Kinetic and Magnetic Resonance Studies of the Glutamate-43 to Serine Mutant of Staphylococcal Nuclease†

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ABSTRACT: The Glu-43 residue of staphylococcal nuclease has been proposed to function as a general base that facilitates the attack of water on the phosphodiester substrate [Cotton, F. A., Hazen, E. E., & Legg, M. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2551–2555]. With DNA as substrate, V_{max} in the glutamate-43–serine (E43S) mutant enzyme is decreased by 2700-fold at pH 7.4 but only 376-fold at pH 9.9. With the wild-type enzyme, V_{max} increases with pH to pH 9.2, above which it becomes less sensitive to further increase in pH, leveling off at pH 9.8. In contrast, V_{max} of the E43S mutant continues to rise, first order in [OH^-], to pH 9.8. Above pH 10 both activities fall irreversibly. Hence the hydroxyl ion can partially replace the effect of Glu-43 on k_{cat}, in accord with the proposed role of Glu-43 as a general base. The inflection point in the curve relating pH to log V_{max} of the wild-type enzyme at pH 9.4 may reflect the ionization of a Ca^{2+}-bound water, or of a Lys or Tyr residue at the active site. The activator Ca^{2+} and the competitive inhibitor Mn^{2+} bind to the E43S mutant an order of magnitude more weakly than to the wild-type enzyme as detected by kinetics and by direct metal binding studies, and approximately one additional water ligand on Mn^{2+} is found in the binary Mn^{2+} complex of the E43S mutant (1.4 ± 0.2) as compared to that of the wild-type enzyme (0.8 ± 0.2). These data suggest that Glu-43 coordinates the divalent cation in the binary enzyme–metal complex but dissociates from the metal to create a water binding site and to function as a general base in the ternary enzyme–metal–DNA complex. While a 2-fold weaker binding of DNA to the Ca^{2+} complex of the E43S mutant than to the wild-type enzyme is found by kinetic studies, an order of magnitude tighter binding of the competitive inhibitor 3′,5′-pdTp to the Mn^{2+} and Ca^{2+} complexes of E43S is found by direct binding studies. Distances from Co^{2+} to phosphorus in the ternary enzyme–Co^{2+}–pdTp complexes reveal coordination of only the 5′-phosphate by Co^{2+} on the wild-type enzyme but coordination of both the 3′- and 5′-phosphates of pdTp on the E43S mutant. This additional ligand to the metal in the ternary pdTp complex of E43S explains both the tighter binding of pdTp and the greater synergy in metal and pdTp binding in the E43S mutant. Smaller structural changes at the active site of functional DNA complexes of the E43S mutant are suggested by the kinetic data.

Mechanisms for the hydrolysis of DNA phosphodiesters catalyzed by staphylococcal nuclease generally include the proposal that Glu-43 functions as a general base (Cotton et al., 1979; Hibler et al., 1987). A recent mechanism based on the X-ray structure of the ternary enzyme–Ca^{2+}–pdTp[1] complex (Cotton et al., 1979), on the stereochemistry of the reaction (Mehdi & Gerlt, 1982), and on the kinetic and thermodynamic effects of mutations of active site residues (Serpersu et al., 1986, 1987, 1988; Hibler et al., 1987) is shown in figure 1. The earliest evidence for the importance of Glu-43 in the mechanism of staphylococcal nuclease was the observation of ≥100-fold lower activity of a semisynthetic enzyme in which Glu-43 was replaced by Asp (Chaiken & Sanchez, 1972). Modern, site-specific mutations of this residue reveal a 190-fold lower k_{cat} of the E43D mutant and 10^3- to 10^4-fold lower maximal activities of the E43N, E43Q, E43A, and E43S mutants (Hibler et al., 1987). While the proposed role of Glu-43 as a general base is reasonable on the basis of its proximity to both inner-sphere and second-sphere water ligands of Ca^{2+} which, in turn, are near enough to the phosphorus to attack it, mutations of Glu-43 also cause remote structural changes in the protein as detected by increases in thermal stability, and by changes in chemical shifts ascribable to 0.4–0.6-Å movements of Val-74 relative to both Phe-34 and Phe-36, which are 18 and 28 Å, respectively, from Glu-43 (Hibler et al., 1987; Wilde et al., 1988).

Although the effects of these small and remote structural changes on the conformation at the active site and on catalytic activity are not clear, they raise the possibility that some, or all, of the 10^4-fold decrease in k_{cat} found with the Glu-43 mutants E43S, E43Q, and E43A may result from conformational changes at the active site rather than from the loss of general-base catalysis. The E43S mutant was chosen for detailed kinetic and structural studies because of its profoundly decreased k_{cat}, and because the low residual activity of the E43Q mutant might have resulted, in part, from the spontaneous deamidation of Gln-43 to generate the wild-type enzyme. The E43A mutant was not chosen since it replaces a polar residue with a nonpolar one. With the E43S mutant, OH^- can partially replace the effect of Glu-43 on k_{cat}, in accord with the proposed role of Glu-43 as a general base. While structural changes at the active site in enzyme–metal–pdTp complexes of the E43S mutant are detected by magnetic resonance methods, smaller alterations are found in functional

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[1] Abbreviation: pdTp, thymidine 3′,5′-diphosphate.

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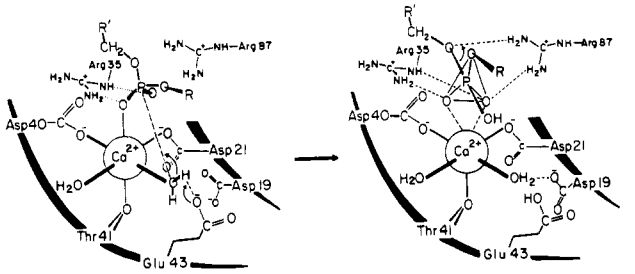

FIGURE 1: Mechanism of phosphodiester hydrolysis catalyzed by staphylococcal nuclease based on structural studies of Ca^{2+}, Mn^{2+}, and Co^{2+} complexes (Cotton et al., 1979; Serpersu et al., 1986, 1987, 1988) and on kinetic studies of enzymes mutated at positions 21, 35, 40, 41, 43, and 87 (Serpersu et al., 1986, 1987; Hibler et al., 1987).

DNA complexes by kinetic analysis. A preliminary report of this work has been published (Mildvan et al., 1988).

Experimental Procedures

Materials

Isolation of the Enzymes. Isolation of the wild-type staphylococcal nuclease from the engineered strain of *Escherichia coli* carrying the expression plasmid pFOG405 was performed as described previously (Serpersu et al., 1986). The construction and sequence analysis of the mutant E43S gene were previously reported (Hibler et al., 1987). *E. coli* carrying the expression plasmid for E43S was grown overnight and diluted into complete MOPS medium, as described earlier (Serpersu et al., 1986), with the exception that the complete MOPS medium contained 1.0 mM KH_2PO_4 rather than 0.1 mM KH_2PO_4. After 30 min of incubation at 37 °C, production of E43S was induced by the addition of isopropyl β-D-thiogalactopyranoside to 1.6 mM final concentration. After 6 h, the cells were harvested by centrifugation and the E43S mutant enzyme was extracted from the periplasmic space and purified by chromatography on Bio-Rex 70 as described for the wild-type enzyme (Serpersu et al., 1986). On the basis of SDS–polyacrylamide gel electrophoresis, both the wild-type and the E43S mutant enzymes were homogeneous.

The nucleotide 3′,5′-pdTp was obtained from P-L Biochemicals. Before use, buffer and nucleotide solutions were passed over Chelex 100 resin to remove trace metals. Salmon sperm DNA was purchased from Sigma, and DNA used in the enzyme assays was denatured by heating for 30 min at 100 °C, followed by rapid cooling on ice (Cuatrecasas et al., 1967).

Methods

Enzyme Assay. The enzymatic activity was measured by observing the absorbance increase at 260 nm as DNA is hydrolyzed (Cuatrecasas et al., 1967). One unit of enzymatic activity is defined as the amount of enzyme causing a change of 1.0 absorbance unit/min at 260 nm in a 1-cm cell. Protein concentrations were determined by the absorbance at 280 nm ($E^{0.1\%}_{1\ cm}$ = 0.93 at neutral pH) (Dunn et al., 1973; Tucker et al., 1978). The assay mixture consisted of 8.75–87.5 μg of DNA and 0.4–4.0 mM $CaCl_2$ in either 40 mM Tris·HCl, pH 7.4, or 40 mM glycine–NaOH, pH 9.86, in a volume of 1.0 mL at 24 ± 1 °C. At pH 7.4, 17.7 μg of E43S was added to start the reaction. At pH 9.86, the reaction was started by the addition of 0.02 μg of wild-type enzyme (M_r = 16 800) or 4.45 μg of E43S (M_r = 16 800), in accord with their differing specific activities. Under all conditions studied, the activities of both the wild-type and E43S mutant enzymes were proportional to their respective protein concentrations, and no hydrolysis of DNA was observed when enzyme was absent. Kinetic parameters of the wild-type enzyme at pH 7.4 were previously determined (Serpersu et al., 1986).

