Site-Directed Mutants of Staphylococcal Nuclease. Detection and Localization by ^{1}H NMR Spectroscopy of Conformational Changes Accompanying Substitutions for Glutamic Acid-43†

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ABSTRACT: The high-resolution X-ray crystal structure of staphylococcal nuclease suggests that the γ-carboxylate group of Glu-43 is directly involved in catalysis as a general base that facilitates the attack of water on the substrate phosphodiester. We have used primer-directed, site-specific mutagenesis to generate aspartate, glutamine, asparagine, alanine, and serine substitutions for this residue. The V_{max}/K_m for the aspartate mutant is reduced 1400-fold and the values for the charge-neutral mutations are reduced 5000-fold relative to the wild-type enzyme. Although these reductions in catalytic efficiency might appear useful in quantitatively estimating the importance of general basic catalysis in the reaction catalyzed by the wild-type enzyme, the thermal stabilities and ^{1}H NMR spectral properties of the mutants suggest that such interpretations are ambiguous. All five mutants have higher melting temperatures for thermal denaturation than the wild-type enzyme, suggesting that the mutants have enhanced thermal stabilities relative to the wild-type enzyme. Chemical shift changes relative to the wild type are observed in both the aromatic and upfield-shifted methyl group regions of the ^{1}H NMR spectra of the aspartate and serine mutants, suggesting the presence of conformational differences between the wild-type and mutant enzymes. That these conformational differences may be large enough to be mechanistically relevant is suggested by comparisons of the magnitudes of nuclear Overhauser effect (NOE) correlations between the aromatic and upfield-shifted methyl group regions observed via two-dimensional nuclear Overhauser effect correlation spectroscopy. The aromatic protons involved in NOE correlations that differ in intensity in the wild-type and mutant proteins have been localized to the three phenylalanine residues present in the protein. Since these phenylalanine residues are at least 15 Å removed from the position of Glu-43 in the structure of the wild-type enzyme, the substitutions for Glu-43 are accompanied by "global" changes in the conformation of the protein molecule. As such, the kinetic parameters measured for the mutant enzymes cannot be used with confidence to deduce the quantitative importance of Glu-43 in catalysis.

Staphylococcal nuclease catalyzes the hydrolysis of single-stranded DNA or RNA in the presence of Ca^{2+} to yield 3′-mononucleotides as products. The structure of this enzyme is well characterized by virtue of the efforts of the laboratories of Anfinsen, Cotton, and Shortle. Anfinsen's laboratory ascertained the linear sequence of the 149 amino acids designated nuclease A (Cone et al., 1971; Bohnert & Taniuchi, 1972). Cotton's laboratory determined the X-ray structure of nuclease A to 1.5-Å resolution (Cotton et al., 1979), thereby permitting identification of the amino acid residues present in the active site. Shortle cloned the gene for nuclease A and determined its DNA sequence (Shortle, 1983), thereby facilitating the application of recombinant DNA methodology to the study of relationships between structure and function. As a result of the efforts of these laboratories, nuclease A is a particularly attractive system for detailed study of the role of specific amino acid residues in catalysis.

The mechanism presently accepted for the hydrolysis reaction catalyzed by nuclease A is based largely upon interpretation of the high-resolution X-ray structure of a complex of the enzyme with Ca^{2+} and the competitive inhibitor thymidine 3′,5′-bisphosphate (pdTp) (Figure 1). The salient features of the mechanism proposed by Cotton and co-workers are as follows (Cotton et al., 1979). The 5′-phosphate of the inhibitor (and, presumably, of the substrate) is coordinated to the essential Ca^{2+}. The γ-carboxylate group of Glu-43 is hydrogen bonded to two water molecules, with one of these being coordinated to the metal ion and the second being hydrogen bonded to one of the phosphoryl oxygens of the bound inhibitor; this carboxylate group is assumed to facilitate the attack of one of the active site water molecules on the phosphate of the substrate by acting as a general basic catalyst. This mechanism predicts that the hydrolysis reaction should proceed with inversion of configuration at phosphorus, and stereochemical experiments reported by this laboratory have verified this expectation (Mehdi & Gerlt, 1982). However, the only evidence implicating Glu-43 in catalysis remains the high-resolution X-ray structure of a nonproductive complex of nuclease A with pdTp; thus, we have sought direct experimental evidence regarding the role of Glu-43 in catalysis.

In this paper we summarize the results of experiments originally designed to probe the function of Glu-43 in the reaction catalyzed by nuclease A. Site-directed mutagenesis of the codon for Glu-43 to codons for aspartate, glutamine, aspara-

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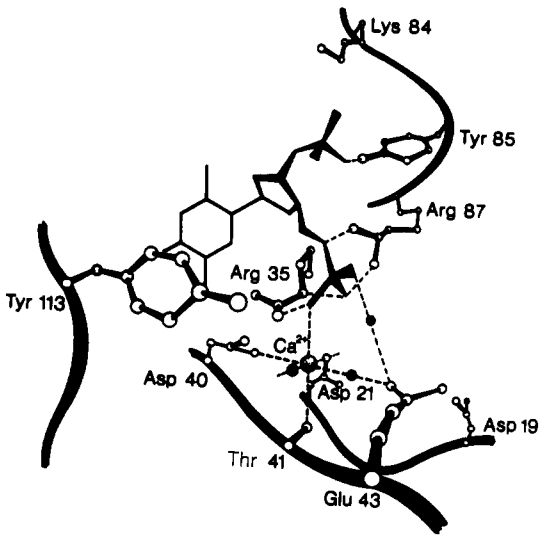

FIGURE 1: Active site structure of nuclease A as derived from the 1.5-Å X-ray coordinates [taken from Cotton et al. (1979)].

gine, alanine, and serine in pONF1, a plasmid that directs the secretion of nuclease A into the periplasmic space of an *Escherichia coli* host (Takahara et al., 1985), has permitted the kinetic and structural characterization of mutant proteins containing substitutions in the position of the putative general basic glutamate residue. All five mutants have V_{max}/K_m values that are reduced from 1400-fold (aspartate) to 5000-fold (glutamine, asparagine, alanine, and serine) relative to the wild-type enzyme. However, these reductions are small compared to the estimated 10^{15}-fold rate acceleration achieved by the wild-type enzyme relative to the uncatalyzed reaction. While these decreases in V_{max}/K_m might be used quantitatively to estimate the importance of general basic catalysis in the reaction catalyzed by the wild-type enzyme, this is not possible given the ^{1}H NMR evidence that the presence of these mutations in the active site has a "global" effect on the conformations of the mutant proteins.

