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Staphylococcal Nuclease Active-Site Amino Acids: pH Dependence of Tyrosines and Arginines by ^{13}C NMR and Correlation with Kinetic Studies†

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ABSTRACT: The pH and temperature dependence of the kinetic parameters of staphylococcal nuclease (EC 3.1.4.7) have been examined with three *p*-nitrophenyl phosphate containing DNA analogues that vary as to 3′-substituent. With wild-type (Foggi variant) nuclease (nuclease wt) and the substrates thymidine 3′-phosphate 5′-(*p*-nitrophenyl phosphate) (PNPdTp), thymidine 3′-methylphosphonate 5′-(*p*-nitrophenyl phosphate) (PNPdTp*Me), and thymidine 5′-(*p*-nitrophenyl phosphate) (PNPdT), k_{cat} remains nearly constant at 13 min^{-1}. However, k_{cat}/K_m with nuclease wt varies considerably: 413, 13, and 0.52 mM^{-1} min^{-1} with PNPdTp, PNPdTp*Me, and PNPdT, respectively. When tyrosine-85 is changed to phenylalanine (nuclease Y85F) by site-directed mutagenesis, k_{cat} is unchanged at about 13 min^{-1}, except with PNPdTp where it drops to 1 min^{-1}. With nuclease Y85F, k_{cat}/K_m is 19.5 and 25 mM^{-1} min^{-1} with PNPdTp and PNPdTp*Me, respectively. With PNPdTp as the substrate, a bell-shaped k_{cat}/K_m vs pH profile is seen with pK_a values at 8.94 and 9.67 in 0.3 M KCl and H_2O. The pK_a at 9.67 disappears, and a new pK_a appears at 10.1 when tyrosine-85 is changed to phenylalanine (nuclease Y85F) or when the substrate 3′-phosphomonoester is changed to a 3′-methylphosphonate (PNPdTp*Me). This suggests that the inflection in k_{cat}/K_m with pK_a at 9.67 arises from ionization of tyrosine-85, which hydrogen bonds to the divalent 3′-phosphomonoester of substrates with this substituent. The enthalpy of ionization of both deprotonation steps in the k_{cat}/K_m versus pH profile is 5 kcal/mol. ^{13}C NMR has been used to determine the pK_a values of the arginine and tyrosine residues. The protein was enriched uniformly with 20% ^{13}C at all carbons and specifically with 90% ^{13}C at the guanidino carbon of the arginine residues. All five arginines in the protein have pK_a values greater than 11.6 in H_2O and 0.3 M KCl. This eliminates arginine as a candidate for the basic catalyst that deprotonates H_2O to facilitate nucleophilic attack on phosphorus. The results do not rule out arginine as a candidate for the acidic catalyst that protonates the 5′-ribose alkoxide prior to product release. The phenolic hydroxyl carbon of tyrosine-85 has been assigned by comparing the ^{13}C NMR spectrum of nuclease wt and nuclease Y85F. The structure of nuclease Y85F is not perturbed significantly by this substitution. Tyrosine-85 has a spectroscopically observed pK_a of 9.53 ± 0.05 in H_2O and 0.3 M KCl, which is similar to the basic-side pK_a of 9.67 seen in the k_{cat}/K_m pH profile. This correlation between pK_a values along with the absence of other candidates indicates that the ionization of tyrosine-85 is the pK_a seen in the k_{cat}/K_m vs pH profile for substrates with a divalent 3′-phosphomonoester. This conclusion is consistent with the proposed role of tyrosine-85 as a hydrogen-bond donor to the 3′-phosphomonoester of substrates poised for exonucleolytic hydrolysis.

Staphylococcal nuclease [ribonucleate (2′-deoxyribonucleate) 3′-nucleotidohydrolase, EC 3.1.4.7] catalyzes the hydrolytic cleavage of ribo- and 2′-deoxyribonucleotides between the 5′-phosphate and the 5′-oxygen of the ribose ring. The enzyme has been studied thoroughly and is a prototype for elucidation of the relationship between protein structure and catalytic activity (Hazen & Cotton, 1978; Tucker et al., 1979a–c). A detailed understanding of the catalytic role of active-site amino acids consistent with the pH dependence of the kinetic parameters (Dunn et al., 1973) has eluded researchers despite the availability of a 1.5-Å resolution X-ray crystal structure with the competitive inhibitor thymidine 3′,5′-diphosphate (pdTp)[1] and the required metal ion, Ca^{2+},

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[1] Abbreviations: pdTp, thymidine 3′,5′-diphosphate; PNPdTp, thymidine 3′-phosphate 5′-(*p*-nitrophenyl phosphate); PNPdT, thymidine 5′-(*p*-nitrophenyl phosphate); PNPdTp*Me, thymidine 3′-(methylphosphonate) 5′-(*p*-nitrophenyl phosphate); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Ches 2-(cyclohexylamino)ethanesulfonic acid; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; ΔH_{ion}, enthalpy of ionization; Mops, *N*-morpholinopropanesulfonic acid; TSP, (trimethylsilyl)propionic acid.

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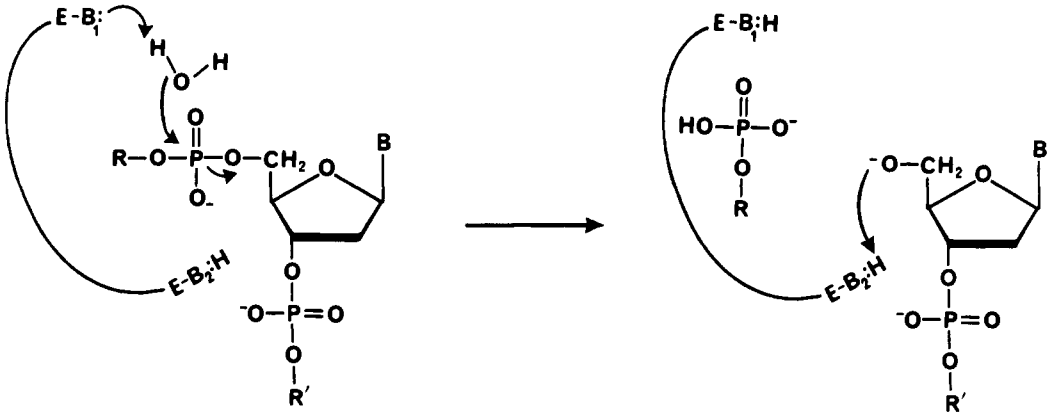

FIGURE 1: Proposed catalytic mechanism for staphylococcal nuclease. To be consistent with the stereochemical course of the reaction, two enzymatic bases are required for catalysis of phosphodiester bond cleavage. Base 1 must be unprotonated to abstract a proton from the water molecule that carries out direct nucleophilic attack on phosphorus. Base 2 must be protonated to donate a proton to the departing 5′-alkoxide. A stepwise process is not to be inferred from the diagram. The two events may in fact occur synchronously.