For the pH studies, the assay mixture was shown to contain saturating amounts of DNA in all measurements on the basis of the observation of indistinguishable rates with both 50 and 85.2 μg of DNA. The concentration of Ca^{2+} was varied over the range 0.4–4.0 mM, and the buffer was either 40 mM Tris·HCl from pH 7.4 to 8.6 or 40 mM glycine–NaOH from pH 8.6 to 10.3 in a total volume of 1.0 mL. The reaction was started by the addition of 0.02 μg of wild-type enzyme or 7.08 μg of E43S. Control experiments at pH 8.6 with both buffers indicated that the change of buffer did not affect the measured activities of the wild-type enzyme or of E43S.

In the kinetic experiments with E43S using Mn^{2+} or Co^{2+} as inhibitor, the assay mixtures consisted of 64–70 μg of DNA, six concentrations of $CaCl_2$ ranging from 0.4 to 4.0 mM, and either four concentrations of $CoCl_2$ (0–1.45 mM) in 40 mM NaTES, pH 7.4, or four concentrations of $MnCl_2$ (0–199 μM) in 40 mM Tris·HCl, pH 7.4, in a volume of 1 mL at 24 ± 1 °C. The reaction was started by the addition of 17.7 μg of E43S to the reaction mixture. Tris buffer was not used in the experiments with Co^{2+} because it is known to bind Co^{2+} (Hall et al., 1962). Instead, TES which has a low affinity for Co^{2+} was used for kinetic and magnetic resonance experiments. Free Co^{2+} and Mn^{2+} concentrations were estimated by assuming similar binding of Co^{2+} and Mn^{2+} to DNA (Hanlon et al., 1978), and by using the dissociation constant (68 μM) and stoichiometry (0.38 ± 0.04 site/DNA phosphorus) found for Mn^{2+} (Slater et al., 1972). In the inhibition studies with Co^{2+}, the results are not very sensitive to the value of this dissociation constant because the concentration of Co^{2+} exceeded the concentration of binding sites on DNA by at least an order of magnitude. The calculated concentrations of free Co^{2+} approximated the concentrations of Co^{2+} in excess of the concentration of binding sites.

In all kinetic experiments velocity was determined from the linear portion of the recorder trace and expressed either as the change in absorbancy per minute per milligram of protein or as s^{-1}, which is calculated by assuming the total change in absorbance = 0.300 for the complete reaction in the presence of 50 μg of denatured DNA (Cuatrecasas et al., 1967) and by estimating the micromolar concentrations of DNA from the micrograms per milliliter with the assumption that the substrate is an average tetranucleotide of molecular weight 1400. Expression of the concentration of DNA in micromolar units also permits comparison of K_M values of DNA with dissociation constants of the substrate analogue pdTp.

Analysis of Kinetic Data. The data obtained from the spectrophotometric enzyme assays at pH 9.86 for wild-type enzyme and E43S and at pH 7.4 for E43S were plotted in double-reciprocal form as initial velocity vs the concentration of free Ca^{2+} and as initial velocity vs the concentration of DNA as previously described (Serpersu et al., 1986, 1987). Secondary plots were made from these two primary plots to obtain K_A^{Ca}, K_M^{DNA}, K_S^{DNA}, K_M^{Ca}, and V_{max} as described earlier (Serpersu et al., 1986, 1987). In the analyses of all of the kinetic data, lines in the primary plots were computed by a weighted least-squares analysis (Cleland, 1979), and the lines in the secondary plots were computed by a linear least-squares analysis.

Water Proton Relaxation Rate Measurements. The longitudinal relaxation rate of water protons was measured with a Seimco pulsed NMR spectrometer at 24.3 MHz as described previously (Mildvan & Engle, 1972) by using the 180°–τ–90° pulse sequence method of Carr and Purcell (1954) and

Table I: Kinetic Parameters of Wild-Type and Mutant (E43S) Staphylococcal Nuclease[a]

enzyme	pH	V_{max} [$\Delta A/(min \cdot \mu g)$]	K_A^{Ca} (μM)	K_M^{Ca} (μM)	K_M^{DNA} (μM)[b]	K_S^{DNA} (μM)[b]	K_I^{Mn} (μM)	K_I^{Co} (μM)
wild type	7.4	$(7.14 \pm 0.04) \times 10^{-1}$	460 ± 60	110 ± 20	2.50 ± 0.6	12.8 ± 0.5	6.8 ± 4.2	350 ± 80
E43S	7.4	$(2.68 \pm 0.13) \times 10^{-4}$	2170 ± 150	950 ± 100	5.81 ± 0.6	28.6 ± 4.3	51 ± 8	570 ± 93
wild type	9.9	7.15 ± 0.1	408 ± 40	317 ± 20	2.43 ± 0.1	3.25 ± 0.2		
E43S	9.9	$(1.90 \pm 0.1) \times 10^{-2}$	6760 ± 600	6060 ± 400	3.25 ± 0.1	6.33 ± 0.2		

[a] The data for the wild-type enzyme at pH 7.4 is from Serpersu et al. (1986). K_M^{Ca} is the Michaelis constant of Ca^{2+} at saturating [DNA], K_M^{DNA} is the Michaelis constant of DNA at saturating [Ca^{2+}], K_A^{Ca} is the K_M of free Ca^{2+} extrapolated to zero [DNA], and K_S^{DNA} is the K_M of DNA extrapolated to zero [Ca^{2+}]. [b] Micromolar concentrations of DNA have been calculated from the micrograms per milliliter by assuming the substrate to be an average tetranucleotide of molecular weight 1400. The factor 1.40 converts these values to units of $\mu g/mL$.

Mildvan and Engle (1972). The observed enhancement of the relaxation rate is defined as $\epsilon^* = (1/T_{1P}^*)/(1/T_{1P})$, where $1/T_{1P}$ is the paramagnetic contribution to the relaxation rate in the presence (*) and absence of the enzyme (Mildvan & Engle, 1972).

The correlation times τ_c for the electron nuclear dipolar interaction in the binary enzyme–Mn^{2+} and ternary enzyme–Mn^{2+}–pdTp complexes were determined by studying the frequency dependence of $1/T_{1P}$ of water protons at 15, 19, 24.3, 30, 38, 48, and 59.8 MHz, on a Seimco pulsed NMR spectrometer equipped with a variable-frequency probe as described above. The τ_c values in the ternary enzyme–Co^{2+}–pdTp complexes were determined by studying the frequency dependence of $1/T_{1P}$ of water protons at 15, 24.3, 42, and 59.8 MHz on the Seimco instrument, at 250 MHz on a Bruker WM250 spectrometer, and at 360 MHz on a Bruker AM360 spectrometer, as described above. Evaluation of τ_c from the frequency-dependent $1/fT_{1P}$ values was done as described earlier (Mildvan & Gupta, 1978; Mildvan et al., 1980). Samples contained in 40 mM TES, pH 7.4, either 0.96 mM wild-type enzyme with equimolar pdTp and 0.81 mM $CoCl_2$ or 0.58 mM E43S with equimolar pdTp and 0.51 mM $CoCl_2$. The observed relaxation rates were corrected for the paramagnetic effects of free Co^{2+} in each system, which was always less than 18% of the total Co^{2+} concentration.

Binding Studies. The concentration of free Mn^{2+} in a mixture of free and bound Mn^{2+} was determined by electron paramagnetic resonance (Cohn & Townsend, 1954) with a Varian E-4 EPR spectrometer. The EPR and proton T_{1P} data were analyzed as previously described (Mildvan & Cohn, 1963, 1966; Mildvan & Engle, 1972; Serpersu et al., 1986, 1987) to determine the stoichiometry of Mn^{2+} ions bound to each enzyme (n), the dissociation constant (K_D), and the enhancement of the binary enzyme–Mn^{2+} complex (ϵ_b). Titrations of binary enzyme–Mn^{2+} complexes with pdTp were analyzed by computer as previously described (Reed et al., 1970; Mildvan & Engle, 1972) to yield dissociation constants and enhancement factors of ternary complexes (ϵ_T). In addition, the binding of Mn^{2+} to the enzyme–pdTp complex was monitored by EPR and also by changes in T_{1P} of water protons, thereby providing independent measurements of the dissociation constants of Mn^{2+} from ternary enzyme–Mn^{2+}–pdTp complexes.

The dissociation constants of Ca^{2+} and Co^{2+} from the binary enzyme–Ca^{2+} and Co^{2+}–pdTp complexes and from the ternary enzyme–Ca^{2+}–pdTp and enzyme–Co^{2+}–pdTp complexes were determined by competition with Mn^{2+}. Measured dissociation constants of Mn^{2+} from the binary and ternary complexes (Serpersu et al., 1987; this work) were then used to calculate the concentrations of residual free enzyme or pdTp or enzyme–pdTp complexes following the displacement of Mn^{2+} by Ca^{2+} or Co^{2+} as observed by the changes in ϵ^* by measuring $1/T_{1P}$ of water protons. In this way the concentrations of bound Ca^{2+} or Co^{2+} in appropriate complexes and their dissociation constants were determined. In addition to $1/T_{1P}$ measurements, EPR was also used to determine the concentrations of free Mn^{2+} in the displacement experiments with Co^{2+} to independently determine the dissociation constants of Co^{2+}.

