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A single cleavage assay for T5 5′→3′ exonuclease: determination of the catalytic parameters for wild-type and mutant proteins

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ABSTRACT

Bacteriophage T5 5′→3′ exonuclease is a member of a family of sequence related 5′-nucleases which play an essential role in DNA replication. The 5′-nucleases have both exonucleolytic and structure-specific endonucleolytic DNA cleavage activity and are conserved in organisms as diverse as bacteriophage and mammals. Here, we report the development of a structure-specific single cleavage assay for this enzyme which uses a 5′-overhanging hairpin substrate. The products of DNA hydrolysis are characterised by mass spectrometry. The steady-state catalytic parameters of the enzyme are reported and it is concluded that T5 5′→3′ exonuclease accelerates the cleavage of a specific phosphodiester bond by a factor of at least 10^{15}. The catalytic assay has been extended to three mutants of T5 5′→3′ exonuclease, K83A, K196A and K215A. Mutation of any of these three lysine residues to alanine is detrimental to catalytic efficiency. All three lysines contribute to ground state binding of the substrate. In addition, K83 plays a significant role in the chemical reaction catalysed by this enzyme. Possible roles for mutated lysine residues are discussed.

INTRODUCTION

T5 5′→3′ exonuclease is a member of a family of sequence related 5′→3′ exonucleases isolated from viral sources or as a domain of prokaryotic polymerases (1). More distantly related eukaryotic and mammalian enzymes also exhibit sequence homology, albeit to a lesser extent, and include the human and mouse Flap endonuclease (*h* or *m*FEN-1) (1). These enzymes catalyse the exonucleolytic hydrolysis of the phosphodiester bonds in a variety of DNA substrates which include single-stranded and double-stranded DNA and DNA–RNA hybrids (RNase H activity). In addition, the 5′→3′ exonucleases also exhibit structure-specific endonucleolytic activity, resulting in the cleavage of DNA flap structures (Fig. 1; 2–4). This structure-specific hydrolysis of bifurcated structures is mainly observed at the junction between double-stranded and single-stranded DNA (2–4). In recognition of this diverse range of activities, it has been suggested that this family of enzymes may be more accurately described as 5′-nucleases (4). *In vivo* the enzymes play a role in DNA replication and repair removing the Okazaki fragments produced during replication.

The X-ray crystal structures of four members of the 5′→3′ exonuclease family have been reported in the absence of DNA substrates (5–8). The structures of T4 RNase H and the 5′-exonuclease domain of *Taq* polymerase both contained areas of disorder (6,8) but the structure of T5 exonuclease revealed the presence of a hole, bound by a helical arch upon a globular domain containing the active site (5). The hole is large enough to accommodate single-, but not double-stranded, DNA and a threading mechanism in which the single-stranded 5′-terminus enters the arch has been proposed (5). A hole is also observed in the most recent structure of a flap endonuclease from *Methanococcus jannaschii* (*Mj*FEN-1) (7). The active site of the T5 enzyme is located in front of the arch and contains a number of conserved acidic residues which co-ordinate to two magnesium ions. Divalent metal ions are an essential cofactor in the reaction. The active site of T5 5′→3′ exonuclease also contains a lysine residue (K83) which is conserved throughout all the eubacterial polymerases and phages (5). In the crystal structure of T5 exonuclease and T4 RNase H, the metal binding sites are separated by distances of 7–8 Å. The structure of the 5′-exonuclease domain of *Taq* polymerase also has a separate binding site for a zinc ion, close to one of the magnesium sites (6). The biological significance of this site is unknown. In the case of *Mj*FEN-1 the distance between the metal sites is 5 Å and again the dimensions of the hole suggest that single-stranded DNA could become threaded through it.

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 similar to that of the 3′→5′ exonuclease domain of the Klenow fragment (6). The large distance between metal ions in the RNase H and T5 5′→3′ exonuclease structures, far beyond the usual separation of

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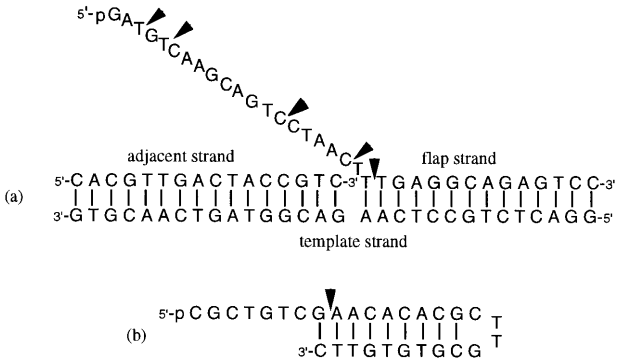

Figure 1. (**a**) The DNA flap structure used in characterisation of the cleavage specificity of T5 5′→3′ exonuclease. The flap structure is composed of a flap strand, a template strand and an adjacent strand. The flap strand is 5′-^{32}P-labelled in these studies. Arrows indicate the sites of reaction. (**b**) The 5′-overhanging hairpin, HP1. The oligonucleotide is 5′-^{32}P-labelled in kinetic studies. An arrow indicates the site of reaction.