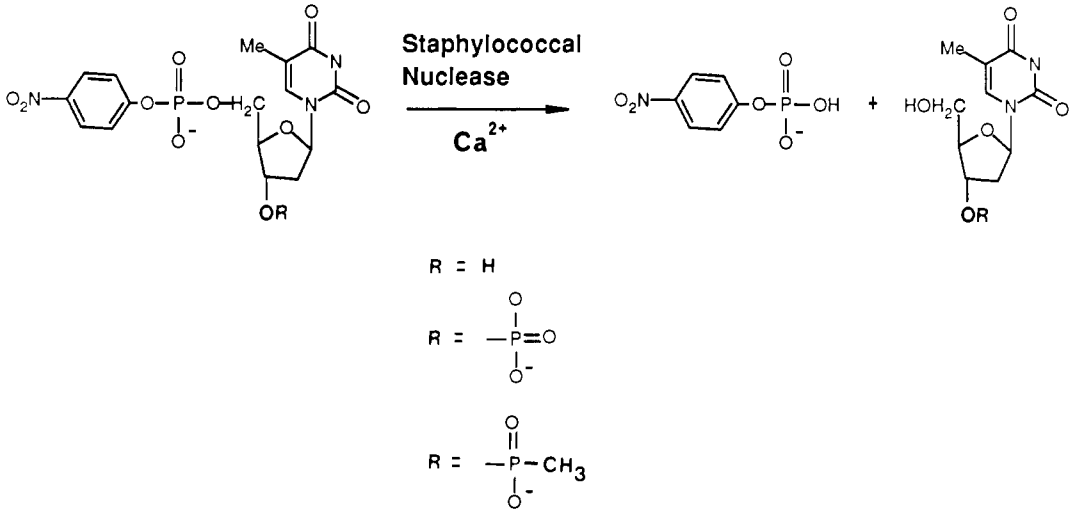

FIGURE 2: Hydrolysis of DNA analogue substrates by staphylococcal nuclease. In the assay employed here, the initial *p*-nitrophenyl phosphate product of substrates without a free 3′-phosphate was converted to *p*-nitrophenol by coupling the nuclease assay with alkaline phosphatase.

bound in the active site (Cotton et al., 1979). Three tyrosines (85, 113, and 115), two arginines (35 and 87), one lysine (84), and various water molecules and carboxyl ligands (Asp 21 and Asp 40) to Ca^{2+} surround the bound inhibitor, pdTp. All of these are possible candidates as participants in substrate binding or phosphodiester hydrolysis. Hydrolysis occurs with inversion of stereochemistry at phosphorus, thus making covalent catalysis by a group on the enzyme or the existence of a metaphosphate intermediate unlikely (Mehdi & Gerlt, 1982). This leaves direct nucleophilic attack of H_2O on the $5'_n$-phosphate to yield a free $3'_{n+1}$-phosphate monoester and $5'_n$-alkoxide as the favored mechanism. Chemical intuition suggests a need for two enzymatic bases (Figure 1): a deprotonated base to deprotonate H_2O in order to facilitate nucleophilic attack on phosphorus and a protonated base to donate a proton to the departing 5′-alkoxide. Cotton et al. (1979) first proposed a mechanism employing these basic tenets, with the carboxyl group of Glu-43 deprotonating the attacking water molecule and the guanidinium ion of Arg-87 protonating the leaving alkoxide. Since the postulated role for Glu-43 occurs before the first irreversible step[2] (thought to be hydrolysis of the phosphodiester bond), the titration of this residue should be observable in the k_{cat}/K_m vs pH profile. Dunn et al. (1973) observed a bell-shaped dependence of k_{cat}/K_m with pK_a values at 8.43 and 9.15 on following hydrolysis of the DNA analog thymidine 3′-phosphate 5′-(*p*-nitrophenyl phosphate) to the product thymidine 3′-phosphate (Figure 2). They observed only one pK_a at 9.01 in the k_{cat} vs pH profile. If the pK_a at 8.43 is the catalytic base (since it must be unprotonated for activity), then what is the second pK_a observed at 9.15? It probably is not the enzymatic group responsible for protonating the departing alkoxide unless the mechanism is concerted, since this occurs after the first irreversible step. Similarly, it cannot be protein denaturation[3] or a titratable group on the substrate (since there are no substrate pK_a values within the range of interest).

In the present paper, we have made use of a nuclease variant produced by site-directed mutagensis to investigate the effect of changing Tyr-85 to Phe (nuclease Y85F). We have found that the mutant lacks the pK_a of 9.15 observed by Dunn et al. (1973). This result suggests that the pK_a at 9.15 belongs

[2] The kinetic parameter k_{cat}/K_m reflects the binding of substrate(s) to free enzyme, conformational changes, and catalytic events that precede the first irreversible step. After this, the commitment to catalysis is infinite and must proceed through product release. Subsequent catalytic steps and the rate of product release will be reflected in k_{cat}.

[3] ^1H NMR studies of nuclease wt (Dr. Eldon L. Ulrich, personal communication) indicate that chemical shift coalescence of amino acids of the same type (as would happen in a denatured protein without significant secondary or tertiary structure) does not occur below pH 11.5 in D_2O and 0.3 M KCl.

to Tyr-85, which participates in binding substrates having a free 3′-phosphate. This conclusion is strengthened by our finding that the pK_a of 9.15 is absent when substrates without a doubly ionized 3′-phosphate are hydrolyzed by nuclease wt. Thus, the substrate and the enzyme have been "mutated" to provide complementary data on catalysis.

We have also used ^{13}C NMR as a spectroscopic probe to monitor the protonation states of the seven tyrosines (three of which, 85, 113, and 115, are in the active site) and five arginines (two of which, 35 and 87, are in the active site) of nuclease. The two active-site arginines are thought to be essential for activity (Cotton et al., 1977, 1979; Serpersu et al., 1987). Examples of kinetically observed pK_a values that unambiguously correspond to the biophysically observed ionization of a single amino acid residue are few. The present study defines the role of Tyr-85 in nuclease with experimental evidence provided by ^{13}C NMR, site-directed mutagenesis, and kinetic studies.

Materials and Methods

Enzymes. Staphylococcal nuclease was produced by *Escherichia coli* carrying the pFOG405 plasmid for nuclease (Shortle, 1983, 1986). Plasmids coding for nuclease wt and nuclease Y85F (Uhlmann & Smith, 1987) were gifts from Professors David Shortle and John A. Smith, respectively. Cells were grown in low-phosphate Mops media to induce expression of the nuclease gene (located downstream from the phosphate-sensitive alkaline phosphatase promoter). Typical yields were 4–8 mg of nuclease/L of cells when grown in a 5-L New Brunswick fermenter. The concentration of nuclease wt was determined spectrophotometrically at 280 nm ($E^{1\%}_{1cm}$ = 9.3). The concentration of nuclease Y85F was determined by Bio-Rad protein assay reagent with nuclease wt as the standard. $E^{1\%}_{1cm}$ = 9.2 for nuclease Y85F. The protein was purified on Bio-Rad 70 resin (Bio-Rad Laboratories) according to the procedure of Shortle (1986). The enzyme was dialyzed twice against 0.3 M NaCl and 50 mM KH_2PO_4 to remove Tris buffer. The final two dialyses were against H_2O. Chelex 100 (1–2 g) was added to all dialysis buffers to remove trace metal ions. Polyacrylamide gel electrophoresis revealed a single band whose migration corresponded to nuclease purchased from U.S. Biochemicals.

