Variation in the Steady State Kinetic Parameters of Wild Type and Mutant T5 5′-3′-Exonuclease With pH

PROTONATION OF Lys-83 IS CRITICAL FOR DNA BINDING*

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T5 5′-3′-exonuclease is a member of a family of homologous 5′-nucleases essential for DNA replication and repair. We have measured the variation of the steady state parameters of the enzyme with pH. The log of the association constant of the enzyme and substrate is pH-independent between pH 5 and 7, but at higher pH, it decreases (gradient −0.91 ± 0.1) with increasing pH. The log of the turnover number increases (gradient 0.9 ± 0.01) with increasing pH until a pH-independent plateau is reached. The T5 5′-3′-exonuclease-catalyzed reaction requires the protonation of a single residue for substrate binding, whereas $k_{cat}$ depends on a single deprotonation as demonstrated by the bell-shaped dependence of log ($k_{cat}/K_m$) on pH. To investigate the role of a conserved lysine (Lys-83), the pH profile of log ($k_{cat}/K_m$) of a K83A mutant was determined and found to increase with pH (gradient 1.01 ± 0.01) until a pH-independent plateau is reached. We therefore conclude that protonation of Lys-83 in the wild type protein facilitates DNA binding. The origin of the pH dependence of the $k_{cat}$ parameter of the wild type enzyme is discussed.

Organisms as diverse as bacteriophage and humans require 5′-nucleases to participate in, and ensure the fidelity of, DNA replication and repair. These enzymes are involved in excising damaged DNA and the removal of Okazaki fragments produced during replication. T5 5′-3′-exonuclease is a virus-encoded member of a family of homologous 5′-nucleases which includes the N-terminal domain of DNA polymerase I proteins (1). More distantly related eukaryotic and mammalian enzymes also exhibit sequence homology, albeit to a lesser extent, and include the human enzyme hFEN-1.† The 5′-nucleases catalyze the exonucleolytic hydrolysis of phosphodiester bonds in a variety of nucleic acid substrates, including single-stranded and double-stranded DNA and DNA-RNA hybrids (RNase H activity).

In addition, the 5′-nucleases also exhibit structure-specific endonucleolytic activity, resulting in the cleavage of DNA flap and pseudo-Y structures (Fig. 1a) (2–4). This structure-specific hydrolysis of bifurcated structures is mainly observed at the junction between double-stranded and single-stranded DNA (2–4). Both exonucleolytic and endonucleolytic hydrolysies proceed with scission of the 3′-oxygen phosphorus bond to generate products terminating in a 3′-hydroxyl group and a 5′-phosphate monoester, respectively (5–7).

The x-ray crystal structures of five members of the 5′-nuclease family have been reported in the absence of DNA substrates (8–12). The structures of T4 RNase H and the 5′-exonuclease domain of Taq polymerase I both contained areas of disorder (10, 12), but the structure of T5 5′-3′-exonuclease revealed the presence of a hole bounded by a helical arch upon a globular domain containing the putative active site (9). The hole is large enough to accommodate single- but not double-stranded DNA. To explain substrate processing, a threading mechanism in which the single-stranded 5′-terminus enters the arch has been proposed (9). Regions through which single-stranded DNA could thread are also observed in the most recently solved structures of MjFEN-1 (11) and PfFEN-1 (8), two further members of the 5′-nuclease family.

The putative active sites of the enzymes are located in front of the holes and contain a number of conserved acidic residues that coordinate two metal ions. Divalent metal ions are an essential co-factor in the reaction. In the crystal structure of T5 exonuclease (Fig. 2) and T4 RNase H, the metal binding sites are separated by distances of 7–8 Å. The structure of the 5′-nuclease domain of Taq polymerase also has a separate binding site for a zinc ion, close to one of the magnesium sites (10). The biological significance of this site is unknown. In the structures of the MjFEN-1 and PfFEN-1, the distance between the metal sites is 5 Å (8, 11).

The putative active sites of T5 5′-3′-exonuclease, T4 RNase H, and Taq polymerase I contain a lysine residue (Lys-83 in the T5 enzyme, Fig. 2) that is conserved throughout all the eubacterial polymerases and phage 5′-nucleases (9, 10, 12, 13). Mutagenesis of the lysine residue produces dramatic effects on both DNA binding and catalysis (7, 14–16). However, the FEN-1 enzymes appear to lack similarly conserved basic residues adjacent to the metal binding sites, and it has been suggested that DNA interactions may be mediated in a different way in these enzymes (8, 11). A tyrosine residue (Tyr-82 in the T5 enzyme, Fig. 2) is also conserved throughout all the eubacterial polymerases and phage 5′-nucleases (13) although, surprisingly, mutation of the corresponding amino acid in T4 RNase H to phenylalanine appears to have little effect on enzyme activity (15).