^{31}P Relaxation Rate Measurements. To determine the paramagnetic effects of Co^{2+} on the relaxation rates of the phosphorus nuclei of 3′,5′-pdTp bound to E43S, 2.0-mL samples were prepared which contained 10 mM TES, pH 7.4, 28 mM NaCl, 20% ^2H_2O for field/frequency locking, 0.39 mM E43S, and 4.18 mM pdTp. Under these conditions 98% of the E43S was in the enzyme–pdTp complex. The solution was then titrated with $CoCl_2$, and the increases in the longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the 3′- and 5′-phosphates of pdTp were measured. The data were analyzed by plotting the increases in the relaxation rates against the increases in concentration of Co^{2+} bound in the ternary E43S–Co^{2+}–pdTp complex. The slopes obtained this way, when multiplied by the total pdTp concentration, yielded the normalized relaxation rates ($1/fT_{1P}$) and ($1/fT_{2P}$), where f is defined as [bound metal ion]/[total nucleotide]. The normalized relaxation rates of the ternary complex were then corrected by subtracting the small paramagnetic effects in the residual binary Co^{2+}–pdTp complex to yield $(1/fT_{1P})_{corr}$ and $(1/fT_{2P})_{corr}$. These small corrections, at most 10%, were based on the known distribution of Co^{2+} among its binary and ternary complexes as calculated from the respective dissociation constants and the measured paramagnetic effects of Co^{2+} on $1/T_1$ and $1/T_2$ of the phosphorus in the binary Co^{2+}–pdTp complex.

^{31}P spectra were obtained at 101.25 MHz by using 12-bit analog to digital conversion, collecting 8K data points over a spectral width of 1500 Hz with an acquisition time of 2.73 s. Routine spectra were acquired by collecting 128–512 transients, by use of a 25-s delay to obtain fully relaxed spectra. The longitudinal relaxation rates ($1/T_1$) of the ^{31}P resonances were measured by the nonselective saturation–recovery method which permitted shorter recycle times. However, in all cases the recycle times were at least $5T_1$. Transverse relaxation rates ($1/T_2$) were derived from the widths of resonances at half-height ($\Delta\nu_{1/2}$), where $1/T_2 = \pi\Delta\nu_{1/2}$.

Results

Kinetic Parameters of the E43S Mutant of Staphylococcal Nuclease. Figure 2 shows a kinetic analysis of the E43S mutant of staphylococcal nuclease at pH 7.4 obtained by systematic variation of the concentrations of the Ca^{2+} activator and the DNA substrate. A comparison of the resulting kinetic parameters with those previously obtained with the wild-type enzyme (Table I; Serpersu et al., 1986) indicates a 2700-fold lower V_{max} with the E43S mutant (Figure 2B,F). The binding of Ca^{2+} to the mutant enzyme is 4.7-fold weaker and to the enzyme–DNA complex is 8.6-fold weaker as revealed by the K_A^{Ca} and K_M^{Ca}, respectively (Figure 2B,C, Table I). Weaker

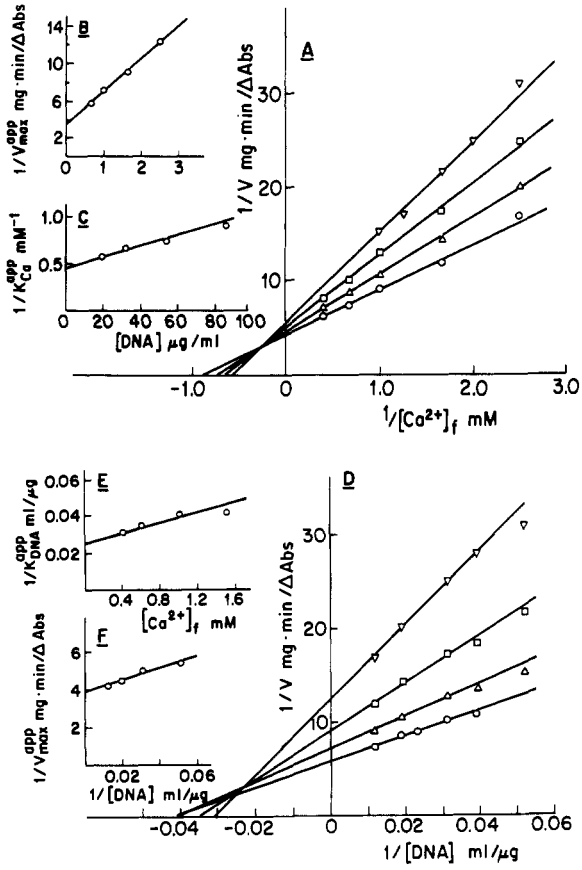

FIGURE 2: Kinetics of activation of staphylococcal nuclease mutant E43S by Ca^{2+} and DNA. (A) Double-reciprocal plot of initial velocity vs Ca^{2+} concentration. DNA concentrations in micrograms per milliliter were 19.2 (▽), 32.0 (□), 53.3 (△), and 85.2 (○). The assay medium also contained 40 mM Tris·HCl, pH 7.4. The reaction, in a total volume of 1.0 mL at 24 °C, was started by the addition of 10 μL of a solution containing 17.7 μg of E43S in the same buffer. The velocity of the reaction is defined as the absorbance change at 260 nm per milligram of enzyme per minute. (B) Secondary plot of extrapolated apparent V_{max} values at infinite DNA concentration from (A) against free Ca^{2+} concentration in double-reciprocal form, to determine K_M^{Ca} and V_{max}. (C) Reciprocal of the extrapolated apparent K_M^{Ca} (K_{Ca}^{app}) obtained from the intercepts on the abscissa from (A) plotted against DNA concentration, to determine the activator constant of Ca^{2+} (K_A^{Ca}) by extrapolation to zero DNA concentration. These secondary plots yielded K_M^{Ca} = 0.95 mM, V_{max} = 0.282 ΔA/(mg·min), and K_A^{Ca} = 2.17 mM. (D) Double-reciprocal plot of initial velocity against DNA concentration. Free Ca^{2+} concentrations in millimolar were 0.4 (▽), 0.6 (□), 1.0 (△), and 1.5 (○). (E) Secondary plot of the reciprocal of the extrapolated apparent K_M^{DNA} values (K_{DNA}^{app}) obtained from the intercepts on the abscissa of (D), against free Ca^{2+} concentrations which yielded, on extrapolation to zero $[Ca^{2+}]$, the dissociation constant of the enzyme–DNA complex (K_S^{DNA}) as 40 μg/mL (28.6 μM). (F) Secondary plot of the extrapolated apparent V_{max} at infinite Ca^{2+} concentration (V_{max}^{app}) against DNA concentrations in double-reciprocal form, which yielded K_M^{DNA} = 8.13 μg/mL (5.81 μM) and V_{max} = 0.253 ΔA/(mg·min). In the primary plots, the data points are shown together with the lines computed by a weighted least squares analysis (Cleland, 1979), and in the secondary plots, the lines are computed by a linear least-squares analysis.

metal binding to the active site of the E43S mutant is also detected by the 7.5- and 1.6-fold larger K_I values for Mn^{2+} and Co^{2+}, respectively (Table I). As found with the wild-type enzyme (Serpersu et al., 1986, 1987), Mn^{2+} and Co^{2+} do not activate the E43S mutant but are linear competitive inhibitors with respect to Ca^{2+} (data not shown). The binding of DNA to the mutant enzyme is weakened by a factor of ~2 as detected kinetically by extrapolation of the apparent K_M^{DNA} to both zero and infinite Ca^{2+} (Figure 2E,F, Table I).

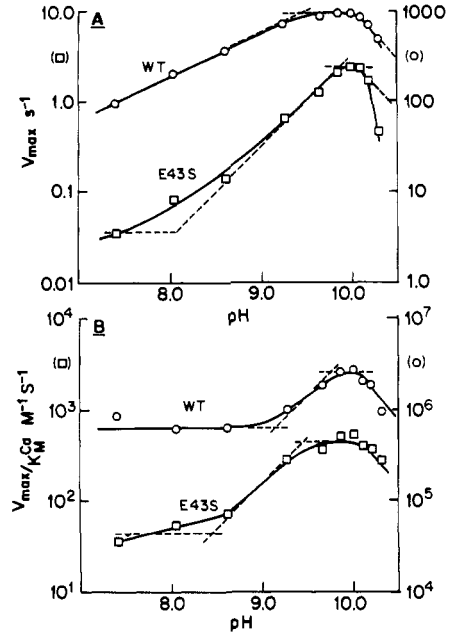

FIGURE 3: Effect of pH on the kinetic parameters of the wild-type and E43S mutant of staphylococcal nuclease. (A) Logarithmic plot of V_{max} against pH for the wild-type enzyme (○) and for the E43S mutant (□). (B) log (V_{max}/K_M) plotted against pH for the wild-type enzyme (○) and for E43S mutant (□). In (A) and (B) the ordinate on the right refers to the values for the wild-type enzyme and on the left to the E43S mutant. Assay media contained 40 mM Tris·HCl from pH 7.4 to 8.6 or glycine–NaOH from pH 8.6 to 10.3, saturating amounts of DNA (50 μg/mL for the wild-type enzyme and 85.2 μg/mL for E43S), and 0.4–4.0 mM Ca^{2+} in a total volume of 1.0 mL at 24 °C. The reactions were started with the addition of either 0.02 μg of wild-type enzyme or 7.08 μg of E43S. V_{max} and K_M for each pH value are obtained from the double-reciprocal plots of initial velocity vs Ca^{2+} concentrations. Intersecting tangents to the curves are shown as broken lines.