To produce nuclease labeled uniformly with 20% ^{13}C at all carbons, *E. coli* was grown on a synthetic medium containing ^{13}C-labeled amino acids isolated from hydrolyzed light-harvesting protein from *Anabaena variabilis* (cyanobacterium) grown on 20% ^{13}C CO_2 as the sole carbon source. The 45% $(NH_4)_2SO_4$ precipitate of *A. variabilis* broken cell suspension was dialyzed to remove sulfate and inorganic phosphate and then extracted with acetone at −15 °C to remove lipid-soluble material and pigments. The remaining protein was hydrolyzed in 6 N HCl at 110 °C for 16 h. The hydrolysate was neutralized with NaOH and then lyophilized. The resulting 20% uniformly ^{13}C-labeled amino acids were used in the low phosphate nuclease inducer medium (Shortle, 1986) in place of casein hydrolysate. Protein specifically labeled with ^{13}C arginine was produced in order to distinguish the arginine resonances from the tyrosine resonances in the ^{13}C NMR spectrum. The medium used to produce this analogue contained arginine synthetically labeled with 90% ^{13}C in the guanidino carbon position (Baillargeon et al., 1980b) in place of unlabeled arginine in a synthetic amino acid mixture with the same molar amino acid composition as commercially available casein hydrolysate.

Transformation of E. coli. Transformation of *E. coli* with the expression vector was accomplished with either the cold $CaCl_2$ or $CaCl_2/RbCl_2$ procedure as described in Maniatis et al. (1982). *E. coli* strain SE6004 was used to express uniformly enriched ^{13}C-labeled nuclease wt and Y85F. To prevent randomization of the ^{13}C label at the guanidino carbon position of arginine, the arginine auxotroph AW624 (gal+, argE−, thi−, ilv−) was used to produce nuclease when the growth medium contained arginine specifically labeled with ^{13}C along with other unlabeled amino acids.

^{13}C NMR Spectra. ^{13}C NMR spectra were obtained on a Bruker AM-500 spectrometer operating at a ^{13}C frequency of 125 MHz with a 5-mm broadband probe. A 70° pulse angle (typically 11 μs) was used with a relaxation delay of 1.5 s. Composite (WALTZ) proton decoupling was applied during acquisition. Exponential multiplication yielding a line broadening of 7 Hz was applied to all spectra. Typically, 6000–10000 transients were accumulated for each spectrum before Fourier transformation. Dioxane served as an internal reference assumed to be 67.475 ppm. The protein concentration was between 2 and 2.5 mM unless otherwise indicated. All spectra were taken in 0.3 M KCl in 90% H_2O with 10% D_2O for the frequency lock. The reported pH was determined by averaging pH values measured before and after data acquisition. The head-space above the solution in the NMR tube was purged with N_2 prior to NMR spectroscopy to minimize the downward drift of the pH due to carbonic acid formation.

Substrates. Thymidine-5′-(*p*-nitrophenyl phosphate) (PNPdT) was obtained as the NH_4^+ salt (Sigma). Thymidine 3′-phosphate 5′-(*p*-nitrophenyl phosphate) (PNPdTp) was synthesized from PNPdT and cyanoethyl phosphate by the procedure of Glinski et al. (1971) and purified on diethylaminoethyl-Sephadex (Pharmacia) (Rammler et al., 1967): ^1H NMR (500 MHz, in D_2O) δ 1.61 (s, 3 H, CH_3), 2.37 (m, 2 H, H_2'), 4.11 (d, 2 H, H_5'), 4.24 (m, 1 H, H_4'), 4.71 (m, 1 H, H_3'), 6.23 (t, 1 H, J = 6.5 Hz, H_1'), 7.22 (d, 2 H, J = 10.1 Hz, CH), 7.51 (s, 1 H, CH), 8.09 (d, 2 H, J = 10.1 Hz, CH).

Thymidine 3′-methylphosphonate 5′-(*p*-nitrophenyl phosphate) (PNPdTp*Me) was synthesized by the dicyclocarbodiimide coupling of thymidine 5′-(*p*-nitrophenyl phosphate) and methylphosphonate in pyridine (Glinski et al. 1971): ^1H NMR (500 MHz in D_2O) δ 1.25 (d, 3 H, J_{P-H} = 17 Hz, CH_3P), 1.61 (s, 3 H, CH_3), 2.39 (m, 2 H, H_2'), 4.14 (d, 2 H, H_5'), 4.24 (m, 1 H, H_4'), 4.80 (m, 1 H, J = 8.7 Hz, H_3'), 6.25 (t, 1 H, J = 7.1 Hz, H_1'), 7.22 (d, 2 H, J = 10.6 Hz, CH), 7.51 (s, 1 H, CH), 8.09 (d, 2 H, J = 10.6 Hz, CH); ^{31}P NMR (202 MHz) δ 35.47 (d of q, J_{P-H} = 17 Hz, J = 8.7 Hz, PCH_3).

Initial Velocity Studies. All assays were buffered with 100 mM Hepes, Tris, or Ches and sufficient KCl to achieve a total ionic strength of 0.3 M. The Ca^{2+} requirement was satisfied with $CaCl_2$. The absorbance of *p*-nitrophenyl phosphate was monitored at 330 nm. The extinction coefficient was determined experimentally to be 9.4×10^3 M^{-1} cm^{-1}. The concentration of *p*-nitrophenyl phosphate substrate analogues was determined with nucleotide pyrophosphatase (EC 3.6.1.9). The production of *p*-nitrophenol was monitored at 405 nm ($\epsilon = 18.3 \times 10^3$ M^{-1} cm^{-1}) until all of the substrate was converted to products. The pH electrode, sample, and pH buffers were all maintained at the desired temperature with a circulating water bath. Hence, it was not necessary to correct the measured pH for differences in temperature between the spectrophotometer and pH measurement. Potassium was used as the counterion whenever possible.

Data Analysis. Hyperbolic substrate saturation curves were fitted to eq 1 by using the HYPERO program of Cleland

$$v = VA/(K_m + A) \quad (1)$$

Table I: Staphylococcal Nuclease Kinetic Parameters with Thymidine 5′-(*p*-Nitrophenyl Phosphate) Substrate Analogues: Wild Type and Tyr-85 Mutated to Phe (Y85F)[a]

nuclease	substrate[b]	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	K_m[c] (mM)	K_{Ca}
wild type	PNPdTp	13.9 ± 1	413 ± 50	0.035 ± 0.009	3.7 ± 0.4
wild type	PNPdTp*Me	14 ± 2	13 ± 2	1.1 ± 0.1	8.6 ± 1.1
wild type	PNPdT	10.2 ± 1	0.52 ± 0.06	19.6 ± 2.4	11.1 ± 1.6
Y85F	PNPdTp	0.98 ± 0.11	19.5 ± 3	0.051 ± 0.01	1.7 ± 0.1
Y85F	PNPdTp*Me	13 ± 1	25 ± 2	0.52 ± 0.7	10.3 ± 1.4

[a] Assay conditions: approximately 0.2 M KCl and 100 mM Ches, pH 9.5, for a total ionic strength of 0.3 M. Temperature = 26 °C. [b] Substrate abbreviations: PNPdTp, thymidine 3′-phosphate 5′-(*p*-nitrophenyl phosphate); PNPdT, thymidine 5′-(*p*-nitrophenyl phosphate); PNPdTp*Me, thymidine 3′-methylphosphonate 5′-(*p*-nitrophenyl phosphate). [c] K_m is for the indicated substrate.