The mechanism of this group of enzymes remains unknown although various proposals have been made. A two-metal ion
mechanism was proposed by Steitz and co-workers (10), similar to that of 3'-5'-exonuclease domain of the Klenow fragment (17). This mechanism involves one metal ion acting as a Lewis acid to generate magnesium-bound hydroxide that attacks the scissile phosphodiester bond. The other metal ion coordinates the 3'-oxygen to activate the leaving group and acts as a Lewis acid coordinating one of the nonbridging oxygens to lower the energy of the penta-coordinate transition state. The structure of 5'-nuclease domain of Taq polymerase contains two magnesium ions and a zinc ion, and the distance between one magnesium and the zinc ion are close to the distance between magnesium ions in 3'-5'-exonuclease domain of the Klenow fragment (10). Although the distance between the metal ions in the four other x-ray structures is variable, in each case it is beyond the typical separation of 4 Å required in two-metal ion mechanisms, appearing to make this mechanistic suggestion less plausible unless gross conformational change accompanies substrate binding (8, 9, 11, 12).

An alternative mechanistic proposal is that the exonuclease and endonuclease activities of this group of enzymes may operate via different mechanisms (16). For the exonucleolytic reaction, it has been suggested that a lysine residue located in the active site of the enzyme (Lys-83 in the T5 exonuclease structure) acts as a general base/acid-activating water to attack the scissile bond and protonating the leaving group oxygen (16). Further biochemical studies are clearly required if the mechanism of this family of enzymes is to be fully elucidated.

Insights into enzyme catalysis can be provided by analysis of the pH-rate profiles of enzymatic reactions. Previous studies of T5 5'-3'-exonuclease determined the pH optimum of this enzyme to be 9.3, using a nonstructure-specific spectrophotometric assay based on the release of acid-soluble nucleotides from high molecular weight DNA (6). This assay primarily detects the products of exonucleolytic cleavage. Treatment of the radiolabeled pseudo-Y structure shown in Fig. 1a with T5 5'-3'-exonuclease at pH 9.3 results in the formation of labeled 19-, 21-, and 14-mer oligonucleotide endonucleolytic products along with exonucleolytic products of approximately 3 and 5 nucleotides in length (3). The pH dependence of the product distribution after exposure of the pseudo-Y structure to enzyme has been quantified (16). Although the total amount of products obtained was found to be invariant, high pH favored the formation of exonucleolytic products, whereas lower pH favored the production of endonucleolytic products. The crossover point where the amount of exonucleolytic products first appeared to exceed endonucleolytic products was about pH 7.

Recently we reported a single-cleavage assay for T5 5'-3'-exonuclease using a 5'-overhanging hairpin substrate, HP1 (Fig. 1b), which enabled the catalytic parameters of this enzyme to be determined for the first time (7). T5 5'-3'-exonuclease catalyzes the structure-specific endonucleolytic hydrolysis of the 5'-overhanging hairpin substrate HP1 (Fig. 1b) with a rate enhancement of at least $10^{18}$ (7). To further aid our understanding of the mechanistic features of the T5 5'-3'-exonuclease-catalyzed reaction, we studied the variation of the catalytic parameters of the enzyme and a K83A mutant with pH. To our knowledge, this is the first detailed analysis of the pH-rate profile of any of the 5'-nuclease family.

**EXPERIMENTAL PROCEDURES**

**Synthesis, Purification, and Characterization of HP1—HP1 (d(CGCTGTCAACACACGGTTGTGCTGTTC) Fig. 1b) was synthesized and purified according to Pickering et al. (7).**

**Preparation of Purification of K83A Mutant Exonuclease—K83A was prepared and purified as previously described (3, 16). The concentration of protein was determined using the assay of Bradford (18).**

**pH Dependence of the Michaelis-Menten Parameters—Separate reaction mixtures of appropriate concentrations of 5'-32P-labeled substrate HP1 (Fig. 1b) in 25 mM buffer, 10 mM MgCl₂, 50 mM KCl, and 0.2 mg/ml BSA were prepared. These reaction mixtures were incubated at 90 °C for 2 min and then allowed to cool to 37 °C. The enzyme stock solution was dissolved in 25 mM buffer, 10 mM MgCl₂, 50 mM KCl, and 0.2 mg/ml BSA and kept on ice until use. The buffers employed to maintain pH were pH 5.6–6.0 MES, pH 6.5–7.0 MOPS, pH 7.0–8.0 HEPES, pH 8.0–8.4 EPS, pH 9.0–10.0 CHES, pH 9.3 potassium glycinate, and pH 10.5 CAPS. Reactions were initiated by addition of enzyme (final concentrations: 25 mM buffer, 10 mM MgCl₂, 50 mM KCl, and 0.2 mg/ml BSA) and briefly vortexed. The final concentrations of the substrate and enzyme were 0.001–1 μM and 3–30 μM, respectively. The progress of the reactions was followed by taking aliquots at appropriate time intervals. Individual samples were quenched by immediate addition to 10 μl of denaturing “stop” mix (95% deionized formamide, 10 mM EDTA, 0.05% xylene cyanol FF, and 0.05% bromphenol blue) and analyzed by 20% denaturing polyacrylamide gel electrophoresis followed by phosphoimaging.**