Effects of pH on the Kinetic Parameters of E43S and Wild-Type Staphylococcal Nuclease. Raising the pH from 7.4 to 9.9 weakens Ca^{2+} binding and slightly tightens DNA binding to both the E43S and wild-type enzymes as detected by small changes (within an order of magnitude) in the extrapolated K_M values for Ca^{2+} and DNA (Table I). Much larger effects of pH on V_{max} are detected. Raising the pH from 7.4 to 9.9 increases the V_{max} of the wild-type enzyme by a factor of 10 and increases that of the E43S mutant by the significantly larger factor of 71, such that the E43S mutant becomes only 376-fold less active than the wild-type enzyme at the higher pH. A detailed study of the effects of pH on V_{max} (Figure 3A) indicates that with the wild-type enzyme V_{max} increases with pH up to pH 9.2, above which it rises significantly less with pH, approaching an asymptote at pH 10. With the E43S mutant, V_{max} continues to increase with pH in proportion to the first power of $[OH^-]$, up to pH 9.8, above which it begins to level off. Above pH 10, both activities fall irreversibly, probably due to denaturation. Thus, as the pH is increased from 9 to 10, the V_{max} of the E43S mutant shows a significantly greater sensitivity to $[OH^-]$ and begins to catch up with the V_{max} of the wild-type enzyme, indicating that hydroxide ion can partially replace Glu-43 in promoting catalysis by staphylococcal nuclease. If alkaline denaturation had not occurred above pH 10, V_{max} of the E43S mutant might well have more closely approached that of the wild type.

Points of inflection in the curves relating V_{max} to pH occur at pH 9.4 ± 0.2 and 10.1 for the wild-type enzyme, and at 9.9 and 10.1 for the E43S mutant (Figure 3A), possibly reflecting pK_A values of essential groups in the respective ternary

Table II: Dissociation Constants of Binary Complexes of Mn^{2+}, Ca^{2+}, and Co^{2+} and Enhancement Factors of Mn^{2+} Complexes

ligand	n^c	K_D^{Mn} or K_I^{Mn} (μM)^c	ϵ_b^e	K_D^{Ca} or K_I^{Ca} (μM)^{c,d}	K_I^{Co} (μM)^{e,f}
wild type	0.95 ± 0.03[a]	416 ± 22[a]			
	1.00 ± 0.05[b]	460 ± 63[b]	8.4 ± 0.7	510 ± 70	
E43S	0.85 ± 0.09[a]	3400 ± 180[a]			
	0.75 ± 0.11[b]	3250 ± 300[b]	5.8 ± 0.4	5400 ± 1600	
3′,5′-pdTp	1.6 ± 0.2[a]	474 ± 50[a]	1.7 ± 0.1	1200 ± 700	209 ± 30

[a] Determined by EPR. The parameters for the wild type are from Serpersu et al. (1986). [b] Determined by $1/T_{1P}$* of protons, using ϵ_b. [c] n is the stoichiometry of metal binding, K_D is the dissociation constant of the binary enzyme–metal complex, and K_I is the dissociation constant of the binary metal–nucleotide complex. [d] Determined by competition with Mn^{2+}, measuring $1/T_{1P}$* of water protons. [e] Determined by EPR and $1/T_{1P}$ of water protons. [f] Determined by competition with Mn^{2+}, measuring free [Mn^{2+}] by EPR and measuring $1/T_{1P}$* of water protons.

enzyme–Ca^{2+}–DNA complexes. A study of the effect of pH on V_{max}/K_M^{Ca} (Figure 3B) reveals inflections at pH 9.8 and 10.0 with the wild-type enzyme, and at 9.4 and 10.1 with the E43S mutant, possibly reflecting pK_A values of essential groups in the respective binary E–DNA complexes. Alternatively, the inflections at pH 10.0 and 10.1 may result from alkaline denaturation.

Binary Mn^{2+} and Ca^{2+} Complexes of the E43S Mutant. The binding of Mn^{2+} to the E43S mutant was detected by two independent methods, EPR, which measures residual free Mn^{2+}, and the enhanced paramagnetic effect of bound Mn^{2+} on the $1/T_1$ of water protons, which measures bound Mn^{2+}. A Scatchard plot based on the EPR data (Figure 4A) reveals 0.85 ± 0.09 binding sites with a dissociation constant, 3400 ± 180 μM, which is 8-fold weaker than that observed with the wild-type enzyme (Table II). Very similar results were obtained by the $1/T_1$ measurements, which yield, in addition, an enhancement factor, ϵ_b, for the binary enzyme–Mn^{2+} complex of 5.8. This value is significantly smaller than that found for the wild-type enzyme (ϵ_b = 8.4, Table II), reflecting a differing liganding environment in the E43S mutant.

The competitive displacement of Mn^{2+} by Ca^{2+} from the binary complex of the E43S mutant was detected by both EPR and $1/T_1$ measurements. Titrations based on the latter effect (Figure 5A) yielded a dissociation constant for the binary enzyme–Ca^{2+} complex of 5.4 ± 1.6 mM (Table II), in reasonable agreement with the kinetically determined K_A^{Ca} of 2.2 ± 0.2 mM (Table I). As with Mn^{2+}, the E43S mutant binds Ca^{2+} an order of magnitude more weakly than does the wild-type enzyme (Table II), in accord with the kinetic measurements (Table I).

Ternary Enzyme–Metal–pdTp Complexes. As shown by direct (Figure 4B) and competitive titrations (Figure 5B), the presence of the substrate analogue 3′,5′-pdTp increases the affinity of wild-type staphylococcal nuclease for Mn^{2+} and Ca^{2+} by factors of 31 and 7.2, respectively, as seen by comparing the K_D values of these cations (Table II) with their K_A' values (Table III). Significantly greater tightening of metal binding by pdTp is observed with the E43S mutant, the presence of pdTp increasing the affinities of E43S for Mn^{2+} and Ca^{2+} by factors of 294 and 23.4, respectively (Table III). Scatchard plots of both the EPR data (Figure 4B) and $1/T_{1P}$ data (not shown) yielded approximately one tight binding site for Mn^{2+} with the K_A' values listed in Table III. The n and K_A' values obtained by both methods showed good agreement. A comparison of the K_A' values determined by binding studies (Table III) with the K_I values of Mn^{2+} determined kinetically (Table I) shows reasonable agreement, considering the structural differences between 3′,5′-pdTp and DNA.

To determine K_3, the dissociation constant of pdTp from the ternary enzyme–Mn^{2+}–nucleotide complex, solutions of enzyme and Mn^{2+} were titrated with pdTp, and changes in the enhancement of $1/T_{1P}$ of water protons were measured (Mildvan & Engle, 1972; Serpersu et al., 1986, 1987). Such

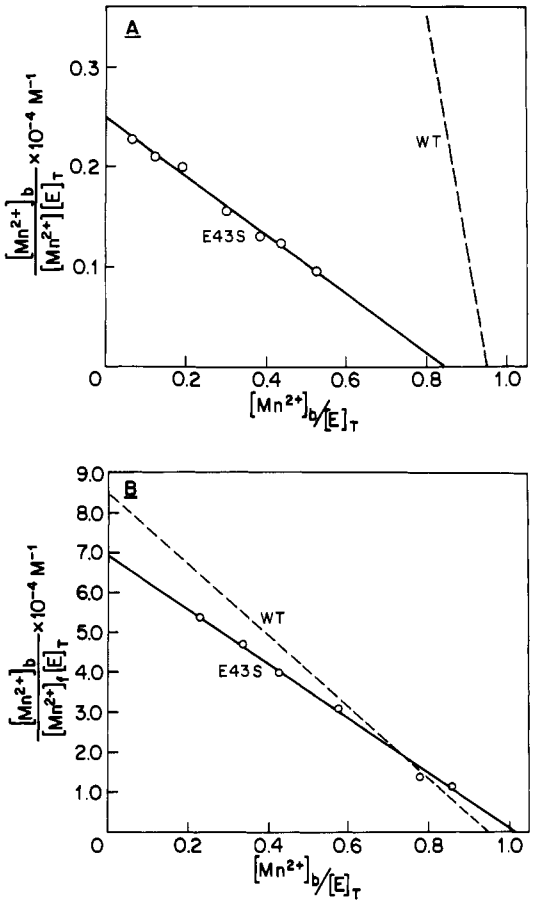

FIGURE 4: Scatchard plots of Mn^{2+} binding to the E43S mutant of staphylococcal nuclease in the absence or in the presence of 3′,5′-pdTp. For comparison, the corresponding plots for the wild-type enzyme are shown (- - -) (Serpersu et al., 1986). (A) Titration of the E43S mutant with Mn^{2+}. The solution contained either 0.707 or 1.49 mM E43S and 40 mM Tris·HCl, pH 7.4. (B) Titration of the E43S mutant with Mn^{2+} in the presence of 3′,5′-pdTp. The solution contained 48.6 μM E43S, 48.8 μM 3′,5′-pdTp, and 40 mM Tris·HCl, pH 7.4. In (A) and (B), the free Mn^{2+} concentration was determined by EPR spectroscopy, and the lines represent linear least-squares fits to the data points. The symbols $[Mn^{2+}]_b$, $[Mn^{2+}]_f$, and $[E]_T$ represent bound Mn^{2+}, free Mn^{2+}, and the total enzyme concentrations, respectively.