(1979b).[4] Substrate saturation curves involving both Ca^{2+} and DNA-analogue substrates were fitted to

$$v = VAB/(K_{ia}K_B + K_BA + K_AB + AB) \quad (2)$$

When substrate concentrations were in the range of k_{cat}/K_m enzymatic reaction conditions (i.e., all substrate concentrations below $^1/_{10}K_m$), the resulting velocities were fitted to the equation for a straight line with the intercept fixed at the origin. The slope of the line is k_{cat}/K_m. This provides a precise estimate of k_{cat}/K_m even though neither k_{cat} nor K_m is known individually.[5] Typically, four substrate concentrations at four different Ca^{2+} concentrations (16 different assays) were employed at each pH.

Substrate saturation curves exhibiting substrate inhibition at high concentration were fitted to

$$v = VA/[K + A + A^{e(2/K_i)}] \quad (3)$$

Bell-shaped k_{cat}/K_m vs pH profiles with separate pK_a values at low and high pH were fitted to eq 4, whereas pH profiles

$$\log (k_{cat}/K_m) = \log [C/(1 + H/K_A + K_B/H)] \quad (4)$$

$$\log (k_{cat}/K_m) = \log [C/(1 + H/K_A)] \quad (5)$$

with only a decrease in k_{cat}/K_m at low pH were fitted to eq 5. In eq 4 and 5, C is the pH-independent parameter, H is the measured hydrogen-ion activity, and K_A and K_B are the observed dissociation constants for the enzymatic base(s) being titrated. When observed pK_a values are closer than 2 pH units apart, eq 4 determines the average of these values precisely, but does not determine either individual pK_a precisely (Cleland, 1977; Segel, 1975).

The observed chemical shifts of pH-dependent ^{13}C resonances (δ observed) were fitted as described by Markley (1975).

Results

The initial velocity kinetic patterns observed with all DNA analogues indicate a sequential rapid equilibrium random kinetic mechanism as reported with DNA as substrate (Serpersu et al., 1986). The kinetic parameters for nuclease hydrolysis of PNPdT, PNPdTp, and PNPdTp*Me are shown in Table I.

Plots of k_{cat}/K_m as a function of pH for nuclease wt with PNPdTp, PNPdT, and PNPdTp*Me are shown in Figure 3. All assays in the present study were carried out at 300 mM ionic strength, contributed mainly by KCl and buffer. As a result, the pK_a values obtained here are consistently higher than those observed by Dunn et al. (1973) at about 50–100 mM ionic strength. A decrease in enzymatic activity at lower pH was observed with all three substrates and both enzyme variants. The low pK_a values of 8.94 and 8.98 found for PNPdTp and PNPdT (Table II) are similar to one another, but that observed for PNPdTp*Me is substantially higher at 9.5. The pK_a on the basic side of the pH profile varies, however, depending upon the identity of the substrate 3′-group and

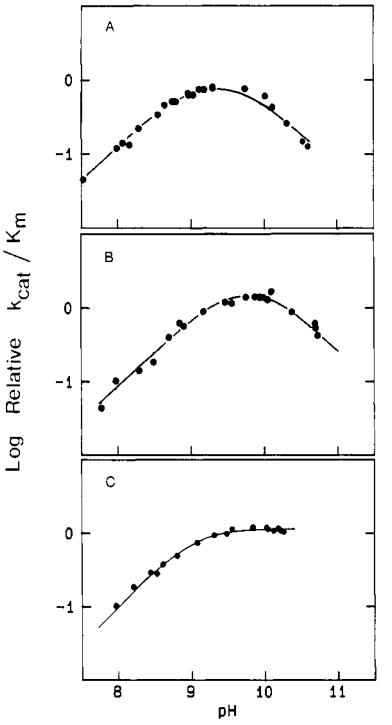

FIGURE 3: log (relative k_{cat}/K_m) vs pH profiles for the DNA substrate analogues (A) PNPdTp, (B) PNPdTp*Me, and (C) PNPdT with nuclease wt at 26 °C and 0.3 M ionic strength. Profiles A and B, which each break with a slope of 1 and −1 on the acidic and basic pH sides, respectively, were fitted to eq 4. Profile C, which breaks with a slope of 1 only on the acidic side, was fitted to eq 5. In each case, the fitted results are shown by a solid line.

Table II: Staphylococcal Nuclease k_{cat}/K_m vs pH Profiles with Thymidine 5′-(*p*-Nitrophenyl Phosphate) Substrate Analogues: Wild Type and Tyr-85 Mutated to Phe (Y85F) at 26 °C[a]

nuclease	substrate[b]	pK_{a1}	pK_{a2}
wild type	PNPdTp	8.94 ± 0.08	9.67 ± 0.10
wild type	PNPdTp*Me	9.50 ± 0.08	10.10 ± 0.07
wild type	PNPdT	8.98 ± 0.09	none
Y85F	PNPdTp	9.04 ± 0.10	10.13 ± 0.10
Y85F	PNPdTp*Me	9.56 ± 0.10	10.10 ± 0.10

[a] Assay conditions: approximately 0.2 M KCl and 100 mM buffer for a total ionic strength of 0.3 M. Temperature = 26 °C. Relative k_{cat}/K_m values were fitted to eq 4 or 5. Error estimates include a propagation of error involved in the determination of k_{cat}/K_m and assay pH. [b] Substrate abbreviations: PNPdTp, thymidine 3′-phosphate 5′-(*p*-nitrophenyl phosphate); PNPdT, thymidine 5′-(*p*-nitrophenyl phosphate); PNPdTp*Me, thymidine 3′-methylphosphonate 5′-(*p*-nitrophenyl phosphate).

[4] Diskettes containing FORTRAN source code for this and other data-fitting programs for the IBM PC are available from C.B.G.

[5] The primary constants k_{cat} and k_{cat}/K_m describe enzymatic rates as a function of substrate concentration. At saturating substrate concentrations, k_{cat} is the dominant term, whereas at very low substrate concentrations, k_{cat}/K_m describes the enzyme rate vs substrate concentration function. K_m is the ratio of k_{cat} and k_{cat}/K_m and does not have to be defined to know k_{cat}/K_m precisely.

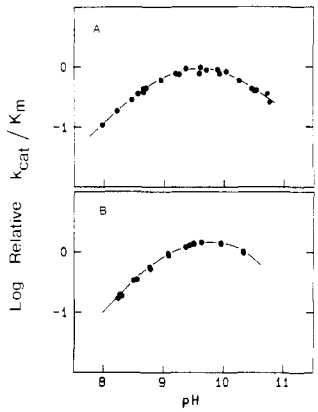

FIGURE 4: log (relative k_{cat}/K_m) vs pH profiles for (A) PNPdTp and (B) PNPdTp*Me with nuclease Y85F at 26 °C and 0.3 M ionic strength. The solid lines represent calculated fitting of the data to eq 4.