Materials and Methods

The recombinant plasmid pONF1, containing the coding sequence for nuclease A directly fused to the coding sequence for the *E. coli ompA* signal peptide, was described previously (Takahara et al., 1985); this fused gene is under the control of the *lac*UV5 promoter and upon induction by lactose directs the secretion of nuclease A into the periplasmic space of an *E. coli* host. Bacteriophage M13mp11 was obtained from Bethesda Research Laboratories. The primer directing the mutation of Glu-43 to Asp-43, d(CACCTGACACTAAGC), was synthesized by P-L Biochemicals (the positions of the mutagenic mismatches are underlined). The primer directing the mutation of Glu-43 to Gln-43, d(GGTTGATACACCCCAGACAAAGCATCC), was generously prepared by Dr. Philip J. Barr, Chiron Corp., Emeryville, CA. The primers directing the mutation of Glu-43 to Asn-43, d(GATACACCTAATACAAAGC), to Ala-43, d(GATACACCAGCAACAAAGC), and to Ser-43, d(GATACACCTTCCACAAAGCATCC), were prepared in this laboratory on a Systec 1450A DNA synthesizer using phosphoramidite chemistry. The universal M13 sequencing primer, d(GTAAAACGACGGCCAGT), was purchased from New England Biolabs. Three primers for DNA sequence analysis of the hybrid preenzyme gene present in pONF1 were also prepared by Dr. Philip J. Barr: d(GCGGATAACAATTTCACACAGG), a 22-mer complementary to a sequence 57 base pairs (bp) upstream of the initiation codon for the hybrid preenzyme; d(GGTCAACCAATGACATTCAGAC), a 22-mer complementary to codons 29–35 of the nuclease A structural gene; and d(GCTGATGGAAAAATGGTAAACG), a 22-mer complementary to codons 94–100 of the nuclease A structural gene. Restriction endonucleases and bacteriophage T_4 DNA ligase were from New England Biolabs. *E. coli* DNA polymerase (Klenow fragment), bacteriophage T_4 polynucleotide kinase, and calf intestinal alkaline phosphatase were purchased from Boehringer. All enzymes were used according to the supplier's instructions.

[*ring*-$^2H_5$]-L-Phenylalanine, [2,6,α-$^2H_3$]-L-tyrosine, and [3,5-$^2H_2$]-L-tyrosine were purchased from MSD Isotopes. All other chemicals were the highest quality commercially available.

Construction of the M13 Template Used for Mutagenesis. The 385-bp *Xba*I–*Hin*dIII restriction fragment containing the coding sequence for the *ompA* leader peptide fused to the first 100 codons of the coding sequence for nuclease A was isolated from pONF1 and ligated with double-stranded M13mp11 that had been digested with *Xba*I and *Hin*dIII and dephosphorylated with calf intestinal phosphatase; the ligation mixture was used to transfect JM101, and the template was identified by sequence analysis of the single strands isolated from colorless plaques.

Mutageneses. The mutageneses to generate the aspartate, glutamine, and asparagine substitutions were performed with the double-primer method described by Norris et al. (1983) using 5′-phosphorylated mutagenic and universal M13 sequencing primers. Following incubation with DNA polymerase and DNA ligase, the dephosphorylated 7.6-kilobase (kb) *Xba*I–*Hin*dIII restriction fragment obtained from pONF1 was added to the reaction mixture; this mixture of DNAs was digested with both *Xba*I and *Hin*dIII. Following treatment with DNA ligase, the reaction mixture was used to transform HB101. Transformants were screened by colony hybridization followed by DNA sequence analysis. Plasmids containing the desired sequences were used to retransform HB101, and the transformants were subjected to colony hybridization and sequencing.

The mutageneses to generate the alanine and serine substitutions were performed with the double-primer method described by Zoller and Smith (1984) using 5′-phosphorylated mutagenic and universal M13 sequencing primers. Plaques obtained from transfection of JM107 with the mutagenic mixture were screened by plaque hybridization followed by DNA sequence analysis. Single strands containing the mutation were used to transfect JM107, and the resulting plaques were subjected to plaque hybridization and sequencing. The *Xba*I–*Hin*dIII fragment containing each mutation was removed from double-stranded M13 and ligated into the 7.6-kb *Xba*I–*Hin*dIII fragment obtained from pONF1.

Each gene coding for a mutant *ompA*–nuclease A fusion protein at residue 43 was sequenced by using the three sequencing primers to verify the presence of only the desired base changes.

The mutants are designated according to the convention in which the identity of wild-type residue (using the single-letter abbreviation) is followed by the residue number, which in turn is followed by the identity of the mutant residue. For example, the mutant in which glutamate (E) at residue 43 is replaced by aspartate (D) is designated E43D.

Isolation of Wild-Type and Mutant Nucleases. HB101 transformed with pONF1 was used as the source of wild-type nuclease A, and HB101 transformed with the various mutant

plasmids was used as the source of the mutant enzymes. One hundred milliliters of an overnight culture of the appropriately transformed HB101 was used to innoculate 4 L of Luria broth supplemented with 25 mg/L ampicillin. Cell growth at 37 °C was monitored by measurement of OD_{590nm}; when the absorbance reached 0.9, production of enzyme was induced by the addition of lactose to a final concentration of 2 mM. After 3 h, the cells were harvested by centrifugation, and the wild-type and mutant proteins were extracted from the periplasmic space and purified by chromatography on Bio-Rex 70 according to the procedure described by Shortle (1986). Approximately 10 mg of electrophoretically homogeneous nuclease is obtained by this procedure. Further purification for the NMR analyses was accomplished by affinity chromatography on pdTp–Sepharose as described by Cuatrecasas et al. (1968). The extinction coefficients of nuclease A and its mutants were assumed to be identical [ϵ_{280nm} = 9.3 $(mg/mL)^{-1}$ cm^{-1}].

Preparation of Deuteriated Samples of Wild-Type Nuclease. Samples of nuclease-containing deuteriated amino acids were prepared by using an expression plasmid, designated pNJS, in which the *Sau*3A restriction fragment bearing the structural gene for the nuclease is ligated in the unique *Bam*HI site of the expression vector pCQV2 (Queen, 1983); the precise sequence of the *Sau*3A fragment and of the vector leads to the production of an N-terminal-modified nuclease in which the heptapeptide Met-Asp-Pro-Thr-Val-Tyr-Ser is appended to the N-terminal alanine of nuclease A. This gene is under the control of the bacteriophage λ P_R promoter; the vector also carries the gene for the cI857 temperature-sensitive mutation of the λ repressor. Following thermal induction of the gene in the *E. coli* host N4830 (whose chromosome contains a lytic-deficient λ that also carries the same temperature-sensitive gene for the repressor) by raising the temperature of the growth medium from 30 to 42 °C, approximately 50% of the total cellular protein accumulates as the N-terminal-modified nuclease.