titrations of E43S are shown in Figure 6 together with a titration of the wild-type enzyme replotted from Serpersu et al. (1986). Computer-fitted curves taking into account all of the complexes present yielded 11.4-fold tighter binding of pdTp in the ternary E43S–Mn^{2+}–pdTp complex as compared to the ternary complex of the wild-type enzyme (Table III). Similarly, pdTp was found to bind 4.7-fold more tightly in the ternary Ca^{2+} complex of the E43S mutant than to that of the wild-type enzyme as determined indirectly by competitive titrations with Mn^{2+} using the relationship $K_3 = K_A'K_S/K_D$ (Table III). The computed values of K_3 and K_S reveal that Mn^{2+} raises the affinities of both wild-type enzyme and the

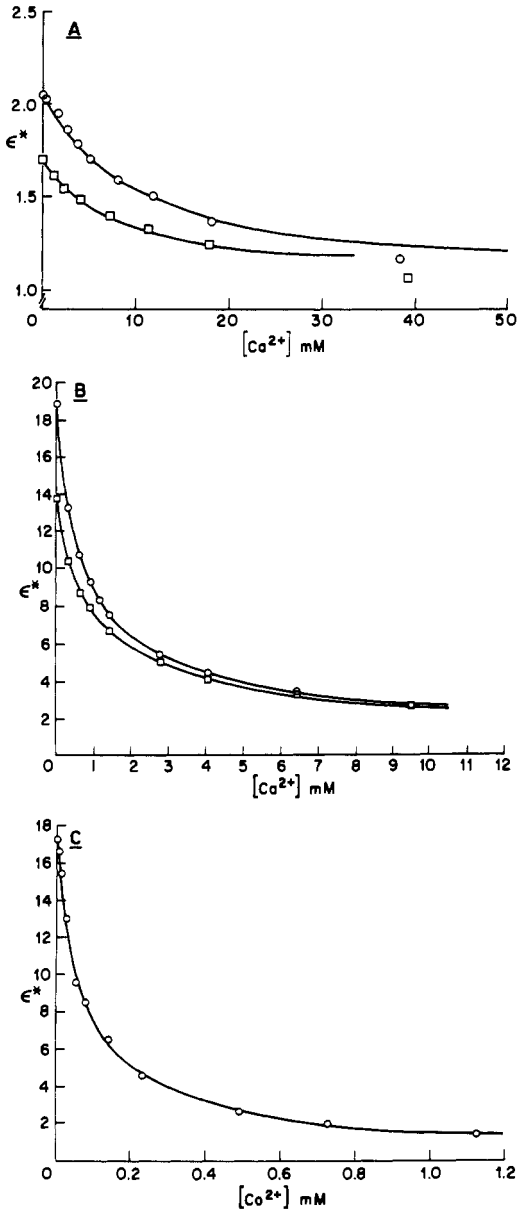

FIGURE 5: Displacement of Mn^{2+} by Ca^{2+} and Co^{2+} in complexes of the E43S mutant of staphylococcal nuclease detected by changes in the enhancement of the effects of Mn^{2+} on $1/T_1$ of water protons. (A) Displacement of Mn^{2+} by Ca^{2+} from the binary Mn^{2+} complex of E43S. Solutions contained 707 μM E43S with either 142 μM Mn^{2+} (○) or 322 μM Mn^{2+} (□), as well as 40 mM Tris·HCl, pH 7.4. From the change in the observed enhancement (ϵ^*), which is proportional to the displacement of enzyme-bound Mn^{2+}, the concentrations of free and enzyme-bound Mn^{2+} were determined. The measured dissociation constant of Mn^{2+} from the binary enzyme–Mn^{2+} complex (Table II) was then used to calculate the concentrations of residual free enzyme following the displacement of Mn^{2+} by Ca^{2+}. In this way the concentration of bound Ca^{2+} and the dissociation constant of Ca^{2+} were determined. (B) Displacement of Mn^{2+} by Ca^{2+} from the ternary E43S–Mn^{2+}–pdTp complex. Solutions contained 40 mM Tris·HCl, pH 7.4, 48.6 μM E43S, 48.8 μM pdTp, and either 24 μM Mn^{2+} (○) or 47 μM Mn^{2+} (□). Free Mn^{2+} concentration was determined as in (A) and the concentration of free E43S–pdTp complex was calculated from the known dissociation constant of Mn^{2+}, which was then used to determine the concentration of free and bound Ca^{2+}. Hence the dissociation constant of Ca^{2+} from the ternary complex (K_A') was evaluated. (C) Displacement of Mn^{2+} by Co^{2+} from the ternary E43S–Mn^{2+}–pdTp complex. Solutions contained 40 mM Na^+TES, pH 7.4, 66 μM E43S, 62 μM pdTp, and 62 μM Mn^{2+}. Dissociation constant of Co^{2+} from the ternary complex was calculated as in (B). In (A–C), to avoid dilution of other components, Ca^{2+} or Co^{2+} additions were made from concentrated stock solutions, which also contained all of the other components of the titration solutions at the same final concentrations. Temperature was 24 °C.

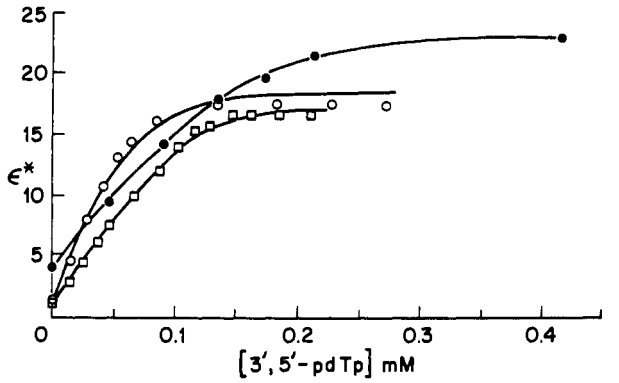

FIGURE 6: pdTp binding studies to Mn^{2+} complexes of wild-type enzyme and E43S mutant of staphylococcal nuclease, measuring the changes in the enhancement (ϵ^*) of the paramagnetic effects of Mn^{2+} on $1/T_1$ of water protons. The data for wild-type enzyme (●) is from Serpersu et al. (1986). For the titration of the E43S mutant enzyme, solutions contained 40 mM Tris·HCl, pH 7.4, and 182 μM E43S with either 63 μM Mn^{2+} (○) or 118 μM Mn^{2+} (□). To avoid dilution of other components, pdTp was added from concentrated stock solutions, which also contained the other components of the titration at the same final concentrations. The data points are shown together with curves computed by using the parameters given in Tables II and III.

E43S mutant for pdTp by significantly different factors of 37.6- and 300-fold respectively, while Ca^{2+} raises the affinities of wild-type and mutant enzyme by differing factors of 7.2- and 23.6-fold, respectively. Thus, in comparison with the wild-type enzyme, the E43S mutant binds Mn^{2+} and Ca^{2+} an order of magnitude more weakly and binds pdTp with comparable affinity in binary complexes, but in ternary complexes, the mutant shows an order of magnitude greater synergy in the metal-enhanced binding of nucleotide and in the nucleotide-enhanced binding of metals (Tables II and III). These compensatory effects result in very similar values of K_2, the dissociation constant of metal–pdTp from the ternary enzyme–metal–pdTp complexes, for the wild-type and E43S mutant enzymes with both Mn^{2+} and Ca^{2+}. Moreover, in the enzyme–Mn^{2+}–pdTp complexes of the wild-type and E43S mutant enzymes, no significant differences in paramagnetic effects of Mn^{2+} on $1/T_1$ of water protons are detected as reflected in similar ϵ_T values, indicating structural similarities in the Mn^{2+} coordination sphere in both enzymes.