**Initial rates of reaction at the various substrate concentrations were determined. This allowed the kinetic parameters to be calculated by nonlinear regression fitting of the data to the Michaelis-Menten equation (Equation 1). All curve fitting was carried out using KaleidaGraph Software (Synergy Software, Reading, PA),**

$$v = \frac{v_{\text{max}}[S]}{[E]_0 + K_s + [S]}$$

(Eq. 1)
where \( v = \text{initial rate}, \ [S] = \text{substrate concentration}, \ \text{and} \ [E] = \text{total enzyme concentration}. \)

The pH dependence of \((k_{\text{cat}}/K_m)_{\text{obs}}\) was fitted to a single ionization using Equation 2 (19),

\[
(k_{\text{cat}}/K_m)_{\text{obs}} = \frac{(k_{\text{cat}}/K_m)_{\text{ESN}}}{K_{\text{ESN}} + [H^+]} \quad \text{(Eq. 2)}
\]

where \(K_{\text{ESN}}\) is the acid dissociation constant of the enzyme-substrate complex; \((k_{\text{cat}}/K_m)_{\text{ESN}}\) is the turnover number at a given pH; and \((k_{\text{cat}}/K_m)_{\text{ESN}}\) is the maximal turnover number of the deprotonated catalytically competent form of the ES complex.

The pH dependence of \((1/K_m)_{\text{obs}}\) was fitted to a single ionization using Equation 3 (19),

\[
\left(\frac{1}{K_m}\right)_{\text{obs}} = \left(\frac{1}{K_m}\right)_{\text{ESN}} \left[\frac{[H^+]}{[H^+] + (K_{\text{ESN}})}\right] \quad \text{(Eq. 3)}
\]

where \(K_{\text{ESN}}\) is an acid dissociation constant of the free enzyme, \((1/K_m)_{\text{ESN}}\) is the association constant of free enzyme and substrate at a given pH, and \((1/K_m)_{\text{ESN}}\) is the maximal association constant for a singly protonated catalytically competent form of the enzyme.

The pH dependence of \((k_{\text{cat}}/K_{\text{ESN}})_{\text{obs}}\) was fitted to a double ionization using Equation 4 (19),

\[
\left(\frac{k_{\text{cat}}}{K_{\text{ESN}}}\right)_{\text{obs}} = \frac{(k_{\text{cat}}/K_m)_{\text{ESN}}}{K_{\text{ESN}}^2 + [H^+]^2} \quad \text{(Eq. 4)}
\]

where \(K_{\text{ESN}}\) and \(K_{\text{ESN}}\) are acid dissociation constants of the free enzyme, \((k_{\text{cat}}/K_{\text{ESN}})_{\text{obs}}\) is the second order rate of reaction at a given pH, and \((k_{\text{cat}}/K_{\text{ESN}})_{\text{obs}}\) is the maximal second order rate constant of the singly protonated catalytically competent form of the enzyme.

**Determination of the pH Dependence of \((k_{\text{cat}}/K_m)_{\text{ESN}}\) for K83A—Reactions were carried out as above with a final concentration of 10 μM K83A, 1 mM 5'-32P-labeled HP1, 25 mM buffer (pH 7.5–8.0 HEPES, pH 8.4 FMPS, pH 9.0–10.5 CHES), 10 mM MgCl2, 50 mM KCl, and 0.2 mg/ml BSA. The pseudo first order rate of reaction \((k_{\text{cat}}/K_{\text{ESN}})_{\text{obs}}\) was determined by plotting the percentage of product against time and fitting Equation 5 by nonlinear regression analysis.

\[
\text{Percentage product} = 100\left(1 - \exp\left(-\frac{b}{amon}\right)\right) \quad \text{(Eq. 5)}
\]

where \([E]\) = total enzyme concentration.

The pH dependence of \((k_{\text{cat}}/K_m)_{\text{obs}}\) was fitted to a single ionization using Equation 6 (19),

\[
\left(\frac{k_{\text{cat}}}{K_m}\right)_{\text{obs}} = \left(\frac{k_{\text{cat}}}{K_m}\right)_{\text{ESN}} \left[\frac{[H^+]}{[H^+] + (K_{\text{ESN}})}\right] \quad \text{(Eq. 6)}
\]

where \(K_{\text{ESN}}\) is the acid dissociation constant of the free enzyme, \((k_{\text{cat}}/K_{\text{ESN}})_{\text{obs}}\) is the second order rate of reaction at a given pH, and \((k_{\text{cat}}/K_{\text{ESN}})_{\text{obs}}\) is the maximal second order rate constant of the singly deprotonated catalytically competent form of the enzyme.