Twenty-five milliliters of an overnight culture of the transformed host grown in M9 minimal salt medium with 0.2% glucose as carbon source and supplemented with 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 50 μg/mL thiamin, 50 μg/mL biotin, and 100 μg/mL concentrations of all the L-amino acids except the isotopically labeled aromatic L-amino acid(s) to be incorporated was used as an inoculum for a 500-mL culture of the same medium. When the absorbance of the culture reached 0.9, 100 mg of the desired isotopically labeled L-amino acid(s) was (were) added; production of the isotopically labeled nuclease was induced 15 min later by shifting the temperature of the growth medium from 30 to 42 °C by the addition of 150 mL of the M9 medium heated to 68 °C containing 200 μg/mL concentrations of all L-amino acids except the previously added isotopically labeled amino acid(s). After 2 hours at 42 °C, the cells were harvested by centrifugation. The isotopically labeled enzyme was isolated from the induced cells by a previously described procedure employing Bio-Rex 70 and affinity chromatographies (Calderon et al., 1985). The extinction coefficient of this N-terminal-modified nuclease was assumed to be ϵ_{280nm} = 1.0 $(mg/mL)^{-1}$ cm^{-1}. Three samples of deuteriated wild-type nuclease were prepared by this procedure: [*ring*-$^2H_5$]phenylalanine, [*ring*-$^2H_5$]phenylalanine plus [2,6-$^2H_2$]tyrosine (deuterium meta to the hydroxyl group), and [*ring*-$^2H_5$]phenylalanine plus [3,5-$^2H_2$]tyrosine (deuterium ortho to the hydroxyl group).

Kinetic Studies. Enzyme activity was quantitated with heat-denatured salmon sperm DNA according to the spectrophotometric assay described by Cuatrecasas et al. (1967b). The 1.0-mL reaction mixtures contained 25 mM sodium glycinate, pH 9.49, $CaCl_2$, and DNA. The dependencies of the observed rates of change in absorbance at 260 nm on the concentrations of DNA and of Ca^{2+} were used as input data for Cleland's program HYPER (Cleland, 1979).

Fluorescence Studies. The thermal denaturations of wild-type and mutant enzymes were studied by measuring the decrease in tryptophan fluorescence that occurs upon unfolding; the experiments were conducted in a SPEX F111A spectrofluorometer by using 295 nm as the excitation wavelength, 325 nm as the emission wavelength, and a sample heating rate of 1 °C/min. Approximately 3 nmol of each enzyme was dissolved in 3 mL of 25 mM potassium phosphate, pH 7.0, containing 100 mM NaCl. The fluorescence values of the folded and unfolded states were estimated in the transition region by linear extrapolations from the values of fluorescence at low and high temperatures, respectively, and the differences from these values were used to calculate the equilibrium constant for unfolding K_u. Each T_m (melting temperature) was obtained from a van't Hoff plot of the logarithm of K_u vs. $1/T$.

^1H NMR Studies. For comparisons of the ^1H NMR spectra of wild-type enzyme and the various mutants, samples of each protein (20.2 mg or 1.2 μmol) were separately dissolved in D_2O, warmed to 40 °C for 15 min, and then lyophilized; the dissolution in D_2O and lyophilization were repeated to exchange the amide protons with solvent D_2O. For comparisons of the spectra of the protiated wild-type enzyme with the various deuteriated versions, samples of each protein (25 mg or 1.5 μmol) were analogously treated. A D_2O solution (0.60 mL) containing 50 mM sodium borate buffer, pH 7.8, 0.1 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), pdTp (1.8 mM for the comparisons of the wild-type and mutant enzymes and 3.75 mM for the comparisons of the protiated and deuteriated wild-type enzymes), and 10 mM $CaCl_2$ was added to each protein sample and lyophilized; the concentrations of Ca^{2+} and pdTp are sufficient to produce greater than 95% saturation of the active site of each enzyme as judged by measurement of the binding of pdTp by quenching of the tyrosine fluorescence. Following dissolution in 0.60 mL of D_2O, each sample was filtered through a glass fiber filter into a high-precision 5-mm NMR tube, and the pH meter reading was readjusted to pH 7.8. The final protein concentration of each sample for comparison of wild-type enzyme and mutants was 2.0 mM; the final protein concentration for comparison of protiated and deuteriated samples of wild-type enzyme was 2.5 mM. All ^1H NMR spectra were obtained at 30 °C on either a Varian XL-400 or a Bruker AM-400 NMR spectrometer. The two-dimensional homonuclear nuclear Overhauser effect correlation (NOESY) spectra were obtained with a 150-ms mixing time.

Results and Discussion

Kinetic Analyses. The kinetic properties of the wild-type and mutant enzymes were quantitated at pH 9.5 (the pH optimum of the wild-type enzyme) in the presence of Ca^{2+} with single-stranded DNA as the substrate. The observed values for V_{max}, K_m, V_{max}/K_m, and K_a for Ca^{2+} are summarized in Table I. All five mutant enzymes have significantly reduced V_{max} values and modestly increased K_m values relative to those measured for the wild-type nuclease. The latter observation demonstrates that the low levels of activity associated with each of the mutants cannot be ascribed to a contaminant by wild-

Table I: Kinetic Parameters for Wild-Type and Mutant Enzymes[a]

enzyme	V_{max} [ΔOD/(min·mg)]	K_m (μg/mL)	V/K[b]	K_a for Ca^{2+} (mM)
wild type	5700 ± 460	15 ± 4	1000	0.53 ± 0.05
E43D	29.9 ± 2.2	113 ± 17	0.7	0.40 ± 0.05
E43N	6.2 ± 0.4	75 ± 12	0.2	0.50 ± 0.12
E43Q	1.9 ± 0.1	43 ± 6	0.1	0.41 ± 0.08
E43A	11.7 ± 0.6	134 ± 13	0.2	1.00 ± 0.19
E43S	4.2 ± 0.1	64 ± 6	0.2	1.21 ± 0.07

[a] Measured at pH 9.49. [b] The value for the wild-type enzyme has been assigned as 1000, and the values for the mutants are relative to that of the wild-type enzyme.

type enzyme. In addition, nondenaturing gel electrophoresis followed by activity staining also demonstrates that the activity associated with each charge-neutral substitution is associated with the mutant protein and not a contaminating protein containing a carboxylate group (wild type or E43D). The K_a values for activation by Ca^{2+} are not significantly altered by the amino acid substitution, with this being consistent with the crystallographic observation that the γ-carboxylate of Glu-43 is not a direct metal ion ligand.

The changes in V_{max}/K_m summarized in Table I are small in comparison to the estimated 10^{15}-fold rate acceleration characteristic of the reaction catalyzed by the wild-type enzyme; this estimate of the catalytic efficiency of the nuclease-catalyzed reaction relative to the nonenzymatic reaction is based upon the reported rate of hydrolysis of diethyl phosphate at alkaline pH and high temperature (Bunton et al., 1960; Kumamoto et al., 1956) and may be subject to an error as large as perhaps 2 orders of magnitude. However, despite this large uncertainty, the conclusion remains that the γ-carboxylate group of Glu-43 is not *entirely* responsible for catalysis. The tempting conclusion suggested by these changes in kinetic parameters is that general basic catalysis by the γ-carboxylate group of Glu-43 contributes approximately 4.5 kcal/mol to the estimated 21 kcal/mol decrease in activation energy for hydrolysis of the phosphodiester bonds in DNA brought about by the nuclease; the remainder of catalytic efficiency would presumably be associated with contributions such as proximity effects associated with substrate and Ca^{2+} binding and electrophilic catalysis by Ca^{2+}, Arg-35, and Arg-87. The remainder of this paper describes structural characterization of the wild-type and mutant proteins in solution that bears upon the validity of these mechanistic interpretations of the decreased catalytic efficiencies of the mutant enzymes.