Table III: Temperature Dependence of Wild-Type Staphylococcal Nuclease k_{cat}/K_m vs pH Profiles with Thymidine 5'-(*p*-Nitrophenyl Phosphate) and Thymidine 3'-Phosphate 5'-(*p*-Nitrophenyl Phosphate)[a]

temp (°C)	substrate[b]	pK_{a1}	pK_{a2}
17.3	PNPdT	9.08 ± 0.10	none
25.4	PNPdT	8.98 ± 0.09	none
33.0	PNPdT	8.90 ± 0.08	none
34.0	PNPdT	8.90 ± 0.08	none
8.9	PNPdTp	9.11 ± 0.09	10.02 ± 0.08
16.0	PNPdTp	9.01 ± 0.08	9.67 ± 0.13
26.0	PNPdTp	8.94 ± 0.07	9.67 ± 0.09
37.0	PNPdTp	8.76 ± 0.06	9.48 ± 0.17
38.5	PNPdTp	8.75 ± 0.12	9.61 ± 0.06

[a] Assay conditions: approximately 0.2 M KCl and 100 mM buffer to give a total ionic strength of 0.3 M. Relative k_{cat}/K_m values were fitted to eq 4 or 5. Error estimates include a propagation of error in the determination of k_{cat}/K_m and assay pH. [b] Substrate abbreviations: PNPdTp, thymidine 3'-phosphate 5'-(*p*-nitrophenyl phosphate); PNPdT, thymidine 5'-(*p*-nitrophenyl phosphate).

the presence or absence of a hydroxyl on the phenyl side chain of residue 85. This pK_a disappears entirely when the substrate has a free 3'-hydroxyl. Figure 4 shows the pH dependence of k_{cat}/K_m for nuclease Y85F. The low pK_a is nearly identical with that for nuclease wt with PNPdTp (Table II). The high-side pK_a increases by 0.5 pH unit when the phenolic hydroxyl from residue 85 is removed.

The temperature dependence of the k_{cat}/K_m vs pH profile for nuclease wt with PNPdT and PNPdTp is reported in Table III (the k_{cat}/K_m vs pH profiles at each temperature are shown in the supplementary material). The pK_a values for both the high and low pK_a value groups decrease with increasing temperature. All five k_{cat}/K_m vs pH profiles with PNPdT were fitted to eq 5. Profiles of k_{cat}/K_m vs pH with PNPdTp were fitted to eq 4. A van't Hoff plot of the pK_a vs $1/T$ with PNPdTp gave the enthalpy of ionization (ΔH_{ion}) as 5.0 ± 1 kcal/mol for the acidic-side pK_a (8.94 at 26 °C) and 5.3 ± 1 for the basic-side pK_a (9.67). A similar plot of the pK_a vs $1/T$ with PNPdT as substrate gave a ΔH_{ion} of 5 ± 1 kcal/mol.

Substrate saturation curves for PNPdTp showed substrate inhibition at high concentrations with both nuclease wt and nuclease Y85F (supplementary material). The degree of inhibition was identical with that of nuclease wt and nuclease Y85F, and the saturation curves were superimposable when the difference in k_{cat} was accounted for.

Figure 5A shows the 1-dimensional ^{13}C NMR spectrum of nuclease wt labeled uniformly with 20% ^{13}C. Many upfield methyl peaks are individually resolved, as are the tyrosine and

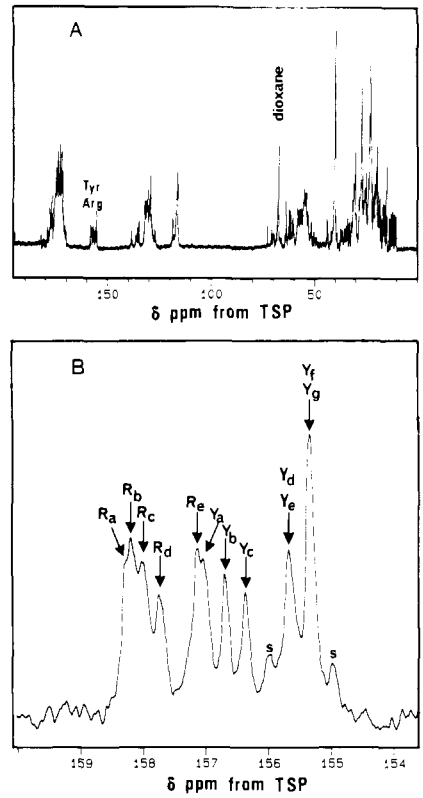

FIGURE 5: ^{13}C NMR spectrum of nuclease wt (20% enriched uniformly with ^{13}C) at 26 °C and pH 8.3. Dioxane (the internal chemical shift reference) was assigned as 67.475 ppm relative to (trimethylsilyl)-propionic acid (TSP). Arginine and tyrosine C^ξ resonances appear between 154 and 165 ppm, as indicated. (A) Full spectrum. (B) Spectrum A expanded to show the tyrosine and arginine region. Peaks identified as arginine C^ξ resonances are indicated by R (see Figure 2) and tyrosine resonances by Y. Peaks indicated by "s" are ^{13}C satellite peaks resulting from ^{13}C–^{13}C coupling from adjacent carbons in the protein.

arginine C^ξ peaks. Figure 5B shows an expansion of the region containing the arginine and tyrosine C^ξ resonances. Satellite peaks are due to ^{13}C–^{13}C scalar coupling observed for ^{13}C carbons that have an adjacent ^{13}C carbon. Statistically, this occurs with only 4% of the total carbon population. ^{13}C NMR selectively observes ^{13}C carbons, however, so that 20% of the observed carbons are directly bonded to another ^{13}C. Hence, two 10% satellite peaks flank each tyrosine C^ξ resonance (two adjacent carbons). The fact that satellite peaks are seen well above the noise in Figure 5B provides evidence, other than main-peak intensity, that resonances Y_d and Y_e and Y_f and Y_g are superimposed at this pH.

To distinguish the arginine resonances from the tyrosine resonances, nuclease was produced containing arginine as the only amino acid enriched with ^{13}C. The arginine C^ξ region of the ^{13}C NMR spectrum of nuclease wt produced by the arginine auxotroph *E. coli* AW624 grown on a synthetic amino acid mixture containing 90% ^{13}C at the guanidino carbon position is shown in the supplementary material. The chemical shift position of the arginine C^ξ resonances is readily discernible from the tyrosine resonances. In addition to the five arginine C^ξ resonances labeled with 90% ^{13}C, unresolved intensities from the peptide carbonyl and aliphatic regions could be observed above the base line (regions not shown). Additional evidence for identification of the peaks as guanidino carbon resonances comes from their characteristic sharpness and slightly increased intensity.