**RESULTS**

**Kinetic Behavior of the Wild Type Enzyme—**The catalytic parameters, \(k_{\text{cat}}\) and \(K_m\), have been determined for wild type T5 5'-3' exonuclease at twelve different pH values. The 32P-labeled 29-mer 5'-overhanging hairpin, HP1 (Fig. 1b), was used as a substrate as it has been previously demonstrated to undergo a single structure-specific enzyme-catalyzed hydrolytic cleavage to generate 8- and 21-mer products (7). Thus, rates recorded in these experiments refer to hydrolysis of a single phosphodiester bond. The concentration of KCl and MgCl2 employed have previously been determined as optimum for the reaction (6, 20). At each pH, initial rates of reaction were determined at twelve substrate concentrations ranging from one-fourth- to 10-fold \(K_m\). Data were then fitted to the Michaelis-Menten equation (Equation 1) using nonlinear regression analysis. For each pH studied, at least three independent experiments have been conducted. At several pH values (pH 7.0 MOPS and HEPES, pH 8.0 HEPES and EEPES, and pH 9.3 CHES and potassium glycinate), independent experiments have varied the buffer component. This was undertaken to control for specific buffer effects. No such effects were observed.

The pH dependence of the individual kinetic parameters \(k_{\text{cat}}\) and \(K_m\) are illustrated in Fig. 5, a and b, respectively. The log of the turnover number, \(k_{\text{cat}}\), increases with increasing pH until a pH-independent plateau is reached at about pH 9. The slope of the curve between pH 5.5–8.4 is equal to 0.9 ± 0.01 indicating a direct dependence of \(k_{\text{cat}}\) on the concentration of hydroxide ions in this pH range. The log of association constant of free enzyme and free substrate, \(1/K_m\), is pH-independent in the range pH 5–7, but decreases with increasing pH. The slope of the curve in the region pH 10.5–8.4 is 0.91 ± 0.1, indicating that \(K_m\) is inversely proportional to hydroxide ion concentration in this pH range. The data shown in Fig. 3, a and b, have been fitted to Equations 2 and 3 which are derived from single ionizations shown in Fig. 4, a and b. Equation 2 assumes that two ionization states of the enzyme-substrate complex are possible, a protonated and deprotonated form, but that only the
FIG. 4. Kinetic schemes including the possible ionization states of enzyme and enzyme-substrate complex used to derive equations for curve-fitting the pH dependence of the catalytic parameters of wild type and K83A mutant T5 5′-3′-exonuclease.

a, the enzyme-substrate complex exists in two ionization states described by the acid dissociation constant $K_{E1}$. Only the deprotonated form of the enzyme-substrate complex is catalytically proficient and catalyzes conversion of enzyme-substrate complex to free enzyme and products with a rate constant of $k_{cat}/K_{E0}$. The relationship of observed $k_{cat}$ at a particular pH to the rate and equilibrium constants is described by Equation 2 under “Experimental Procedures.”

c, the enzyme exists in three ionization states described by the acid dissociation constants $K_{E1}$ and $K_{E2}$. Only the singly protonated form of the enzyme is catalytically competent and promotes the reaction with a maximal second order rate constant of $k_{cat}/K_{E0}$. The relationship of observed $k_{cat}/K_{E0}$ at a particular pH to the rate and equilibrium constants is described by Equation 4 under “Experimental Procedures.”

d, the mutant enzyme, K83A, exists in only two ionization states with the acid dissociation constants $K_{E1}$. Only the deprotonated form of the enzyme can undergo the reaction with a maximal second order rate constant of $k_{cat}/K_{E0}$. The relationship of observed $k_{cat}/K_{E0}$ at a particular pH to the rate and equilibrium constants is described by Equation 6 under “Experimental Procedures.”

deprotonated form is catalytically competent (Fig. 4a).

Fitting the $k_{cat}$ data (Fig. 3b) reveals a value of $(k_{cat}/K_{E0})$, the rate constant which describes conversion of the deprotonated catalytically competent form of the enzyme-substrate complex to enzyme and products, to be $100 \pm 16$ min$^{-1}$, and a $pK_a$ of $8.3 \pm 0.10$ for the enzyme-substrate complex. The $R$ factor for this curve fit is 0.99. Equation 3 assumes that two ionization states of the free enzyme are possible but only a singly protonated form is capable of binding substrate (Fig. 4b). Fitting the $1/K_m$ data yields a value of $(K_{m0})$, the dissociation constant of a protonated catalytically competent form of the enzyme, to be $11 \pm 2$ nM and a $pK_a$ of $8.3 \pm 0.14$ for the free enzyme. The $R$ factor for this curve fit is 0.96. Thus, association of the enzyme and the DNA substrate depends on the deprotonation of a group in the free enzyme with a $pK_a$ of 8.3, whereas $k_{cat}$ depends on the deprotonation of a group in the enzyme-substrate complex with a $pK_a$ of 8.3.