Thermal Denaturation. Wild-type nuclease A undergoes a reversible thermal denaturation that allows a thermodynamic description of the unfolding process (Calderon et al., 1985). We have examined the thermal stability of nuclease A and each of the five mutants by monitoring the quenching of the fluorescence of the single tryptophan residue (at position 140) in the absence of the active site ligands Ca^{2+} and pdTp. The data, reproduced in Figure 2, reveal that each mutant enzyme has a higher T_m than the wild-type enzyme. The T_m for the charge-conserved aspartate mutant is 1.0 °C higher than that of the wild-type enzyme (54.6 vs. 53.6 °C) while the T_m's for the uncharged replacements are even higher (56.3, 56.8, 56.9, and 58.6 °C, respectively, for the glutamine, alanine, asparagine, and serine mutants). These increases in T_m may suggest that the equilibrium between the folded and unfolded forms favors the folded form in the various mutant proteins more than in the wild-type protein. If we assume that the differences in melting temperatures result from differences in conformation, the temperature dependence of the unfolding does not allow distinction of whether the suspected structural changes associated with the introduction of each point mutation occur in the folded or unfolded form (or both) of the mutant enzymes.

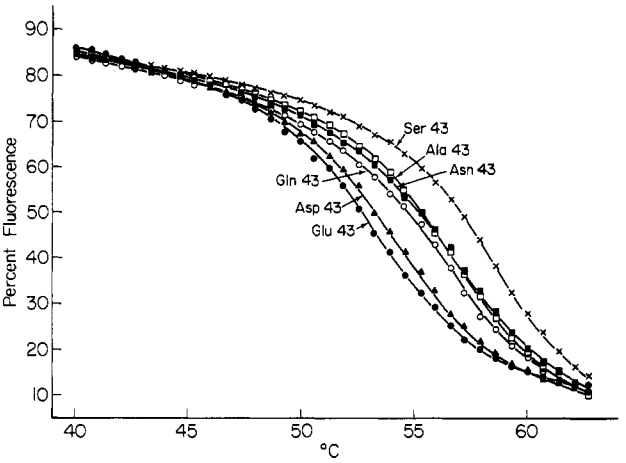

FIGURE 2: Thermal denaturation curves for the wild-type and mutant enzymes. Experimental details are described in the text.

^1H NMR Spectroscopy. In principle, ^1H NMR spectroscopy can detect conformational differences between related proteins and allow a qualitative and, perhaps, quantitative description of structural differences in solution; thus, we have used one- and two-dimensional ^1H NMR experiments to ascertain whether the suspected conformational changes occur in the folded forms of the mutant enzymes. We have focused our attention on selected regions of the high-field (400-MHz) ^1H NMR spectra of the wild type and two mutants, E43D, the charge-conserved replacement, and E43S, the thermally most stable neutral mutant; the one-dimensional ^1H NMR spectra of the remaining neutral mutants are qualitatively identical with that observed for E43S.

The ^1H NMR data presented here are of the aromatic and the upfield-shifted methyl group regions of the spectra of the wild-type enzyme, E43D, and E43S since these regions are relatively simple. The aromatic region contains the resonances associated with protons (total of 56) of the single tryptophan, three phenylalanines, four histidines, and seven tyrosines; the methyl group region contains the resonances associated with the methyl groups (total of 78) of the fourteen alanines, five isoleucines, eleven leucines, four methionines, ten threonines, and nine valines, with about nine of the resonances being shifted upfield of the main aliphatic envelope. We have taken extreme care in obtaining these spectra: samples to be compared were dialyzed at equal protein concentrations in the same vessel of water prior to lyophilization and dissolution in buffer, the protein concentrations of the various samples were as identical as possible, and the pH's of the samples were carefully adjusted immediately prior to spectral acquisition. As a necessary control we have observed that two independently isolated samples of wild-type enzyme have identical spectra in these regions.

We have acquired spectra of the wild-type enzyme and both mutants in the absence and presence of the active site ligands Ca^{2+} and pdTp. The aromatic regions of spectra of E43D, E43S, the wild-type enzyme, and the difference spectra for the two mutants relative to the wild-type enzyme are compared in the absence of ligands in Figure 3 and in the presence of ligands in Figure 4. In the absence of ligands relatively modest changes in the spectra are observed; in the presence of ligands the changes are more pronounced. In the absence of ligands changes in chemical shifts are clearly visible for the protons

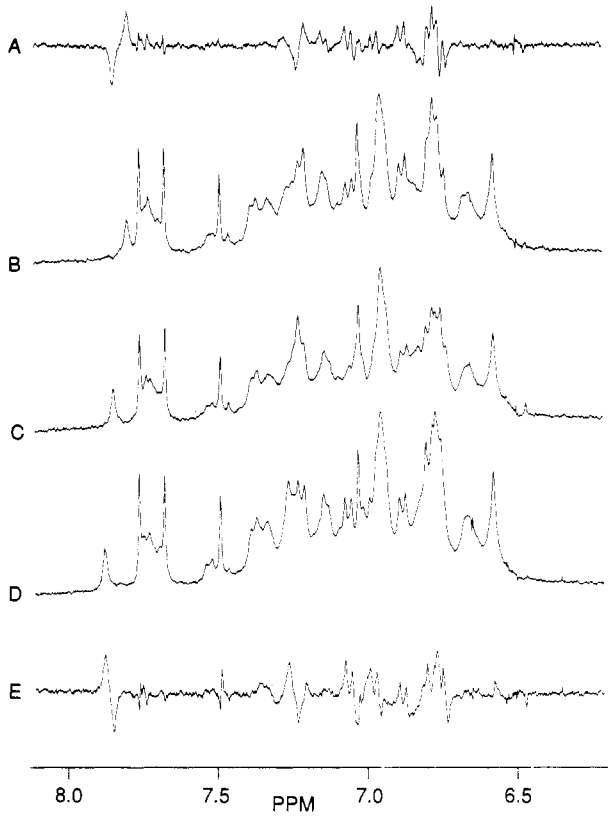

FIGURE 3: Comparison of aromatic regions of ^1H NMR spectra of wild type, E43D, and E43S in the absence of Ca^{2+} and pdTp. The middle spectrum (spectrum C) is that of the wild-type enzyme, and the spectra immediately above (spectrum B) and below (spectrum D) spectrum C are those of E43D and E43S, respectively. The top spectrum (spectrum A) is the difference between E43D and wild type, and the bottom spectrum (spectrum E) is the difference between E43S and wild type.