The ^{13}C NMR spectrum of nuclease wt was obtained at 13 pH values between pH 7.31 and 10.60. The arginine and

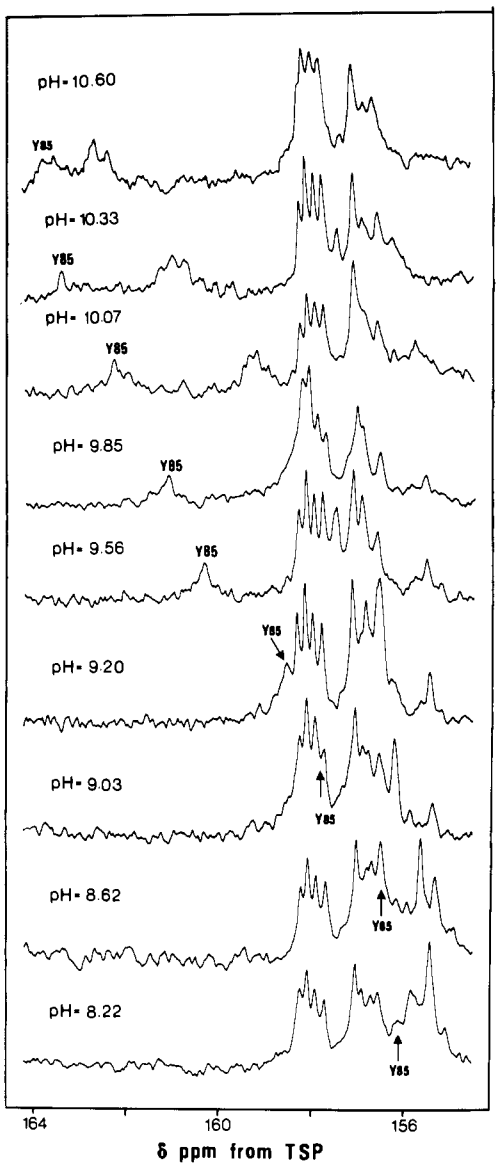

FIGURE 6: Tyrosine/arginine C^{ζ} region of selected ^{13}C NMR spectra illustrating the pH dependence of chemical shifts. All spectra were recorded at 26 °C and 0.3 M total ionic strength. The peak assigned to tyrosine-85 is indicated at each pH.

tyrosine region is shown at selected pH values in Figure 6. All ^{13}C NMR spectra were recorded in 90% H_2O (10% D_2O) so the measured pH is a true indication of the hydrogen ion activity. Of the five arginine resonances, none titrates between pH 7.31 and 10.60 (Figure 7). This suggests that all have pK_a values higher than 11.6 (since a 10% change in protonation state could be detected in the present study).[6] The pH dependence of the tyrosine chemical shifts is shown in Figure 8. Three tyrosine resonances are unperturbed by a change in pH below pH 10.2. Three others undergo small or incomplete spectroscopic titration of their NMR signals in the region of interest. Two of the tyrosines (Y_e and Y_f) have similar pK_a values of 10.1 ± 0.2 and Hill coefficients of 1.2 ± 0.2. Because neither tyrosine Y_e nor Y_f completes its titration in the pH range examined, the error in the determination of these values is large. Tyr-85 (Y_d) titrates with a pK_a of 9.53 ± 0.05 and a Hill coefficient of 0.98 ± 0.09 (Figure 8). The low pH chemical shift plateau of Tyr-85 is 155.75 ppm, and the high

[6] The normal titration range of an arginine guanidino C^{ζ} carbon is 2.0 ppm (Baillargeon, 1980a).

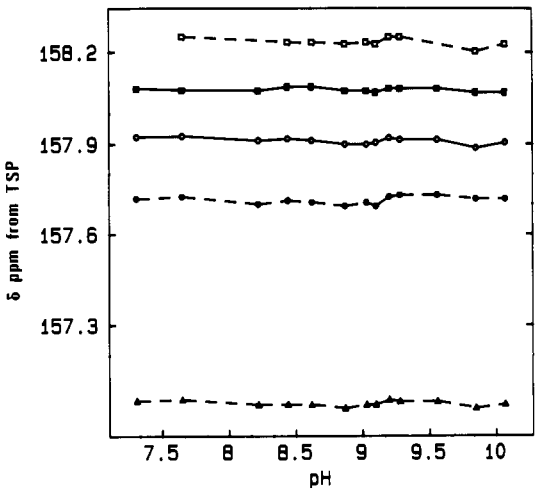

FIGURE 7: ^{13}C NMR chemical shift vs pH for the five arginine C^{ζ} resonances identified in nuclease wt. None of the five arginine resonances titrates with a pK_a less than 11.6. (□) R_a; (■) R_b; (○) R_c; (●) R_d; (△) R_e.

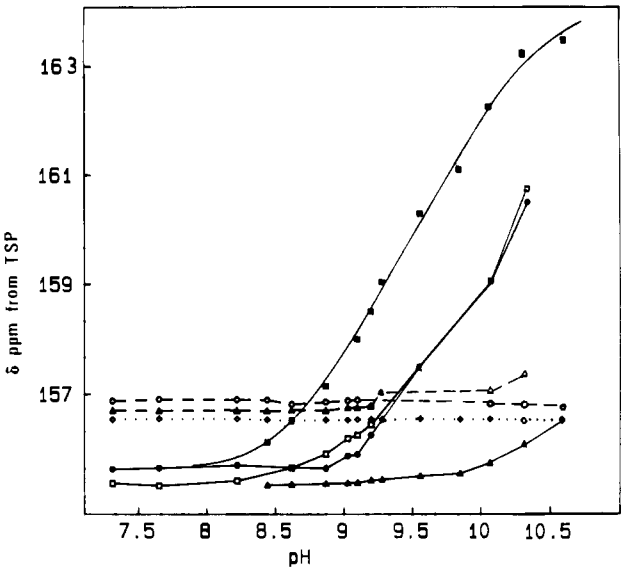

FIGURE 8: ^{13}C NMR chemical shift vs pH for the seven tyrosine resonances identified in nuclease wt. The signal assigned to tyrosine-85 (Y_d) yields a normal titration curve with pK_a = 9.53 ± 0.05. The solid line in this case represents fitting of the data according to the method of Markley (1975). The curves shown for other groups simply connect experimental points. (○) Y_a; (△) Y_b; (◇) Y_c; (■) Y_d; (●) Y_e; (□) Y_f; (▲) Y_g.

pH chemical shift plateau is 164.3 ppm. A 10 ppm chemical shift change is typical for a tyrosine C^{ζ} resonance upon deprotonation of the phenolic hydroxyl.

The Tyr-85 resonance was assigned by comparing the ^{13}C spectra of nuclease wt and nuclease Y85F. The ^{13}C spectrum of nuclease Y85F labeled uniformly with 20% ^{13}C at pH 9.42 and 0.3 M KCl is shown in the supplementary material. The position of the phenolic hydroxyl carbon of Tyr-85 in nuclease wt is indicated. Similar comparisons were made at pH 7.89, 9.14, and 9.37 (data not shown). In each case, the resonance assigned to Tyr-85 in nuclease wt was absent in the spectrum of nuclease Y85F. The positions of the arginine and other tyrosine resonances were the same in nuclease Y85F as in nuclease wt.

Discussion

Mononucleotide DNA analogues have advantages over DNA as substrates when one is attempting to dissect the molecular events of catalysis. Calf-thymus and salmon-sperm

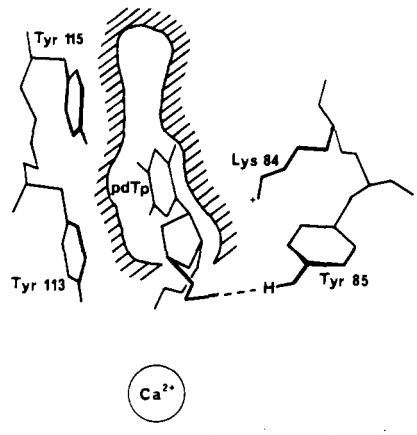

FIGURE 9: Schematic diagram of the active site of staphylococcal nuclease illustrating the hydrogen-bonding interaction between Tyr-85 and the 3′-phosphate of the substrate. When the phenolic hydroxyl of Tyr-85 (pK_a = 9.6) is removed, as in nuclease Y85F, an interaction appears between the 3′-phosphate and a group with a pK_a of 10.1. When neither a 3′-phosphate nor a 3′-phosphomonoester is present, this interaction on the 3′-side of the ribose ring is absent. The perspective shown is a true spatial representation of the groups indicated. It was obtained by examining the X-ray coordinates of Cotton et al. (1979).