Frequency Dependence of $1/T_{1P}$ of Water Protons with Binary and Ternary Mn^{2+} Complexes of the E43S Mutant. As previously shown for Mn^{2+} complexes of the wild-type enzyme and for several liganding mutants of staphylococcal nuclease, determination of the correlation time τ_c for the Mn^{2+}–water proton dipolar interaction leads to an accurate estimate of q, the number of residual water ligands on enzyme-bound Mn^{2+}, consistent with values found by electron spin-echo modulation. Since the latter method is not limited to fast-exchanging water ligands, all of the water coordinated by Mn^{2+} on staphylococcal nuclease appears to be in fast exchange on the NMR time scale (Serpersu et al., 1987, 1988). Accordingly, the $1/T_{1P}$ method was used to determine q for the binary enzyme–Mn^{2+} and ternary enzyme–Mn^{2+}–pdTp complexes of the E43S mutant. As controls, the q values for the same complexes of the wild-type enzyme were redetermined (Table IV) and were found to agree with those previously reported (Serpersu et al., 1987). In comparison with Mn^{2+} bound to the wild-type enzyme which has 0.75 ± 0.2 water ligand, Mn^{2+} bound to the E43S mutant has 0.6 ± 0.3 additional water ligand. This difference represents, within error, an extra water ligand on the mutant since only 0.8 ± 0.2 Mn^{2+} binding sites on the E43S mutant were found (Table

Table III: Dissociation Constants (μM) and Enhancement Factors of $1/T_{1P}$ in Ternary Enzyme–Metal–pdTp Complexes of Wild-Type and Mutant E43S Staphylococcal Nucleases[a]

enzyme	metal	K_A' EPR	K_A' $1/T_{1P}$[b]	K_S $1/T_{1P}$[c]	K_3 $1/T_{1P}$[c]	K_2 $1/T_{1P}$[c]	ϵ_T[c]
wild type	Mn^{2+}	11.0 ± 0.6	17.0 ± 2.0	94 ± 38	2.5 ± 1.0	2.2 ± 0.9	24.8 ± 0.4
E43S	Mn^{2+}	14.6 ± 0.7	8.0 ± 0.8	66 ± 17	0.22 ± 0.06	1.6 ± 0.5	22.0 ± 2.7
wild type	Ca^{2+}		71 ± 8	94 ± 38	13.1 ± 5.8	5.6 ± 1.9	
E43S	Ca^{2+}		228 ± 70	66 ± 17	2.8 ± 0.8	12.6 ± 8.9	
wild type	Co^{2+}	62.0 ± 20.4	31.2 ± 10.6	94 ± 38		21.0 ± 13	
E43S	Co^{2+}	16.7 ± 2.4	11.9 ± 1.8	66 ± 17		4.5 ± 1.7	

[a] The dissociation constants of the ternary and relevant binary complexes of enzyme (E), metal (M), and ligands (L) are defined as follows (Mildvan & Cohn, 1966): K_1 = [M][L]/[M–L]; K_D = [E][M]/[E–M]; K_2 = [E][M–L]/[E–M–L]; K_A' = [E–L][M]/[E–M–L]; K_3 = [E–M][L]/[E–M–L]; K_S = [E][L]/[E–L]. Note that $K_1K_2 = K_3K_D = K_A'K_S$. The parameters for Mn^{2+} and Ca^{2+} complexes of the wild-type enzyme are from Serpersu et al. (1986). [b] Determined by $1/T_{1P}$* of water protons in Mn^{2+} titrations and displacement of Mn^{2+} by titration with Ca^{2+} or Co^{2+}. [c] Determined by computer analysis of pdTp titrations (Reed et al., 1970; Mildvan & Engle, 1972). Although an independent measurement of K_S by competitive fluorescence titrations using 3′,5′-ϵpAp could not be made with E43S because this mutant did not quench the fluorescence of ϵpAp, good agreement in K_S values computed from $1/T_1$ titrations in which the concentration of Mn^{2+} differed by a factor of 2 indicates K_S to be well determined.

Table IV: Analysis of the Frequency Dependence of the Longitudinal Relaxation Rates of Water Protons in the Presence of Mn^{2+} Complexes of the Wild-Type and E43S Mutant of Staphylococcal Nuclease[a]

complex	$1/fT_{1P}$ ($\times 10^{-6}$ s^{-1})	τ_c ($\times 10^9$ s)	$f(\tau_c)$ ($\times 10^9$ s)	B ($\times 10^{-20}$ s^{-2})	τ_ν ($\times 10^{12}$ s)	q	error (%)
wild type–Mn^{2+}	2.26	2.17	5.87	0.63	1.58	0.75	3.5
wild type–Mn^{2+}–pdTp	7.47	2.67	6.87	0.60	1.33	2.12	3.2
E43S–Mn^{2+}	4.03	2.11	5.75	0.56	1.88	1.37	2.5
E43S–Mn^{2+}–pdTp	6.20	2.53	6.60	0.63	1.33	1.83	3.2

[a] The normalized longitudinal relaxation rates of water protons ($1/fT_{1P}$), where $f = [Mn^{2+}]_b/[H_2O]$, as a function of precession frequencies of protons (ω_I) and unpaired electrons (ω_S) were analyzed according to the following equations (Mildvan & Gupta, 1978; Mildvan et al., 1980): $1/fT_{1P} = q(C/r)^6 f(\tau_c)$, $f(\tau_c) = 3\tau_c/(1 + \omega_I^2\tau_c^2) + 7\tau_c/(1 + \omega_S^2\tau_c^2)$ and $1/\tau_c \sim 1/\tau_S = B[\tau_\nu/(1 + \omega_S^2\tau_\nu^2) + 4\tau_\nu/(1 + 4\omega_S^2\tau_\nu^2)]$, where q is the number of fast-exchanging water ligands, C is a product of physical constants, equal to 812 $Å/s^{1/3}$ for Mn^{2+}–proton interactions, r is the metal nucleus distance, τ_c is the dipolar correlation time, τ_S is the longitudinal electron spin-relaxation time, B is the zero-field splitting parameter, and τ_ν is a time constant for motion of the water ligands which modulates B. The data were collected at 15, 19, 24.3, 30, 38, 48, and 59.8 MHz, and the normalized relaxation rates $1/fT_{1P}$, the dipolar correlation times τ_c, and the correlation functions $f(\tau_c)$ are given for 24.3 MHz.

II). The ternary pdTp complexes of both the wild-type and the E43S mutant enzymes have approximately two residual water ligands on the bound Mn^{2+} (Table IV, Figure 1).

Cobalt to Phosphorus Distances in the Ternary E43S–Co^{2+}–pdTp Complex. The ^{31}P NMR spectrum of 3′,5′-pdTp consists of a doublet (J = 7.8 Hz) 3.93 ppm downfield from 85% H_3PO_4 in 20% ^2H_2O, assigned to the 3′-P, and a triplet (J = 3.4 Hz) at 4.18 ppm, assigned to the 5′-P by the structures of the multiplets and by proton decoupling at 4.8 ± 0.2 and 3.9 ± 0.2 ppm from DSS, respectively, in the proton spectrum (Serpersu et al., 1988). We have previously observed slow exchange of pdTp from ternary staphylococcal nuclease–Mn^{2+}–pdTp complexes, but fast exchange, compared to $1/T_{1P}$, from ternary Co^{2+} complexes due to the lower effective magnetic moment of Co^{2+}. Hence Co^{2+} rather than Mn^{2+} was used to measure distances from the metal ion to the phosphorus atoms of 3′,5′-pdTp (Serpersu et al., 1988).

As previously found with the wild-type enzyme and with two other active site mutants (Serpersu et al., 1988), Co^{2+} occupies the Ca^{2+} binding site of the E43S mutant of staphylococcal nuclease. Kinetic experiments with E43S (data not shown) indicate that Co^{2+} is a linear competitive inhibitor with respect to Ca^{2+} (K_I = 570 μM, Table I). Active site binding of Co^{2+} is further supported by the competitive displacement of Mn^{2+} by Co^{2+} from the ternary enzyme–Mn^{2+}–pdTp complex as detected by decreases in $1/T_{1P}$ of water protons (Figure 5C) and by increases in the intensity of the EPR spectrum of free Mn^{2+} (data not shown). The dissociation constants of Co^{2+} from the ternary enzyme–Co^{2+}–pdTp complexes of the wild-type and E43S mutant enzymes (Table III) are an order of magnitude tighter than the respective K_I values of Co^{2+} (Table I), indicating a stronger interaction of Co^{2+} with the enzyme–pdTp than with the enzyme–DNA complexes.