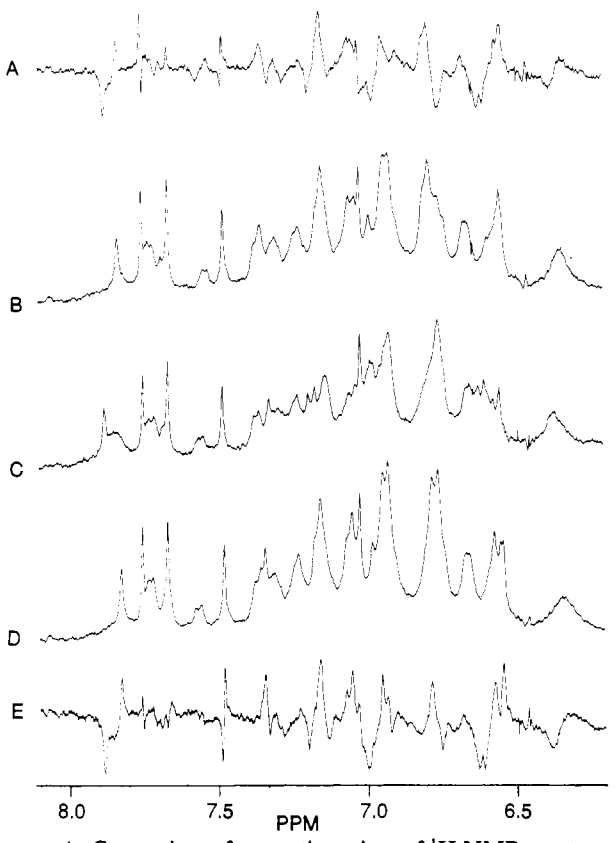

FIGURE 4: Comparison of aromatic regions of ^1H NMR spectra of wild type, E43D, and E43S in the presence of Ca^{2+} and pdTp. The middle spectrum (spectrum C) is that of the wild-type enzyme, and the spectra immediately above (spectrum B) and below (spectrum D) spectrum C are those of E43D and E43S, respectively. The top spectrum (spectrum A) is the difference between E43D and wild type, and the bottom spectrum (spectrum E) is the difference between E43S and wild type.

of a single histidine as well as multiple, more upfield differences that can be associated primarily with protons associated with two tyrosine residues [as judged by superposing the aromatic regions of two-dimensional homonuclear correlation (COSY) spectra of the wild-type and mutant enzymes]. We assume that the histidine whose 2- and 4-protons experience chemical shift changes is His-46. Whereas the changes associated with His-46 are reasonable by virtue of its sequence and spatial proximity to residue 43, the changes in the tyrosine resonances reflect changes remote from the immediate vicinity of residue 43 (the seven tyrosines are at positions 27, 54, 85, 91, 93, 113, and 115, with tyrosines-54, -85, and -113 being the most proximal at approximately 10 Å).

We have also compared the aromatic regions of the spectra of wild-type enzyme and of K48A and Y85F, catalytically active mutants of residues that are located on the surface, since these comparisons serve as necessary and interesting controls for the spectra shown in Figures 3 and 4 (the V_{max} of K48A is the same as that of wild-type enzyme, and that of Y85F is increased about 50%). In the presence of ligands the aromatic region of the spectrum of K48A is identical with that of the wild-type enzyme (top spectra of Figure 6), although in the absence of ligands some differences in tyrosine resonances are noted (top spectra of Figure 5). In the absence of ligands the aromatic region of the spectrum of Y85F is sufficiently identical with that of the wild-type enzyme that the difference spectrum allows the assignment of the resonances associated with the tyrosine in the wild-type enzyme and the phenylalanine in the mutant (bottom spectra of Figure 5). In the presence of ligands the difference spectrum is very complicated (bottom spectra of Figure 6); this is a consequence of our unpublished conclusion that the hydroxyl group of Tyr-85 (as well as the ε-ammonium group of Lys-84) is very important in binding the 3′-phosphate group of pdTp.

We hypothesize that the explanation for the marked chemical shift differences observed in the aromatic regions of the spectra of E43D and E43S but not in the spectra of K48A and Y85F (at least under certain conditions) are related to the solvent accessibility of these residues. On the basis of the available X-ray coordinates for the wild-type enzyme Lys-48 is about 85% exposed to solvent and Tyr-85 is greater than 90% exposed. In contrast, the side chain of Glu-43 is only about 25% exposed. Thus, the substitutions made at residue 43 alter either the position or magnitude of a largely buried charge, and we believe that the energetic consequences of these changes can be propagated through the structure; electrostatic energies are only inversely related to the distance between the charges and the intervening dielectric constant. (The solvent accessibility calculations were performed and provided to us by Dr. Dennis A. Torchia, National Institutes of Health, Bethesda MD.)

The upfield-shifted methyl regions of the spectra of the wild-type enzyme, E43D, and E43S are reproduced in Figure 7. Integrations of the spectra of the wild-type enzyme and E43D are consistent with the presence of nine methyl resonances upfield of 0.7 ppm whereas integration of that of E43S suggests the presence of only eight methyl resonances in this region. Comparison of the spectrum of each mutant relative to that of the wild type reveals that the chemical shifts of most of the resonances are altered in the mutant enzymes, with these

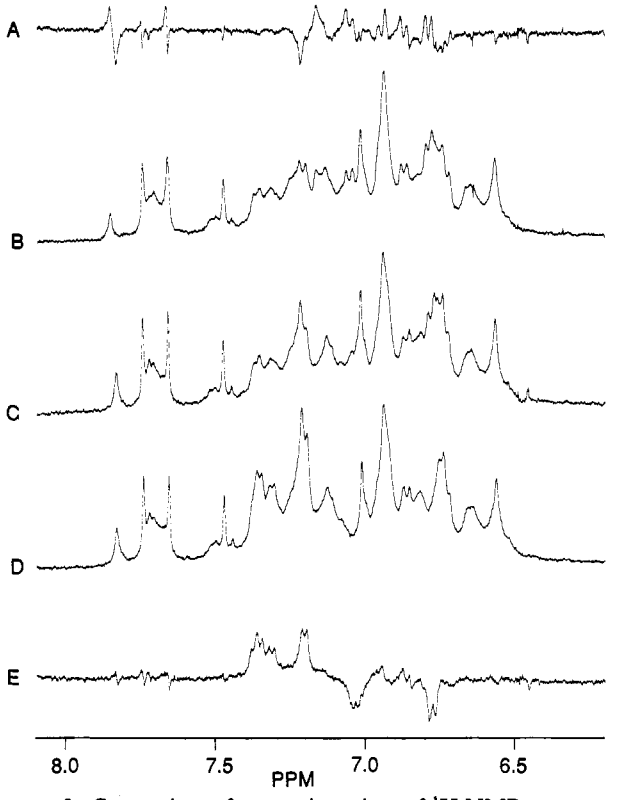

FIGURE 5: Comparison of aromatic regions of ^1H NMR spectra of wild type, K48A, and Y85F in the absence of Ca^{2+} and pdTp. The middle spectrum (spectrum C) is that of the wild-type enzyme, and the spectra immediately above (spectrum B) and below (spectrum D) spectrum C are those of K48A and Y85F, respectively. The top spectrum (spectrum A) is the difference between K48A and wild type, and the bottom spectrum (spectrum E) is the difference between Y85F and wild type.