DNA are heterogeneous and thus offer a spectrum of sites for nuclease action. Once endonucleolytic cleavage has occurred, processive exonucleolytic cleavage is possible. Subsequent catalytic events without complete dissociation of the enzyme from the DNA strand could make DNA a "sticky" substrate (i.e., catalytic events would occur faster than substrate dissociation), thus displacing the observed pK_a values in the k_{cat} vs pH profile from their true thermodynamic values. Relatively poor substrates, such as the mononucleotide DNA analogues used in this study, should yield truer enzymatic and substrate pK_a values in the k_{cat}/K_m vs pH profile.

Kinetic Parameters. In the crystal structure, the 3′-phosphate of pdTp forms a hydrogen bond to the phenolic hydroxyl of Tyr-85 (Cotton et al., 1979). The conventional view has been that the sole role of Tyr-85 is to enhance binding of nucleotide and DNA substrates. In line with this hypothesis is the expectation that the catalytic rate should be independent of the interaction with residue 85. The results of Hibler et al. (1987), which showed that with DNA as the substrate the ratio of V_{max} for nuclease Y85F/nuclease wt is 1.5, are in rough agreement with this. The present data indicate, however, that the situation is more complex. Nuclease wt has a turnover rate of about 13 min^{-1} with the three substrates examined. The observed rates are similar to those reported previously by Cuatrecasas et al. (1969) under somewhat different conditions. When the residue at position 85 is changed from tyrosine to phenylalanine (nuclease Y85F), the turnover number with PNPdTp*Me as the substrate is unchanged, whereas that with PNPdTp is reduced 14-fold. The k_{cat}/K_m values with nuclease wt confirm that substrate binding is weakened by abolishing the interaction between the phenolic hydroxyl of Tyr-85 and the 3′-phosphate of the nucleotide. Current results suggest that exonucleolytic hydrolysis is promoted by the presence of the hydrogen bond observed in the X-ray structure (Figure 9) between the phenolic hydroxyl of Tyr-85 and the 3′-phosphate of the nucleotide.

Despite the absence of the hydroxyl group on the side chain of residue 85 in nuclease Y85F, the identity of the 3′-group still influences substrate binding. Since the kinetic parameters with PNPdTp*Me as substrate are similar with both nuclease wt and nuclease Y85F, Tyr-85 appears not to interact with this substrate. When the 3′-group of the substrate is a dianionic phosphate (PNPdTp), k_{cat}/K_m is only diminished 21-fold relative to nuclease wt (as opposed to a 794-fold diminution with nuclease wt and PNPdT). This result suggests that another group on the enzyme substitutes for the specific interaction between Tyr-85 and the dianionic 3′-phosphate. This suspicion is confirmed by the presence of an altered basic-side pK_a value of 10.1 rather than 9.7 in the hydrolysis of PNPdTp by nuclease Y85F as compared to nuclease wt.

Critical questions to address are (1) whether the pK_a at 9.7 (26 °C) seen in the k_{cat}/K_m profile for nuclease wt is significantly different from the pK_a of 10.1, (2) whether different groups, rather than the same functional group with an altered pK_a, are responsible for the pK_a values of 9.7 and 10.1, and (3) whether all three pK_a values of 10.1 determined from the pH dependence of k_{cat}/K_m with different substrates and enzyme variants (Table II) arise from the same group.

Examination of the temperature dependence of the k_{cat}/K_m vs pH profile for nuclease wt with PNPdTp as the substrate (Table III and supplementary material) shows that the basic-side pK_a remains at about 9.7 ± 0.1 between 16 and 38.5 °C. When two ionizing groups have pK_a values closer that 2 units, it is difficult to determine either value accurately, although their average can be determined precisely.[7] Since the acid-side pK_a appears to be largely unaffected by the nature of the 3′-substituent of the substrate or the substitution at residue 85 (Table II), its value can be fixed to allow for a more precise determination of the basic-side pK_a. Values for the basic-side pK_a determined in this way were not significantly different from those (Table II) determined by letting both pK_a values float. The above considerations provide a good level of confidence that pK_a values of 9.7 and 10.1 can be discriminated.

The slope of the data points that define the ascending limb (acidic side) of the pH profile is constant at +1; this indicates the participation of a single group in the deprotonation step. The slope of the data points that define the descending limb (basic side) varies slightly from −1 to −1.2, also indicating the participation of only a single group in the reprotonation step in the k_{cat}/K_m vs pH profile. Under no circumstances was a slope greater than −1.2 observed.

The pK_a of 9.7 has been tentatively assigned to deprotonation of Tyr-85. The evidence for this assignment rests on the agreement between the kinetically derived pK_a and that obtained spectroscopically by ^{13}C NMR with nuclease wt. In addition, this coincident pK_a is altered (kinetic parameters) or missing (NMR data) with the mutant nuclease Y85F. Having assigned the pK_a of 9.7 to Tyr–85, one is forced to conclude that the kinetically derived pK_a values of 10.1 observed with nuclease Y85F arise from a group other than Tyr-85. Candidates for this pK_a include Tyr-113, Tyr-115, and Lys-84 (Figure 5); histidines can be ruled out by virtue of their much lower pK_a values (Alexandrescu et al., 1988), and the thymidine base has a pK_a of 10.5 ± 0.1 at 0.3 M ionic strength and 25 °C (determined by ^{13}C NMR titration of PNPdT in 90% H_2O/10% D_2O). The fact that no basic-side pK_a is observed with PNPdT (which lacks a 3′-phosphate) as the substrate (Table I) agrees with the hypothesis that this

[7] When two pK_a values are closer than 2 pH units, it is not possible a priori to rule out reverse protonation in the catalytically competent form of the enzyme (i.e., the group with the lower pK_a must be unprotonated and that with the higher pK_a must be protonated). There is no evidence suggesting this is the case with nuclease, however. Because the temperature coefficient of both pK_a values in the k_{cat}/K_m vs pH profile are identical (present study), the two cases described above could not be distinguished by an investigation of the temperature dependence of the k_{cat} pH profile.

pK_a arises from specific interaction between an enzyme group and the phosphate at the 3'-position of the substrate rather than interaction between the enzyme and conserved portions of the substrate, as would be the case with the thymidine base, Tyr-113, or Tyr-115.

The substrate inhibition with PNPdTp noted by Dunn et al. (1973) is not eliminated by removal of the phenolic hydroxyl of Tyr-85 (nuclease Y85F). This suggests that Tyr-85 is not involved in the interaction responsible for substrate inhibition. Although no substrate inhibition is observed with PNPdT, the K_m is so high that inhibitory concentrations may not have been reached.