The pH dependence of log $(k_{cat}/K_{m})$ is illustrated in Fig. 3c. The second order rate constant first increases and then decreases with increasing pH, demonstrating that the rate of conversion of free enzyme and substrate to free enzyme and products is dependent on both a deprotonation and protonation event. The bell-shaped curve fits, with an $R$ factor of 0.95, to the double ionization described by Equation 4 derived from the scheme illustrated in Fig. 4c. Equation 2 is derived by considering that the enzyme is present in three ionization states but that reaction only proceeds when the enzyme is in a singly protonated form and the second order rate constant, which describes the conversion of free enzyme and substrate to free enzyme and products, is $(k_{cat}/K_{E0})$ (19). One of the acid dissociation constants of the free enzyme ($K_{E1}$, or $K_{E2}$) is experimentally determined as $pK_a$ 8.3 by the pH dependence of $1/K_m$, thus $K_{E1}$ was defined during curve fitting. This yields $(k_{cat}/K_{m0}) = (7.8 \pm 1.6) \times 10^3$ M$^{-1}$ min$^{-1}$ and a second ionization of the free enzyme with a $pK_a$ value of 8.4 \pm 0.1. Defining $K_{E2}$ during curve fitting yields, within experimental error, the same second $pK_a$ value of $8.3 \pm 0.1$.

Kinetic Behavior of the K83A Mutant—Kinetic parameters of the K83A mutant have been previously determined to be $K_m = 28$ nM and $k_{cat} = 9.9 \times 10^{-2}$ min$^{-1}$ at pH 9.3 with HP1 as the substrate (7). The very high $K_m$ of this mutant prohibited full analysis of the catalytic parameters at a range of pH values. Instead, rate constants were determined at eight different conditions of pH under pseudo first order reaction conditions where $K_m > [E] > [S]$. Under these conditions, the normalized first order rate constant is equal to $k_{cat}/K_m$ (Equation 5). A plot of the variation of log $(k_{cat}/K_m)$ versus pH is shown in Fig. 5a. In contrast to the pH dependence of the second order rate constant for the wild type enzyme, the data describe a single, rather than a double, ionization. The second order rate constant for K83A increases with pH until a pH-independent plateau is reached. Between pH 7.5 and 8.5, the curve has a slope of $1.01 \pm 0.01$, indicating that $k_{cat}/K_m$ is directly proportional to...
hydroxide ion concentration in this pH range. The most notable feature of this data is the strong resemblance to the pH rate profile for $k_{\text{cat}}$ for the wild type enzyme as shown in Fig. 5b. These data have been fitted to a single ionization using Equation 6, which is derived from the scheme illustrated in Fig. 4d, in which the free enzyme exists in two ionization states, but only the deprotonated form is catalytically competent. This yields an optimum $(k_{\text{cat}}/K_m)_D$ value as $(4.0 \pm 1.0) \times 10^{-3}$ M$^{-1}$ min$^{-1}$ and a $pK_a$ of 8.7 $\pm$ 0.1 for the free K83A mutant enzyme.

**DISCUSSION**

The data reported here demonstrate that the second order rate constant of the wild type enzyme-catalyzed reaction varies with pH, with two groups titrating, one being protonated the other being deprotonated. The slope of the pH dependence of log $(1/K_m)$ and log $k_{\text{cat}}$ are both integral, suggesting a discreet ionization of a single functional group, with the former parameter reaching a maximum upon protonation and the later on deprotonation. The residues titrated have $pK_a$ values of 8.3 and 8.4 in the free enzyme and the enzyme-substrate complex, respectively. Thus, guided by a consensus sequence obtained from sequence alignment of phage and prokaryotic enzymes (13), we were able to select Lys-83 as a reasonable candidate for the ionizing amino acid.

**The pH Dependence of K83A**—In the x-ray structure of T5 5'-3'-exonuclease Lys-83 is located at the bottom of the helical arch close to metal site 1 (9). The distance between the e-amino group of Lys-83 and metal site 1 in the T5 structure is 5 Å. This residue is strictly conserved through all the phage and prokaryotic 5'-nucleases (13). An x-ray structure of this mutant is available (16) and is very similar to the wild type protein. Two studies have already characterized the effect of this mutation to alanine on T5 5'-exonuclease activity (7, 16). In a gel retardation assay, in the absence of magnesium ions, the $K_d$ of this mutant bound to a pseudo-Y structure (Fig. 1a) was found to be increased by a factor of 40 relative to the unmodified protein (16). At pH 9.3, the catalytic parameters of this mutant with HP1 as the substrate were found to be $K_m = 28 \pm 0.4$ μM and $k_{\text{cat}} = 9.9 \pm 0.1 \times 10^{-2}$ min$^{-1}$, some 700-fold increased and 1000-fold decreased, respectively, relative to the wild type enzyme (7). Interestingly, on analysis of the product distribution of the pseudo-Y substrate (Fig. 2a) at pH 9.3, K83A was found to produce only endonucleolytic products, but not exonucleolytic trimeric and pentameric products (16).