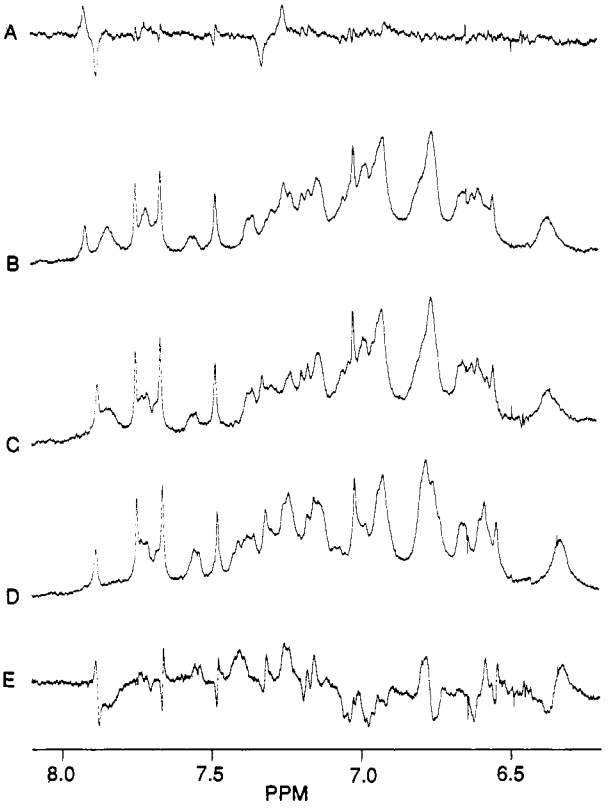

FIGURE 6: Comparison of aromatic regions of ^1H NMR spectra of wild type, K48A, and Y85F in the presence of Ca^{2+} and pdTp. The middle spectrum (spectrum C) is that of the wild-type enzyme, and the spectra immediately above (spectrum B) and below (spectrum D) spectrum C are those of K48A and Y85F, respectively. The top spectrum (spectrum A) is the difference between K48A and wild type, and the bottom spectrum (spectrum E) is the difference between Y85F and wild type.

differences being most simply explained by altered geometric relationships of the associated methyl groups to aromatic rings, peptide carbonyl bonds, and/or a metal ion.

The changes in conformation suggested by the upfield-shifted methyl regions of E43D and E43S have been further investigated by phase-sensitive NOESY spectroscopy (States et al., 1982) of the wild-type and mutant proteins. While these two-dimensional spectra are qualitatively similar in appearance and intensity since they were obtained with samples identical in protein concentration, examination of "slices" of these spectra reveals significant differences in intensities of cross peaks that further confirm the presence of conformational differences in the mutant proteins and potentially permit quantitation of the conformational alterations. Slices from these spectra illustrating such differences are reproduced in Figure 8: the spectra in the left panel depict an NOE correlation from an aromatic proton having a chemical shift of 7.33 ppm and serve as controls since the intensity and frequency of the single NOE cross peak observed in each sample are nearly identical; the spectra in the right panel describe the NOE cross peaks from aromatic proton(s) having chemical shift(s) of 7.03 ppm and reveal that the mutant enzymes have correlations between these aromatic protons and upfield-shifted methyl groups at −0.03 ppm and at 0.43 ppm that are reduced in intensity by a factor of 2. One trivial explanation for the changes observed in the intensities of the correlations in the slices in Figure 8 is increased chemical shift dispersion in the aromatic regions of the spectra of the mutant enzymes due to small changes in chemical shifts of multiple protons that are spatially adjacent to the upfield-shifted methyl groups; however, integrations of the intensities of the correlations

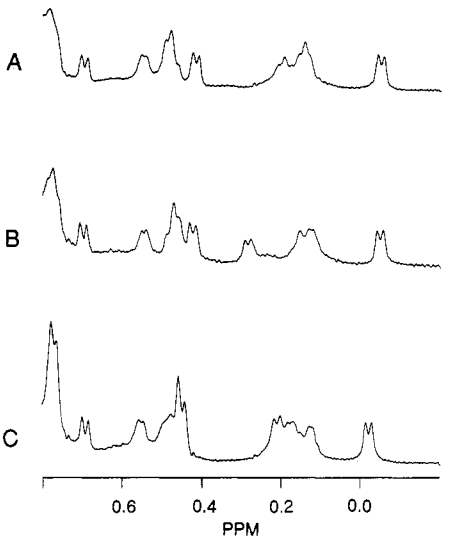

FIGURE 7: Comparison of upfield-shifted methyl group regions of ^1H NMR spectra of E43D (spectrum A), E43S (spectrum B), and wild type (spectrum C) in the presence of Ca^{2+} and pdTp.

present in all slices that contain these cross peaks indicate that the decreases that are apparent in the slices of the spectra of the mutant enzymes accurately reflect the decreases in total intensity and, therefore, are unlikely to result from changes in the chemical shift(s) of the aromatic proton(s). In addition, the NOESY spectra of other mutant enzymes that show altered chemical shifts for the upfield-shifted methyl groups retain correlations to a single chemical shift (and, presumably, a single proton) in the aromatic region of the spectrum.

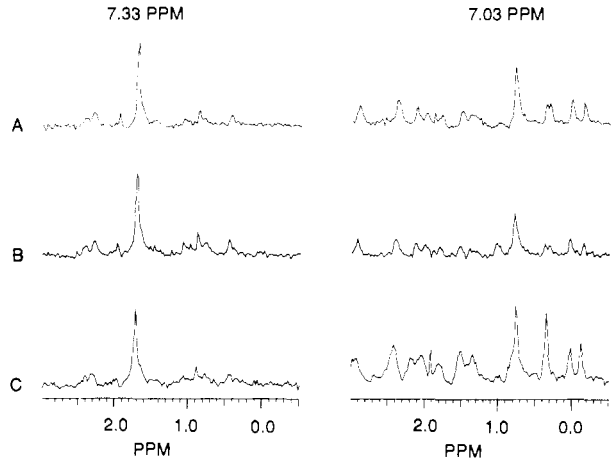

FIGURE 8: Slices from phase-sensitive NOESY spectra of wild type (spectra C), E43S (spectra B), and E43D (spectra A) showing the NOE correlations from 7.33 ppm to the methyl resonances (left panel) and from 7.03 ppm to the methyl resonances (right panel).

The magnitude of an NOE cross peak depends upon several factors: the mixing time, the magnetic field strength, the overall and internal motion correlation times of the protein, the distances between the protons correlated by the NOE cross peak and other protons, and the internuclear distance between the correlated protons. Our NOE data were obtained under identical experimental conditions including both field strength and mixing time. An appreciable change in the overall motion correlation time is very unlikely since the wild-type and mutant proteins are all folded at 30 °C (Figure 2) and presumably have similar conformations, at least in the vicinity of the active site, since all are catalytically active.

The apparent conformational differences between the wild-type and mutant proteins could (and probably to some extent do) involve changes in the internal correlation times of protons of the amino acid residues in the various proteins. Such changes could arise from motional effects that are localized to the site of the amino acid substitution (e.g., a greater degree of side chain motion may be possible when a smaller side chain is introduced at residue 43) or longer range effects that could be associated with changes in the flexibility of larger regions of the protein (e.g., residue 43 is located on a structural loop that shows some disorder in the X-ray structural determination). In the extreme, the internal correlation times of many of the residues could be altered by the amino acid substitution (recall the significant changes in T_m produced by the substitutions). However, the changes associated with the cross peaks shown in the right panel of Figure 8 (factors of 2) would require that the internal motion correlation times of the associated protons change by perhaps as much as a factor of 10 (or more) in the mutant proteins; large changes in internal motion correlation times are likely to result only from significant changes in the potential energy surface, and we assume this would arise only from very large conformational changes that are unlikely for the same reasons used to argue that the overall motion correlation time is likely to be unchanged.