Uhlmann and Smith (1987) also compared the kinetic parameters of nuclease wt and nuclease Y85F. They reported a 40-fold diminution of k_{cat}/K_m with PNPdTp as substrate upon replacement of Tyr-85 with Phe. This result is somewhat higher that the (21 ± 6)-fold change reported here (Table I). Uhlmann and Smith interpreted this difference in terms of the loss of a hydrogen bond between a negatively charged acceptor and neutral donor. The situation is more complicated, however, because a new interaction appears to take over (as indicated by the pK_a of 10.1) for the missing Tyr-85. Thus, interpretation of k_{cat}/K_m reatios for nuclease wt and nuclease Y85F with PNPdTp may be inappropriate.

Temperature Dependence of pK_a Values. The enthalpy of ionization of kinetic parameter pK_a values can be highly diagnostic for certain types of groups. The observed ΔH_{ion} (5 kcal/mol) for the pK_a values in the k_{cat}/K_m profile is very close to the theoretical value (6 kcal/mol) for the phenolic hydroxyl of tyrosine (Edsall & Wyman, 1958). This is consistent with Tyr-85 being the basic-side pK_a at 9.67 (at 26 °C).

Interpretation of the experimental ΔH_{ion} (5 kcal/mol) for the acidic-side pK_a is less straightforward. All amino bases would have a higher ΔH_{ion} (9–12 kcal/mol) than that measured. Hence, lysines and arginines appear to be ruled out. The protein has no sulfhydryls. Tyrosine can be eliminated because none of the seven tyrosines has a pK_a less than 9.5. None of the four histidines has a pK_a value (measured by ^1H NMR in D_2O and corrected for H_2O) close to 9.0 in 0.3 M KCl (Alexandrescu et al., 1988). This leaves a carboxylate (with an elevated ΔH_{ion} possibly because of an accompanying conformational change) as a candidate on the basis of ionization enthalpy. However, a carboxylate base (most likely Glu-43) also seems unlikely since the pK_a of 8.94 is much too high for a carboxylate exposed to solvent. Assuming that the mechanism of hydrolysis proposed by Cotton is correct, then this pK_a might correspond to a Ca^{2+}-bound H_2O molecule that is the attacking nucleophile at phosphorus. However, H_2O, either free, coordinated to the metal ion, or coordinated to Glu-43, should have ΔH_{ion} greater than 12 kcal/mol.

^{13}C NMR. Mutagenesis is a selective form of chemical modification, and assignments based on such changes will be valid only if the amino acid replacement does not affect the observed chemical shift of other residues or the conformation of the protein in a way that would obscure the analysis. The pK_a values observed in the k_{cat}/K_m vs pH profile for nuclease Y85F reflect extrapolation to zero substrate, where the independent ^1H NMR studies of Markley (Grissom et al., 1986) and Bolton (Hibler et al., 1987) indicate a similarity of structure for nuclease wt and nuclease Y85F. Although the one-dimensional ^1H NMR spectra of nuclease wt and nuclease Y85F are similar in the absence of Ca^{2+} and pdTp, they differ substantially upon the addition of Ca^{2+} and pdTp (Hibler et al., 1987). This result suggests it may not be feasible to base the assignment of tyrosine in the ternary complex (nuclease:pdTp:Ca^{2+}) on spectral changes accompanying replacement of this residue. The spectroscopically determined pK_a value for the free enzyme should correspond to k_{cat}/K_m conditions (i.e., extrapolation to zero substrate); the spectroscopically determined pK_a value for an enzyme–inhibitor complex should correspond to that determined kinetically for the central enzyme–substrate complex.

Chemical intuition does not suggest arginine as a logical candidate for the basic catalyst that facilitates attack on phosphorus and appears in the k_{cat}/K_m vs pH profile at 8.94 or 9.67. This expectation is confirmed by the finding that none of the five arginine guanidino carbon resonances shift below pH 10.6. Studies by Mildvan, however, suggest that Arg-35 and Arg-87 each have a central role in catalysis (Serpersu et al., 1987). A second enzymatic base (this one protonated) is required to complete the catalytic cycle. The current study does not preclude Arg-35 or Arg-87 from being a candidate for the enzymatic proton donor to the departing ribose 5'-alkoxide. If arginine serves this role, its pK_a must be above 11.6. A pK_a in the range normally expected for the guanidino group would be acceptable for protonating an alkoxide with a pK_a near 16.

Conclusion. The pH dependence of kinetic parameters can suggest functional groups that participate directly in catalysis. Nuclease contains 60 functional groups (including the carboxyl and amino termini) that can ionize between pH 2 and 12. Remarkably, the ionization of only two groups affects activity near the pH optimum. NMR spectroscopy probes the pH dependence of all residues in a protein, and assignment of the NMR signals to individual amino acids makes it possible to correlate protein structure with catalytic function. The present study has provided kinetic and biophysical evidence suggesting a role for Tyr-85 in exonucleolytic catalysis and identified Tyr-85 as the basic-side pK_a expressed in the k_{cat}/K_m vs pH profile. Further study will be required to elucidate other enzyme–substrate interactions that contribute to catalysis and account for the pK_a at 10.1 that appears when the phenolic hydroxyl of Tyr-85 is removed.

Acknowledgments

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Supplementary Material Available

Figures showing the temperature dependence of log (relative k_{cat}/K_m) vs pH for the substrates PNPdT and PNPdTp, the substrate saturation curves for PNPdTp exhibiting substrate inhibition with nuclease wt and Y85F, the ^{13}C NMR spectrum of nuclease wt specifically labeled with 90% ^{13}C in the C^{ζ} position, and the ^{13}C NMR spectrum of nuclease Y85F at pH 9.42 (5 pages). Ordering information is given on any current masthead page.

Registry No. EC 3.1.4.7, 9013-53-0; PNPdT, 2304-08-7; PNPdTp, 24418-11-9; PNPdTp*Me, 118375-50-1; L-Tyr, 60-18-4; L-Arg, 74-79-3; L-Phe, 63-91-2.

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Mechanistic Studies on Phenylalanine Hydroxylase from *Chromobacterium violaceum*. Evidence for the Formation of an Enzyme–Oxygen Complex†

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ABSTRACT: Steady-state kinetic analysis of pterin-dependent phenylalanine hydroxylase from *Chromobacterium violaceum* indicated that the enzyme follows a partially ordered reaction mechanism. The data suggested that oxygen is the first substrate to bind to the enzyme. This result was further supported by rapid-quench experiments in which the enzyme–oxygen complex was trapped to yield product. Additional support for the presence of an enzyme–oxygen complex was derived from magnetic susceptibility measurements of molecular oxygen in the presence and absence of cuprous phenylalanine hydroxylase. The magnetic susceptibility of dissolved oxygen decreased in the presence of the enzyme, supporting a direct oxygen–metal interaction.

Phenylalanine hydroxylase (EC 1.14.16.1) catalyzes the reaction

$$H_4\text{-pterin} + \text{phenylalanine} + O_2 \rightarrow \text{tyrosine} + H_2O + H_2\text{-pterin}$$

The process requires a tetrahydropterin cofactor that is oxidized by two electrons during substrate hydroxylation. Phenylalanine hydroxylase (PAH)[1] from rat liver and from *Chromobacterium violaceum* are both metalloenzymes. Mammalian PAH contains tightly bound non-heme iron

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[1] Abbreviations: PAH, phenylalanine hydroxylase; $DMPH_4$, 6,7-dimethyltetrahydropterin; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Phe, L-phenylalanine; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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