), wild-type SNase (middle), and R35K (top) labeled with ring-deuteriated tyrosine and phenylalanine labeled with deuterium in the ortho positions (h_3-F).

7.4 ppm, F76 meta; 7.0 ppm, F34 ortho and meta; 6.9 ppm, F76 para; 6.8 ppm, F34 para. The intensities of the F34 ortho correlations increase while the F76 ortho and meta decrease in R35K relative to wild type. The contributions of the ortho protons relative to the meta and para can be separated by comparing slices from two-dimensional spectra obtained for completely protiated proteins, proteins with the tyrosines deuteriated and the ortho positions of the phenylalanines deuteriated (h_3-F), and proteins with the tyrosines deuteriated and the meta and para positions of the phenylalanines deuteriated (h_2-F). The slices for the h_3-F samples are shown in Figure 10 and for the h_2-F samples in the supplementary material.

Analogous data for the upfield-shifted methyl group of Leu 25 (0.11 ppm in wild-type SNase) have been analyzed (the data are present in the supplementary material). For example, for this methyl group in the hydrophobic core, the NOE data indicate that the F34 meta proton–L25 methyl group distance is shorter in R35K than in wild-type SNase.

Our analysis of R35K indicates that the same residues previously identified to be conformationally altered in E43D are also altered in R35K. However, the absolute values of the changes that have been detected are larger for E43D than for R35K. It is interesting to note that in some cases the algebraic sign of the change which has been detected differs for the two mutant proteins.

Conclusions. Thermal denaturation and both one- and two-dimensional ^{1}H NMR spectroscopy have provided evidence that the conformations of R35K and R87K differ from that of wild-type SNase. Given the number of cationic and anionic groups in the active site of liganded SNase, these changes in conformation should result primarily from movement of the positive charge caused by replacing the side chain of arginine with the shorter side chain of lysine, with the other cationic and anionic groups within the solvent-sheltered active site electrostatically responding to this change. (Alternatively, the differing helix-forming potentials of lysine and arginine could provide other structural reasons for conformational readjustments in the vicinity of the substitution.) While changes in NOE correlations involving amino acid residues in the hydrophobic core have again been detected and this region is removed from the active site, our previous experience with E43D allows the prediction that these changes are caused by mechanistically significant alterations in active site conformation. Thus, the large changes in kinetic parameters that have been measured cannot be used with confidence to qualitatively or quantitatively study the roles of Arg 35 and Arg 87 in catalysis. We regard the lysine substitutions to be more subtle than substitutions involving uncharged amino acids if electrostatic interactions within the highly charged active site are important; thus, given our present observations and past experiences, we predict that these residues cannot be mutated without effect on the conformation of the active site. Since our opinion is that structural changes preclude any mechanistic interpretations of the observed levels of catalytic activity, the caveat recently raised regarding the potential for transcriptional and translational errors to cause uncertain interpretation of low levels of catalytic activity is irrelevant to our conclusions (Schimmel, 1989).

Our conclusion that pdTp binds to R35K but very weakly, if at all, to R87K may be compared with the results obtained for the glycine substitutions for Arg 35 and Arg 87 (Serpersu et al., 1987). Mildvan and his co-workers found that the V_{max} values for both R35G and R87G were at least 35 700-fold lower than that measured for wild-type SNase and concluded that both Arg 35 and Arg 87 interact with the pentacoordinate transition state that occurs on the reaction coordinate. Mildvan and his co-workers also reported that the dissociation constant of pdTp from the ternary nucleotide–Ca^{2+}–enzyme complex was increased approximately 100-fold in R35G but was indistinguishable from the value measured for wild type in R87G. These kinetic results were used to infer that Arg 87 does not interact directly with pdTp or the substrate complex with DNA. The V_{max} values for both R35K and R87K are also significantly less than that of wild type.