The magnitude of an NOE correlation is dependent not only on the distance between the two protons of interest (A and B) but also on the distances between each of these two protons and all other protons. In practice, protons further away than about 5 Å from A and B can be ignored. As shown elsewhere (Massefski & Bolton, 1985; Bothner-By & Noggle, 1979; Keepers & James, 1984), both the rate of buildup of an NOE between two protons and its equilibrium value depend on the positions of all other protons. A factor of 2 change in the magnitude of an NOE correlation between A and B with A also dipolar coupled to a third proton C requires that the distance between A and C would need to change dramatically if the distance between A and B is unchanged; if the assumption is made that the distance between A and B does not change and that the change in the NOE between A and B is solely due to a change in the distance between A and C, the percentage change in the distance between A and C must be greater than 15% and perhaps even greater than 25%. The importance of this effect in the interpretation of our data is lessened by using short mixing times, such as the 150-ms time we have used for these spectra obtained at 400 MHz.

If the assumption is made that the overall and internal correlation times and the internuclear distances other than that between A and B remain unchanged, the factor of 2 changes observed in the NOEs shown in Figure 8 imply that the distance between the correlated protons has increased by approximately 12% (the intensity of an NOE correlation is inversely related to the sixth root of the distance separating the protons that are dipolar coupled). Since interresidue NOEs can be detected between two protons whose separation is between 3 and 5 Å, 2-fold changes in NOEs such as those described in this article (and other analogous changes that have not been discussed in detail) could, therefore, be associated with changes in interproton distance between 0.4 and 0.6 Å. While the contributions associated with changes in correlation times cannot be evaluated without relaxation data and while changes in distances to other protons cannot be evaluated without deuteriation of such protons, the only possible explanation for the observed changes in intensities of NOE correlations is conformational differences between the wild-type and mutant proteins.

The positions of the conformational differences between the wild-type and mutant enzymes identified by the data shown in Figure 8 have been localized within the structure of the wild-type enzyme by comparing the NOESY spectra of samples of wild-type enzyme that are deuteriated in the phenyl rings of the three phenylalanine residues and in both pairs of protons in the phenolic rings of the tyrosine residues (seven in nuclease A but eight in the N-terminal-modified version of the nuclease used in these labeling experiments). The aromatic regions of the spectra of the three deuteriated enzymes used in these comparisons are shown in Figure 9; the spectrum on the bottom is the protiated enzyme and the remaining spectra are those of enzyme deuteriated in the phenylalanine residues, enzyme deuteriated in the phenylalanine residues plus ortho to the hydroxyls of the tyrosine residues, and enzyme deuteriated in the phenylalanine residues plus meta to the hydroxyls of the tyrosine residues. Comparisons of these spectra reveal very high levels of isotopic enrichment. Difference spectra generated from these spectra can be used to localize the resonances associated with the phenylalanine residues and with the tyrosine residues in the protiated sample; such difference spectra are compared with the spectrum of the protiated enzyme in Figure 10.

NOESY spectra describing the correlations between the aromatic protons and the upfield-shifted methyl region have been obtained for these deuteriated wild-type enzymes and compared with that of protiated enzyme. These comparisons allow the aromatic partner in the NOE differences revealed by the data shown in Figure 8 to be assigned to type of aromatic residue. The data reveal that most of the NOE correlations between the aromatic protons and the upfield-shifted methyl groups involve the three phenylalanine residues. Most of the remaining NOE correlations are associated with protons

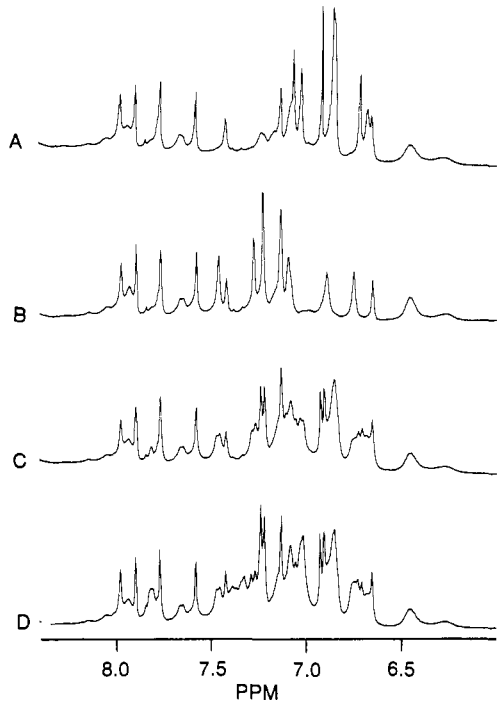

FIGURE 9: Aromatic regions of ^1H NMR spectra of protiated and the three deuteriated samples of wild-type enzyme in the presence of Ca^{2+} and pdTp. From bottom to top, the spectra are as follows: (D) protiated enzyme; (C) enzyme deuteriated with [*ring*-$^2H_5$]-phenylalanine; (B) enzyme deuteriated with [*ring*-$^2H_5$]phenylalanine plus [2,6-$^2H_2$]tyrosine; and (A) enzyme deuteriated with [*ring*-$^2H_5$]phenylalanine plus [3,5-$^2H_2$]tyrosine.

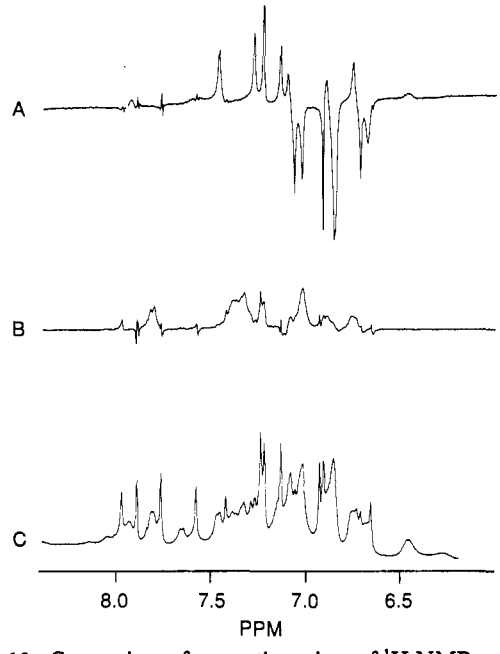

FIGURE 10: Comparison of aromatic regions of ^1H NMR spectra of protiated wild-type enzyme (spectrum C), the difference spectrum between protiated wild-type enzyme and wild-type enzyme deuteriated with [*ring*-$^2H_5$]phenylalanine showing the spectrum of the resonances associated with the phenylalanine residues (spectrum B), and the difference spectrum between wild-type enzyme deuteriated with [*ring*-$^2H_5$]phenylalanine plus [2,6-$^2H_2$]tyrosine and wild-type enzyme deuteriated with [*ring*-$^2H_5$]phenylalanine plus [3,5-$^2H_2$]tyrosine showing the chemical shifts of the ring 3- and 5-protons (positive peaks) and of the ring 2- and 6-protons (negative peaks) (spectrum A).