The corresponding mutation has also been investigated in other 5'-nucleases. With Tag DNA polymerase I, mutation of the equivalent residue, Lys-82 (K82A), produced 25% of the activity of the wild type protein on XmaIS-digested plasmid DNA. (14) The equivalent mutant, K87A in T4 RNase H, lacked the ability to hydrolyze a flap substrate and demonstrated reduced ability to bind DNA in a gel retardation assay (15). Thus previous results, with the T5 enzyme and other related 5'-nucleases, strongly argue that Lys-83 plays an important role in the reaction catalyzed by phage and prokaryotic 5'-nucleases.

The variation of $k_{\text{cat}}/K_m$ with pH for the K83A mutant strongly resembles the titration of the wild type parameter $k_{\text{cat}}$ (Fig. 5, a and b). The curve has an integral slope suggesting the discrete ionization of a single residue. The mutant appears to lose any pH dependence in the $1/K_m$ parameter. This suggests that the mutated amino acid is responsible for the variation of the association constant with pH in the wild type enzyme. We therefore conclude that binding of DNA to wild type T5 5'-3'-exonuclease requires the protonation of Lys-83. Both the single ionization of $k_{\text{cat}}/K_m$ for K83A and the double ionization $k_{\text{cat}}/K_m$ for the wild type enzyme fit well to the derived equations, Equation 6 and Equation 2 yielding $pK_a$ values of 8.7 (single ionization) and 8.3 and 8.4 (double ionization), respectively, for the free enzymes. These values are in reasonable agreement with one another and suggest that the same residue is being ionized in the mutant and the wild type protein.

Based on the previously observed absence of exonucleolytic activity with the K83A mutant, it has been suggested that Lys-83 has a $pK_a$ of approximately 7 (crossover point between excess of exonucleolytic products over endonucleolytic products derived from the pseudo-Y substrate) and plays the role of general base/acid catalyst, activating water for nucleophilic attack on the phosphorus center and protonating the 3'-alkox- yde leaving group in the exonucleolytic reaction (16). If this were also the case in the endonucleolytic reaction studied here, then the $k_{\text{cat}}$ parameter of the wild type enzyme might be expected to show a bell-shaped pH dependence. Alternatively, if attack of the nucleophile was slower than breakdown of the pera-coordinate intermediate to products, then the pH dependence would be expected to follow a single ionization with an integral positive slope (i.e. the step dependent on the general base would be rate-limiting), but the $k_{\text{cat}}$ parameter of K83A mutant which lacks the putative general base/acid would be expected to be pH-independent. Furthermore, if chemistry is not the rate-determining step in the wild type T5 5'-3'-exonu- clease-catalyzed reaction, mutation of a critical amino acid to one which cannot play the role of general base/acid would be expected to decelerate this rate to such an extent that the chemical step would be rate-limiting. Thus, the pH dependence of the K83A mutant allows the possibility of Lys-83 acting as a general base/acid to be ruled out in the case of the endonucleolytic reaction.

At pH 9.3, the catalytic efficiency of the K83A mutant is decreased by a factor of 10$^2$. While some of this reduction can be attributed to a reduction in affinity of the enzyme for its DNA substrate as attested to by the increased $K_m$ at least a 1000-fold reduction in $k_{\text{cat}}$ is also observed (7). Thus, Lys-83 provides a factor of 10$^3$ of the 10$^{15}$-fold rate enhancement provided by the T5 enzyme at pH 9.3. This reduction in $k_{\text{cat}}$ is manifest throughout the pH range tested in the experiments described here, indicating that Lys-83 also plays a role in the endonu- clease-catalytic reaction but not as general acid or base. Based upon the juxtaposition of Lys-83 and the metal sites, it has previously been suggested that Lys-83 could play the role of electrostatic catalyst, serving to lower the energy of the penta-coordinate intermediate or transition state of the reaction by charge neutralization or the formation of short, strong hydrogen bonds (7). A similar role is ascribed to a lysine residue in the phosphoryl transfer reactions catalyzed by RNase A and EcoRV (21–23). If this is the case, then why does the K83A pH log $k_{\text{cat}}/K_m$ profile not differ from the wild type enzyme log $k_{\text{cat}}$ profile (Fig. 5b), as the role of electrostatic catalyst would rely on the ε-amino group being protonated? One possible explanation for this is that the rate determining step of the chemical process is attack of water, hydroxide, or a nucleophile on the phosphorus center and that this remains the case even with the removal of the electrostatic catalyst. A second explanation is that Lys-83 in the enzyme-substrate complex ionizes outside the pH range (5.5–10.5) measured in this experiment.