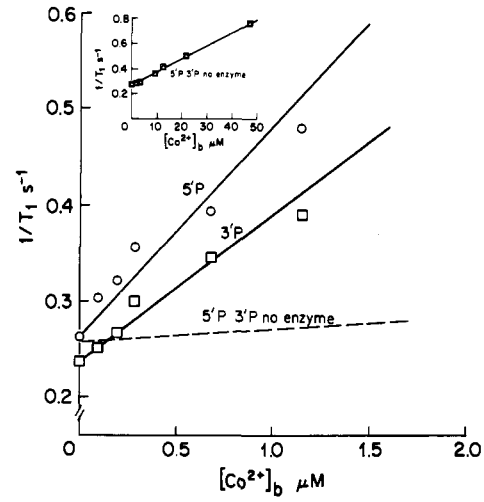

FIGURE 7: Paramagnetic effects of Co^{2+} on $1/T_1$ of the phosphorus resonances of pdTp in the absence and in the presence of the E43S mutant of staphylococcal nuclease. Components present were as follows: 4.18 mM pdTp and 0.39 mM E43S (□, 3′-P; ○, 5′-P); or (inset) 3.63 mM pdTp (△, 3′-P; □, 5′-P). Other components present were 28 mM NaCl, 10 mM Na^+TES, pH 7.4, and 20% ^2H_2O. Dashed line represents the expanded plot of the inset for comparison. ^{31}P spectra were obtained at 101.25 MHz by using 64–512 scans with 8K or 16K data points and 12-bit A/D conversion. Spectral widths used were 1000 or 5000 Hz with acquisition times of 4.1 and 1.6 s, respectively. Recycle times ranged from 6 to 25 s and were always at least $5T_1$. T = 24 °C.

Titrations with $CoCl_2$ measuring $1/T_1$ of the 3′ and 5′ ^{31}P resonances of pdTp in the absence or presence of the E43S mutant indicate that the enzyme enhances the paramagnetic effects of Co^{2+} on $1/T_1$ of both phosphorus nuclei (Figure 7). The enhancement factors, obtained from the ratios of slopes in the titration curves of Figure 7, were 24.4 and 17.1 for the

Table V: Corrected ^{31}P Relaxation Rates, Correlation Times, and Co^{2+} to Phosphorus Distances in Ternary Complexes of Wild-Type Enzyme and the Mutant E43S

complex	$(1/fT_{1P})_{corr}$ (s^{-1})		$(1/fT_{2P})_{corr}$ ($\times10^{-4}$ s^{-1})		B^a ($\times10^{-23}$ s^{-2})	τ_v^a ($\times10^{13}$ s)	$\tau_c^{a,b}$ ($\times10^{12}$ s)	$f(\tau_c)^c$ ($\times10^{12}$ s)	r^d (Å)	
	5′-P	3′-P	5′-P	3′-P					5′-P	3′-P
wild type–Co^{2+}–pdTp	1443 ± 132	81.5 ± 8	2.15 ± 0.46	1.29 ± 0.64	9.42	5.96	0.77 ± 0.11	7.19 ± 0.93	2.7 ± 0.4	4.4 ± 0.7
E43S–Co^{2+}–pdTp	851 ± 119	581 ± 75	13.3 ± 4.8	9.4 ± 2.8	11.97	0.89	1.93 ± 0.24	13.97 ± 1.75	3.3 ± 0.5	3.5 ± 0.5

[a] Determined by the frequency dependence of the normalized longitudinal relaxation rates ($1/fT_{1P}$) of water protons at 15, 24.3, 42, 59.8, 250, and 360 MHz, and analyzed according to the equations given in Table IV, with a C value of 895 ± 180 for Co^{2+}–proton interactions and 662 ± 133 for Co^{2+}–^{31}P interactions. [b] τ_c at 5.87 T, i.e., at 250 MHz for protons and 101.3 MHz for ^{31}P. [c] $f(\tau_c)$ calculated for ^{31}P at 101.3 MHz. [d] Errors shown are in the absolute distances, which include a 14% contribution due to anisotropic g values of Co^{2+} and a 9%–14% contribution due to experimental errors in the measurements of $1/fT_{1P}$, τ_c, and K_A'. Errors in distances are smaller than errors in the measured parameters, due to the sixth root relationship in the Solomon–Bloembergen equation. If the orientation of the g tensor of Co^{2+} were the same in both of the ternary complexes, the errors in the relative distances would be ±3%.

5′-P and 3′-P of pdTp, respectively, reflecting a stronger interaction of enzyme-bound Co^{2+} with the 5′-P as previously found with the wild-type enzyme. The corresponding enhancement factors for the wild-type enzyme were 37.9 and 8.8 for the 5′-P and 3′-P, respectively (Serpersu et al., 1988). A comparison of the enhancement factors on the two enzymes indicates that on the E43S mutant the enzyme-bound Co^{2+} interacts more weakly with the 5′-P and more strongly with the 3′-P than on the wild-type enzyme.

The concentrations of Co^{2+} in the binary Co^{2+}–pdTp complexes and in the ternary enzyme–Co^{2+}–pdTp complexes were calculated from the K_1 and K_A values (Tables II and III), permitting an evaluation of the normalized relaxation rates $(1/fT_{1P})_{corr}$ and $(1/fT_{2P})_{corr}$ for the 3′- and 5′-P in each ternary complex (Table V). In all cases $(1/fT_{2P})_{corr}$, which sets a lower limit to $1/\tau_M$, exceeded $(1/fT_{1P})_{corr}$ by more than an order of magnitude, establishing that $(1/fT_{1P})_{corr}$ is the relaxation rate of the bound ligand and is therefore suitable for distance calculations. Table V gives the correlation times determined from the frequency dependence of $1/T_{1P}$ of water protons and the calculated Co^{2+} to P distances in the ternary complexes.

As previously found for the wild-type enzyme, the measured distances from bound Co^{2+} to the 5′-P and 3′-P of pdTp of 2.7 and 4.4 Å, respectively, are consistent with a bidentate inner-sphere complex of the 5′-phosphate and a predominantly second-sphere complex of the 3′-phosphate with an intervening water ligand (Serpersu et al., 1988). On the E43S mutant, the measured distance of 3.3 Å from enzyme-bound Co^{2+} to the 5′-P of pdTp is in excellent agreement with distances found by X-ray crystallographic analyses of four monodentate inner-sphere Co^{2+}-phosphate complexes (3.31 ± 0.04 Å; Serpersu et al., 1988). Similarly, the 3.5-Å distance from Co^{2+} to the 3′-P of pdTp on the E43S mutant indicates a predominantly inner-sphere monodentate complex. The 6% greater distance from Co^{2+} to the 3′-P than to the 5′-P on the E43S mutant probably represents a real difference since it results from a significantly greater enhancement factor (Figure 7) and is beyond the 3% error in the relative distances (Table V). It could result from the averaging of 68% of a monodentate inner-sphere complex (3.31 Å) and a 32% contribution from a second-sphere complex with an intervening water ligand (4.75 Å). The latter distance is based on X-ray structures of two second-sphere Co^{2+} (H_2O) phosphate complexes (Serpersu et al., 1988).

Discussion

The observed kinetic effects of mutating Glu-43 of staphylococcal nuclease to Ser are in accord with the proposal that Glu-43 functions as a general base (Figure 1). Thus the loss of this residue in the E43S mutant resulted in a 2700-fold decrease in the V_{max} at pH 7.4, as compared to that of the wild-type enzyme, but in only a 376-fold loss in V_{max} at pH 9.9, indicating that $[OH^-]$ can partially replace the effect of Glu-43 in catalysis. This effect is most clearly seen between pH 9.2 and 9.8 where V_{max} of the E43S mutant remains first order in $[OH^-]$ while V_{max} of the wild-type enzyme has become much less sensitive to $[OH^-]$ (Figure 3A), suggesting that when assisted by Glu-43, the attacking water molecule is largely deprotonated at pH 9.2. In the E43S mutant, the attacking water may have a higher pK_A, and OH^- might either deprotonate it or directly attack the DNA phosphodiester from solvent, due to a more accessible active site. The present data do not distinguish among these alternatives. Analogous effects of $[OH^-]$ on V_{max} of the D102N mutant of trypsin were found. In this mutant, His-57 apparently cannot function as a general base (Sprang et al., 1987; Craik et al., 1987).

The inflection point at pH 9.4 ± 0.2 in log V_{max} vs pH for the wild-type enzyme (Figure 3A) is similar to that observed by Dunn et al. (1973) with the synthetic substrate deoxythymidine 3′-phosphate 5′-(*p*-nitrophenyl phosphate) and may reflect the ionization of water coordinated to Ca^{2+}, or of Lys or Tyr residues at or near the active site. Both Lys-84 and Tyr-85 have been implicated in the binding of substrates by chemical modification (Cuatrecasas et al., 1968) and by directed mutagenesis of these residues (Grissom et al., 1986).[2] The fact that this inflection is absent or occurs at a higher pH with the E43S mutant supports the hypothesis that it represents the pK_A of the attacking water.

The inflections at pH 10.0–10.1 in log V_{max} and in log (V_{max}/K_M^{Ca}) for both the wild-type and mutant enzymes (Figure 3) may result from alkaline denaturation of the enzyme at pH values greater than 10. In the absence of such denaturation, V_{max} for the wild type enzyme might remain zero order in $[OH^-]$ while V_{max} of the E43S mutant might remain first order in $[OH^-]$.