ortho to the hydroxyl groups of tyrosine residues; this conclusion is based on the NOESY spectra of the remaining deuteriated enzymes. The bases for these statements are shown in Figure 11, in which the slices at 7.03 ppm from the NOESY

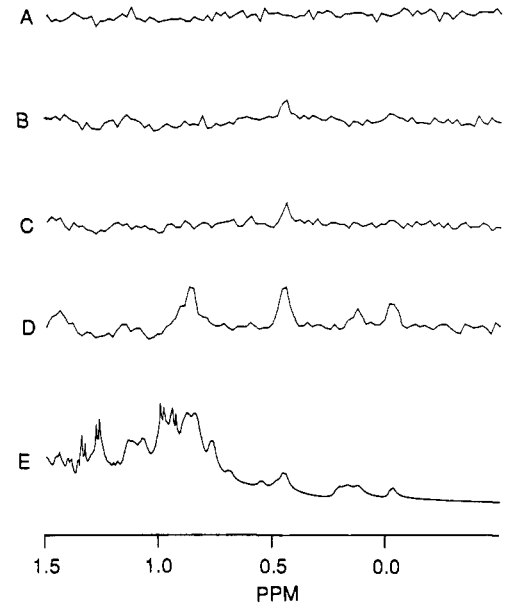

FIGURE 11: Comparison of one-dimensional ^1H NMR spectrum of the methyl region (−0.5 to 1.5 ppm) and slices from the NOESY spectra of protiated and deuteriated wild-type enzyme in the presence of Ca^{2+} and pdTp showing the NOE correlations from 7.03 ppm to the same methyl region. Spectrum E is the one-dimensional spectrum, and the slices (bottom to top) are from NOESY spectra of protiated enzyme (spectrum D), enzyme deuteriated with [*ring*-$^2H_5$]phenylalanine (spectrum C), enzyme deuteriated with [*ring*-$^2H_5$]phenylalanine plus [2,6-$^2H_2$]tyrosine (spectrum B), and enzyme deuteriated with [*ring*-$^2H_5$]phenylalanine plus [3,5-$^2H_2$]tyrosine (spectrum A).

spectra of the protiated enzyme and the three deuteriated enzymes are compared with the one-dimensional spectrum of the upfield-shifted methyl region of the protiated wild-type enzyme. These slices reveal that the resonances at −0.03 ppm and at 0.43 ppm having NOE correlations that are reduced by factors of 2 in the mutant enzymes are spatially adjacent to phenylalanine protons and that the methyl group associated with the resonance at 0.43 ppm *may* also be proximal to protons ortho to a tyrosine hydroxyl group. A COSY spectrum of the wild-type enzyme reveals that these two upfield-shifted methyl groups are scalar coupled to the same resonance at 1.44 ppm, although the presently available data do not allow association of these resonances to a valine or leucine residue.

Since the nuclease contains only three phenylalanine residues, the NOE data for the deuteriated enzymes place considerable restrictions on the locations of the aliphatic residues that experience changes in both chemical shift and magnitudes of NOE correlations from aromatic protons in the mutant enzymes. Phe-61 is located approximately 15 Å from Glu-43 in the structure of the wild-type enzyme; Leu-103 and Leu-108 are spatially proximal to Phe-61. Phe-34 and Phe-76 are located approximately 18 and 28 Å, respectively, from Glu-43; Leu-25 and Val-74 are spatially proximal to these phenylalanine residues as well as to Tyr-27. (The longest dimension of the nuclease molecule is about 35 Å.) With the exception of Tyr-27, all of these residues are located within a structural region that can be regarded as the hydrophobic core of the nuclease molecule. Although the NOE data do not yet allow unequivocal assignment of the phenylalanine and aliphatic amino acid residues that are related by the altered NOE correlations in the mutant enzymes, the necessary localization of these residues to regions of the wild-type enzyme that are distant from residue 43 provides evidence that the introduction of amino acid substitutions for Glu-43 is accompanied by at least one wide-ranging conformational change within the protein molecule. The upfield-shifted methyl regions in both

E43D and E43S (Figure 7) reveal additional chemical shift changes suggestive of other conformational changes that are likely to be located in the region of the hydrophobic core; for that matter, many chemical shift changes are evident in all regions of the spectra of E43D and E43S, although the conformational changes responsible for changes in the more complex regions of the spectra will not be as easy to localize as those in the well-resolved upfield-shifted methyl region.

The NMR spectra we have presented for the wild-type and mutant enzymes describe their average conformations in the presence of Ca^{2+} and pdTp. The differences that are induced by the mutations may simply arise from a change in the distribution of conformations available to each protein; i.e., an "active" conformation may be accessible to the mutant proteins, but the proportion of this conformation may be much less than in the wild-type protein. If this explanation is correct, the decreases in V_{max}/K_m we have observed for the mutants may arise from the energetic requirements to alter the distribution such that the conformation capable of catalyzing the hydrolysis reaction is favored. In any event, *the relationship of these conformational changes to the kinetic properties of the mutant enzymes is unknown.*

Conclusions. The qualitative comparison of the conformations of the wild-type and mutant versions of nuclease A accomplished by ^1H NMR spectroscopy leads to the unfortunate conclusion that the changes in kinetic parameters measured for E43D and E43S relative to the wild-type enzyme (Table I) cannot be confidently attributed to the energetic consequences of removing a general basic catalyst since they may also include contributions of unknown magnitude from the conformational changes in the mutant proteins. We are extending our NMR studies so that the magnitudes of the conformational changes that we have detected can be better quantitated as well as uniquely assigned to specific sites in the protein molecules. However, our observations illustrate that the active site mutations we have made in staphylococcal nuclease do cause conformational changes remote from the position of the mutation and contrast with recent crystallographic characterizations of mutants of other proteins (Howell et al., 1986; Katz & Kossiakoff, 1986). In addition, our results also serve as a clear caution to those who seek quantitative interpretation of kinetic prameters obtained for mutants of staphylococcal nuclease (Serpersu et al., 1987) and perhaps other proteins in the absence of high-resolution information about solution conformation.

Added in Proof

Two additional isotopically labeled samples of the wild-type enzyme have been studied in which perdeuteriated valine and perdeuteriated leucine have been separately incorporated. Comparison of the upfield-shifted methyl regions of the ^1H NMR spectra of these samples with that of the protiated enzyme both demonstrates a high degree of isotopic incorporation of the deuteriated amino acids into valine and leucine residues and, more importantly, allows type assignment of the methyl resonances at 0.43 and −0.03 ppm to valine. This type assignment of these resonances suggests that the conformational change we have detected can be further restricted to the vicinity of Val-74, Phe-34, and Phe-76, which as noted above is well removed from the position of the amino acid substitutions at residue 43. These data will be described in detail in a future publication.

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