A definitive explanation for the differing results on R35K and R87K relative to R35G and R87G concerning pdTp binding is uncertain, but our denaturation and ^{1}H NMR studies suggest that conformational changes accompany the lysine substitutions for both arginine residues. As we have

argued, it is likely for electrostatic reasons that conformational changes also accompany glycine substitutions and that the different results obtained for the lysine and glycine substitutions indicate that these substitutions have different effects on the conformation of the active site. We believe that these conformational alterations preclude the quantitative mechanistic interpretation put forth previously (Serpersu et al., 1987). (We note that the quantitative interpretations made by Serpersu et al. *are* subject to the problem raised by Schimmel.)

We have already concluded that in the active site of SNase aspartate is not a structurally conservative substitution for the putative general base Glu 43 (Hibler et al., 1987; Wilde et al., 1988). On the basis of the data presented in this article we now conclude that lysines are not structurally conservative substitutions for the putative electrophilic catalysts Arg 35 and Arg 87. It is not feasible to critically examine the consequence of every possible substitution at each of the positions; however, we believe that any substitution for a charged residue in the active site of SNase will be accompanied by a conformational change. While this conclusion may not be particularly surprising to structural enzymologists, it should serve as yet another warning to mechanistic enzymologists that the use of site-directed mutagenesis to quantitate structure/function relationships in catalysis must be accompanied by careful assessment of whether mechanistically probing substitutions are accompanied by structural changes.

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Supplementary Material Available

Aromatic (Figure 1) and upfield-shifted regions (Figure 2) of the spectrum for titration of wild-type SNase with Ca^{2+}, upfield-shifted regions of the spectra for the titrations of R35K (Figure 3) and R87K (Figure 4) with Ca^{2+}, upfield-shifted region of the spectrum for titration of the binding complex of the wild-type SNase and Ca^{2+} with pdTp (Figure 5), aromatic (Figure 6) and upfield-shifted regions (Figure 7) of the spectrum for titration of the binary complex of R35K and Ca^{2+} with pdTp, upfield-shifted region of the spectrum for titration of the binary complex of R87K and Ca^{2+} with pdTp (Figure 8), and two-dimensional nuclear Overhauser effect correlation spectra (Figures 9–12) (12 pages). Ordering information is given on any current masthead page.

References

Adams, J., Johnson, K., Matthews, R., & Benkovic, S. J. (1989) *Biochemistry 28*, 6611.

Bohnert, J. L., & Taniuchi, H. (1972) *J. Biol. Chem. 247*, 4457.

Cotton, F. A., Hazen, E. E., & Legg, M. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2551.

Cuatrecasas, P., Fuchs, S., & Anfinsen, C. B. (1967) *J. Biol. Chem. 242*, 3063.

Fuchs, S., Cuatrecasas, P., & Anfinsen, C. B. (1967) *J. Biol. Chem. 242*, 4768.

Hibler, D. W., Stolowich, N. J., Reynolds, M. A., Gerlt, J. A., Wilde, J. A., & Bolton, P. H. (1987) *Biochemistry 26*, 6278.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488.

Loll, P., & Lattman, E. E. (1989) *Proteins 5*, 183.

Perry, K. M., Onuffer, J. J., Grittelman, M. S., Barmat, L., & Matthews, R. C. (1989) *Biochemistry 28*, 7961.

Schimmel, P. (1989) *Acc. Chem. Res. 22*, 232.

Serpersu, E. H., Shortle, D., & Mildvan, A. S. (1987) *Biochemistry 26*, 1289.

Stanczyk, S. M., Bolton, P. H., Dell'Acqua, M., Pourmotabbed, T., & Gerlt, J. A. (1988) *J. Am. Chem. Soc. 110*, 7908.

Takahara, M., Hibler, D. W., Barr, P. J., Gerlt, J. A., & Inouye, M. (1985) *J. Biol. Chem. 260*, 2670.

Torchia, D. A., Sparks, S., & Bax, A. (1989) *Biochemistry 28*, 5509.

Wilde, J. A., Bolton, P. H., Dell'Acqua, M., Hibler, D. W., Pourmotabbed, T., & Gerlt, J. A. (1988) *Biochemistry 27*, 4127.