X-ray studies of the ternary staphylococcal nuclease–Ca^{2+}–pdTp complex reveal the Ca^{2+} to be coordinated by the 5′-phosphate of pdTp, the carboxylates of Asp-21 and Asp-40, the amide carbonyl group of Thr-41, and two water ligands. The carboxylate groups of Asp-19 and Glu-43 are in the second coordination sphere (Figure 1; Cotton et al., 1979). Water proton relaxation, pulsed EPR, and ^{31}P relaxation studies indicate very similar ligand arrangements in the inner coordination spheres of the ternary enzyme–Mn^{2+}–pdTp and enzyme–Co^{2+}–pdTp complexes (Serpersu et al., 1986, 1987, 1988). In the binary enzyme–Mn^{2+} complex, however, water

[2] M. A. Reynolds, L. Harpold, and J. A. Gerlt, unpublished observations.

relaxation and pulsed EPR studies detected only one water ligand on Mn^{2+}, requiring the enzyme to contribute five ligands to the metal in order to preserve octahedral coordination. The nearby residues Asp-19 and Glu-43 are reasonable candidates for the two additional metal ligands in the binary enzyme–metal complex (Table IV; Serpersu et al., 1988). Of course, in the ternary complex, Glu-43 must be released from the metal in order to function as the general base, thereby creating a liganding site for the attacking water molecule (Serpersu et al., 1988; Mildvan & Serpersu, 1988). The present kinetic (Table I) and binding studies (Table II) detect an order of magnitude weaker binding of Ca^{2+} and Mn^{2+} to the active site of staphylococcal nuclease in the E43S mutant, probably due to loss of a direct metal ligand. The detection of an additional water ligand on Mn^{2+} in the binary Mn^{2+} complex of the E43S mutant (Table IV) is consistent with this view. Weaker binding of both Mn^{2+} and Ca^{2+} in ternary DNA complexes of the E43S mutant detected by kinetics (Table I) and by binding studies with Ca^{2+} (Table III) could well result from the loss of a strong hydrogen bond from Glu-43 in the second coordination sphere to an inner-sphere water ligand of the metal.

Slightly weaker binding of DNA to the E43S mutant is suggested by 2-fold increases in K_S^{DNA} and K_M^{DNA} (Table I), but the small magnitude of these effects indicates little interaction of Glu-43 with DNA either in the binary enzyme–DNA or ternary enzyme–Ca^{2+}–DNA complexes. Opposite and large effects of the E43S mutation on pdTp binding are detected, tightening the binding of this ligand in the ternary Mn^{2+} and Ca^{2+} complexes by factors of 11.4- and 4.7-fold, respectively, as reflected in decreases in K_3 (Table III). Moreover, the E43S mutant shows an order of magnitude greater synergy than does the wild-type enzyme in the metal-enhanced binding of pdTp (K_3/K_S) and in the pdTp-enhanced binding of metals (K_A'/K_D) (Tables II and III). Hence the absence of the negatively charged Glu at position 43 permits tighter binding of pdTp to the enzyme–metal complex and greater tightening of metal binding to the enzyme by pdTp. The differing binding behavior of the DNA substrate and the pdTp substrate analogue likely results from additional interactions betwen the enzyme and the DNA (Cuatrecasas et al., 1967).

A structural explanation for the tighter binding of pdTp by the mutant enzyme is provided by the ^{31}P relaxation studies of the ternary enzyme–Co^{2+}–pdTp complexes. On the wild-type enzyme, direct coordination of only the 5′-phosphate of pdTp by enzyme-bound Co^{2+} is observed, while in the E43S mutant the coordination of both the 5′- and the 3′-phosphates of pdTp is observed (Table IV). Apparently, the absence of the anionic Glu-43 in the second coordination sphere of the metal permits the 3′-phosphate of pdTp to enter the inner coordination sphere. This additional ligand from pdTp to the metal can explain the observed increase in affinity for pdTp and the greater synergy of metal and pdTp binding on the E43S mutant. These effects are not observed with DNA, which has no dianionic phosphate to offer as a second ligand, but only phosphodiester monoanions, which are poor metal ligands (Bean et al., 1977). In the X-ray structure of the ternary enzyme–Ca^{2+}–pdTp complex of the wild-type enzyme, direct coordination of the 5′-phosphate is detected, while the 3′-phosphate is 9.45 Å from the metal, placing it in the third coordination sphere (Cotton et al., 1979). Our finding of a second-sphere distance of 4.4 Å from Co^{2+} to the 3′-phosphate of pdTp in the ternary enzyme–Co^{2+}–pdTp complex may result from a difference in solution versus crystal structure[3] or from the smaller and more electrophilic character of Co^{2+} as compared to Ca^{2+}.

Although pdTp donates both the 3′-phosphate and 5′-phosphate as ligands to Co^{2+} on the E43S mutant and donates only the 5′-phosphate to Co^{2+} on the wild-type enzyme, two water ligands on Mn^{2+} are detected in both ternary enzyme–Mn^{2+}–pdTp complexes (Table IV), suggesting that one of the protein ligands of E43S has dissociated from the metal, permitting the coordination of an additional water. Alternatively, the ternary Co^{2+} complex of E43S may differ from that of the Mn^{2+} complex. The former alternative is more likely since recent X-ray studies of the ternary enzyme–Ca^{2+}–pdTp complex of the E43D mutant reveal that Asp-40 has dissociated from the metal.[4] The present binding and nuclear relaxation studies thus reveal structural differences at the active site in the ternary enzyme–metal–pdTp complex of the E43S mutant in comparison with that of the wild-type enzyme. However, significantly smaller structural differences are detected in functional DNA complexes by kinetic analysis.

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This paper is dedicated to the memory of Emil Thomas Kaiser (1938-1988), a leader in the field of mechanistic enzymology. We are grateful to David Shortle for helpful advice and to Pat Loll and Eaton Lattman for permitting us to quote their preliminary X-ray data.

Registry No. 3′,5′-pdTp, 2863-04-9; Glu, 56-86-0; Ser, 56-45-1; Ca, 7440-70-2; Mn, 7439-96-5; Co, 7440-48-4; staphylococcal nuclease, 9013-53-0.

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[3] In the crystal lattice, Lys-71 from a neighboring molecule of staphylococcal nuclease lies between the 3′- and 5′-phosphates of pdTp, possibly perturbing their interactions with the metal (P. Loll and E. Lattman, private communication, 1988).

[4] P. Loll and E. Lattman, private communication, 1988.

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Proton Nuclear Magnetic Resonance Studies on the Variant-3 Neurotoxin from *Centruroides sculpturatus* Ewing: Sequential Assignment of Resonances†

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ABSTRACT: We report the sequential assignment of resonances to specific residues in the proton nuclear magnetic resonance spectrum of the variant-3 neurotoxin from the scorpion *Centruroides sculpturatus* Ewing (range southwestern U.S.A.). A combination of two-dimensional NMR experiments such as 2D-COSY, 2D-NOESY, and single- and double-RELAY coherence transfer spectroscopy has been employed on samples of the protein dissolved in D_2O and in H_2O for assignment purposes. These studies provide a basis for the determination of the solution-phase conformation of this protein and for undertaking detailed structure–function studies of these neurotoxins that modulate the flow of sodium current by binding to the sodium channels of excitable membranes.

The venom from the scorpion *Centruroides sculpturatus* Ewing (range southwestern U.S.A.) is a complex mixture that contains a large number of small basic proteins (MW ~ 7000) which are responsible for the neurotoxic effects (Watt et al., 1978; Watt & Simard, 1984). This venom, like that from other species of scorpions, is unique in that the various toxins present in the venom can differ markedly in their toxicity to a given species, despite their closely related chemical properties and similar modes of action. In addition, the toxins display a degree of target specificity: e.g., some may be more toxic to mammals than to insects while the others are more toxic to insects than to mammals (Watt et al., 1978; Watt & Simard, 1984; Zlotkin et al., 1972a,b). Toxicity of the scorpion venoms and the isolated toxins is a consequence of the depolarizing action of the toxins on membranes of excitable cells. A broad range of receptive excitable cells, e.g., neurons (central and peripheral) and muscle (skeletal and smooth), are depolarized and, in the case of nerve endings, depolarized with the release of neurotransmitters (Rochat et al., 1979; Zlotkin et al., 1978). Results of electrophysiological studies and pharmacological assays suggest that the scorpion toxins could be divided into two different groups, the scorpion α- and β-toxins, that bind to two distinct receptor sites on the sodium channels of excitable membranes (El Ayeb et al., 1986; Wheeler et al., 1983; Meves et al., 1982). The α-toxins bind to the sodium channels in a voltage-dependent manner and prolong the inactivation of the sodium current, an activity that is also shared by the sea anemone toxins (Romez et al., 1976). The binding of the β-toxins, which enhance the flow of sodium inward current, is voltage independent. Among the various toxic proteins isolated and characterized from the venom of *C. sculpturatus* Ewing, CsE-v1 to CsE-v6 (also referred to as variants 1–6) and CsE-V prolong inactivation of the sodium current, whereas CsE toxins I, III, IV, VI, and VII affect sodium activation (Meves et al., 1982; Simard et al., 1986).

The above results suggest the possibility of intriguing conformation–function relationships that contribute to the ob-

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