If Lys-83 plays the role of general acid/base catalyst in the exonucleolytic reaction, but a critical role in substrate binding and electrostatic catalysis in the endonucleolytic reaction, then it follows that the enzyme must be capable of catalyzing phosphodiester hydrolysis by two different chemical mechanisms. This possibility has been suggested recently (16). An alternative explanation for the phenomena of loss of exonucleolytic activity on the pseudo-Y substrate with K83A is that the ener-
getic contribution that Lys-83 plays to lower the activation energy of the reaction is more critical in the exonucleolytic reaction than the endonucleolytic reaction. As the 5′-end of the DNA substrate is threaded through the helical arch of the enzyme, the T5 enzyme appears well equipped to interact with the single-stranded DNA via both electrostatic and stacking interactions as the helical arch of the T5 enzyme contains a large number of potentially positively charged and aromatic residues. The energy of these interactions could be realized not only to enhance the stability of the ground state enzyme-substrate complex, but also to lower the energy of the transition state of the reaction. In the case of exonucleolytic cleavage, the length of the threaded DNA is shorter, and therefore fewer of these contacts will be realized. This may have a detrimental effect on both substrate binding and catalysis and make the energetic contribution of Lys-83 more critical for production of low molecular weight products. To date, there is not an x-ray crystal structure of any of the 5′-nucleases in the presence of DNA. Such a structure should be able to assist in distinguishing between these possible explanations for the role of Lys-83 in exonucleolytic catalysis.

The pH Dependence of $k_{\text{cat}}$—If the chemical step is rate-limiting in the endonucleolytic reaction catalyzed by T5 5′-3′-exonuclease, then the results obtained here indicate that the chemical reaction directly involves a moiety with a $pK_a$ of 8.3 in the enzyme-substrate complex. Two general mechanisms are possible for enzyme-catalyzed hydrolysis of phosphate diesters; either water directly attacks the scissile phosphodiester bond or an amino acid residue acts as a nucleophilic catalyst and attacks phosphorus subsequently to be displaced by water. As the stereocchemical course of the 5′-nuclease-catalyzed reaction has yet to be determined, either mechanistic pathway remains a possibility. Thus, the $pK_a$ observed in the pH rate profile could be the $pK_a$ of a general base that activates water for direct attack, the $pK_a$ of a nucleophile which attacks the phosphorus center, or the $pK_a$ of a general base which activates the nucleophile for attack. Possible candidates for these roles are discussed later.

Because we have not carried out pre-steady state kinetics on the T5 5′-3′-exonuclease-catalyzed reaction, we are unable to be certain that chemistry is the rate-limiting step. Other possibilities for the rate-limiting step in the reaction include conformational change or product release. Furthermore, there is the possibility that the $pK_a$ observed for the $k_{\text{cat}}$ term is a kinetic or apparent $pK_a$, which results from a change in rate-limiting step with pH. The latter explanation seems unlikely as the pH rate profiles of wild type $k_{\text{cat}}$ and K83A $k_{\text{cat}}/K_m$ closely resemble one another although the K83A mutant catalyzes the reaction with a severely impaired rate. Thus, if a change in rate-limiting step with pH was taking place, then the mutation K83A must be capable of producing equivalent effects on both of these steps.

Characterization of hFEN-1 using a flow cytometry assay based on a flap structure has determined the $k_{\text{cat}}$ of this enzyme to be 6.6 min$^{-1}$ (24). It has been suggested that a magnesium-dependent conformational change may be the rate-limiting step in the reaction of the human enzyme in this assay (24). This conclusion is based upon the observation that the rate constant observed is not large enough to account for rates of the exonuclease activities of DNA polymerase and reverse transcriptase. As the $k_{\text{cat}}$ of T5 5′-3′-exonuclease is over an order of magnitude higher than that of the human enzyme, it is not necessary to invoke such a rate-limiting step in this case. Nevertheless, a rate-limiting conformational change remains a possibility. Binding of DNA by the human enzyme is accompanied by an increase in α-helical content of the enzyme (25). In an archaeal FEN-1 homologue, the molecular basis of this conformational change has recently been suggested to be the condensation of a disordered loop around the 5′-end of the DNA strand (8). In the T5 x-ray structure, this region is completely ordered in the form of a helical arch, consisting of two α-helices. A salt bridge exists between the nonconserved residues Arg-93 and Asp-101 at the apex formed between the two helices. In the structures of the FEN-1 enzymes, the loop region is longer and contains one (mFEN-1) or two shorter α-helices (pFEN-1) and a region of random coil (8, 11). In all the 5′-nuclease structures reported to date, the loop region contains both lysine and arginine residues (8–12). Thus, any conformational change step would be expected to be pH-dependent, as protonation of lysine residues would be required to interact with the negatively charged DNA backbone. However, such a process would be expected to produce a nonintegral pH dependence (titration of several residues) with a negative slope (protonation). This does not appear to fit with the data described here that produce a positive integral slope. Another possibility is that the pH titration of $k_{\text{cat}}$ is the consequence of the ionization of a residue necessary for the formation of a critical contact that stabilizes the loop region. If the helical arch is actually disordered prior to substrate binding and is observed in the x-ray structure of the T5 enzyme because of forces of crystal packing, then a positive integral slope could be the result of ionization of a critical residue for formation of the Arg-93 to Asp-101 salt bridge. As the $pK_a$ of the β-carboxylic acid of aspartic acid is 3.86 (26), this would have to be raised by 4.5 units in the environment of the enzyme-substrate complex. This appears to be unlikely but cannot be completely ruled out.

An alternative possibility for the rate-limiting step in the T5 5′-3′-exonuclease-catalyzed reaction is product release. The reaction catalyzed by SNase is thought to be limited in this way above pH 7.3 and displays a nonintegral pH dependence with a positive slope (27). The origins of this pH dependence for SNase appear to lie in the interaction between the lysine residues of the Ω-loop of this enzyme with DNA. In a mutant of the enzyme where this loop has been deleted, an integral dependence of $k_{\text{cat}}$ on hydroxide ion concentration was observed (27). Because the pH rate profile for $k_{\text{cat}}$ observed here is of an integral slope, it is unlikely that this can represent the deprotonation of numerous residues involved in interactions with DNA. We cannot, however, rule out the possibility that the deprotonation of a single residue is of critical importance in product release although this residue cannot be Lys-83 in the endonucleolytic reaction.

If chemistry is rate-limiting for the $k_{\text{cat}}$ parameter, then what are the candidate ionizing functions? The direct participation of Lys-83 as a general base/acid can be ruled out in the endonucleolytic reaction by these experiments. Another candidate amino acid, which might be expected to titrate with a $pK_a$ of 8.3 in the enzyme-substrate complex, and could act as a nucleophile or general acid/base catalyst, is the conserved residue Tyr-82 (Ref. 2) ($pK_a$ free amino acid side chain = 10.07; Ref. 26). However, the pH rate profile of log $k_{\text{cat}}/K_m$ for Y82F is bell-shaped and when fitted to Equation 2 yields similar $pK_a$ values to the unmodified protein.2 Thus, Tyr-82 can also be ruled out as playing an important mechanistic role. The other conserved amino acids which may be expected to titrate with change in pH are aspartic and glutamic acids (Fig. 2). The $pK_a$ values for these free amino acids are 3.86 and 4.25, respectively (26), and would therefore require great perturbation by the

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2 T. J. Pickering, D. Patel, J. A. Grasby, and J. R. Sayers, unpublished results.
protein and metal ions to be changed to 8.3 in the enzyme-substrate complex. Indeed, the presence of metal ions next to these residues might be expected to lower these values and not increase them. One possibility which deserves consideration is that water is activated by binding to one of the two essential metal ion co-factors. The pK_a of [Mg(H_2O)_6]^{2+} is 11.42 (28) and would thus have to be lowered in the environment of the protein by 3 pK_a units. Metal ions acting as a source of OH^- is a feature of several proposed mechanisms for enzymes that catalyze phosphoryl transfer using either one or more metal ions (29) such as SNase (27), EcoRV (30), Nuclease P1 (31), 3'-5'-exonuclease domain of the Klenow fragment (17), and some ribozymes (32). Recently, it has been suggested that 3'-5'-exonuclease-catalyzed phosphodiester bond hydrolysis involves attack of an OH^- ion from aqueous solution around the protein rather than a general base catalysis by an active site residue or Lewis acid catalysis by a metal (33). However, this mechanism can be eliminated in the case of T5 5'-3'-exonuclease because attack of hydroxide ion would cause the pH dependence of log k_cat to increase with a slope of 1 throughout the entire pH range tested in these experiments (5.5–10.5) as the pK_a of water is well outside this range. Evidence that either inner sphere metal ion coordination to the protein or the substrate or the ability of the ligand sphere of the metal to catalyze proton transfer is provided by our recent observation that the inert complex Co(NH_3)_6^{3+} does not support T5 5'-3'-exonuclease catalysis.3

In conclusion, we have demonstrated that the endonucleolytic reaction catalyzed by T5 5'-3'-exonuclease requires the protonation of Lys-83 for substrate binding, whereas the rate-limiting step of the reaction requires a deprotonation event with a pK_a of 8.3 in the enzyme-DNA complex. Lys-83 also contributes to catalytic reaction, presumably by electrostatic interactions, but its pK_a is not within the pH range tested or its replacement with alanine does not affect the rate-limiting step in the reaction. It appears that it is unlikely that the deprotonation observed in the k_cat pH-profile is associated with either a change in rate-limiting step, conformational change, or product release although these possibilities cannot be ruled out entirely. We tentatively suggest that the pK_a of 8.3 may be that of [Mg(H_2O)_6]^{2+} in the enzyme-substrate complex. Future mechanistic proposals for the endonucleolytic activity of T5 5'-3'-exonuclease and other 5'-nucleases will have to explain the data reported here. Determination of the pH rate profiles of other members of the 5'-nuclease family should provide insights into similarities between the kinetic pathways and mechanisms of this family of enzymes.

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