4 Å in two metal ion enzymes, appear to make this less plausible. In addition, recent mutagenesis data of putative metal binding residues in *h*FEN-1 suggest that one metal site may be required for DNA binding whereas the other metal may play a catalytic role (9). An alternative proposal is that the lysine residue located in the active site of the enzyme (K83 in the T5 exonuclease structure) acts as a general base/acid activating water to attack the scissile bond and protonating the leaving group oxygen (10). Further biochemical studies are required if the mechanism of this family of enzymes is to be fully elucidated.

Studies of the structure–function relationships and mechanistic details of nuclease reactions are greatly facilitated by the ability to accurately measure catalytic parameters. However, in the case of exonucleases these experiments are often problematic due to the occurrence of multiple reactions with most nucleic acids substrates. Furthermore, physico-chemical studies such as determination of the stereochemical courses of enzyme reactions demand substrates with a unique site of cleavage. Spectrophotometric assay of the reduction of high molecular weight DNA to acid-soluble smaller nucleotides is the most common method for analysing the catalytic activity of 5′→3′ exonucleases and their mutants (11). These assays quantify the rates of cleavage of a heterogeneous DNA population at numerous sites simultaneously and are remote from the *in vivo* natural substrate.

These problems have been overcome in the case of *h*FEN-1, by the development of a flow cytometry assay (12). A 3′-biotinylated template strand and a 5′-fluorescent flap strand (Fig. 1) form a flap structure which is immobilised on a streptavidin-coated microsphere. The fluorescent nature of this assay allows continuous rapid real time detection. The substrate in this reaction can be cleaved either one nucleotide distal or proximal to the single-stranded/double-stranded DNA junction although the assay measures only the first cleavage event at whichever site it occurs. It is not known whether enzyme catalysed hydrolysis at these two sites proceeds with identical or different rates. This assay has been employed to measure the individual rate constants of the *h*FEN-1 reactions and to quantify the effects of mutations (9,12,13). However, due to the solid support used in this assay, it suffers from the disadvantage that the substrate concentration is heterogeneous and is therefore limited to single turnover analysis.

In order to aid the determination of accurate catalytic parameters for T5 5′→3′ exonuclease and mutants of the enzyme and to facilitate further mechanistic studies, we have developed a single cleavage substrate which undergoes a structure-specific enzyme catalysed reaction. Here, we report the catalytic parameters of the wild-type enzyme and three lysine mutants using this novel substrate. This has allowed us to reach conclusions about the magnitude of the rate enhancement of phosphodiester hydrolysis provided by T5 5′→3′ exonuclease and to suggest roles for the mutated lysines in the reaction pathway.

MATERIALS AND METHODS

Synthesis, purification and characterisation of HP1

The synthetic oligonucleotide hairpin substrate (HP1) was synthesised using an ABI/Perkin Elmer model 394 DNA/RNA synthesiser using standard reagents and conditions. Following deprotection, the oligonucleotide was purified by ion exchange HPLC (DNAPak PA-100 column; buffer A, 20 mM Tris–HCl, pH 6.8, 1 mM $NaClO_4$, 25% formamide; buffer B, 20 mM Tris–HCl, pH 6.8, 400 mM $NaClO_4$, 25% formamide) or by 20% denaturing PAGE. Following HPLC the oligonucleotide was desalted by dialysis. After PAGE the product-containing band was visualised by UV shadowing and excised and then eluted in 0.5 M NH_4OAc, 1 mM EDTA and desalted using a Sep-Pak Cartridge (Waters). The hyperchromicity of HP1 was calculated using standard methods (14) and demonstrated to be 40%. The extinction coefficient used for HP1 was thus 280.1 $cm^2/\mu mol$.

Preparation of purification of mutant exonuclease

Mutants of 5′→3′ T5 exonuclease were prepared and purified according to Garforth and Sayers (3) and Garforth *et al.* (10). The concentration of proteins was determined using the assay of Bradford (15).

Electrospray ionisation mass spectral analysis of cleavage products of HP1

5′-Phosphorylated HP1 (30 μM) was incubated at 37°C in a solution of 25 mM CHES (pH 9.3), 50 mM KCl and 10 mM $MgCl_2$. T5 5′-nuclease was then added to a concentration of 60 nM, initiating the reaction. After 10 min the reaction was quenched by the addition of EDTA (50 mM) and then heated at 95°C for 2 min, to denature the enzyme. Salts were removed using a Sep-Pak C18 column. The oligonucleotide sample was then washed with 5 column vols of a 0.1 M triethylammonium acetate solution (pH 7.0). The sample was then desalted by washing with 10 column vols of dH_2O and eluted with 1.5 ml of a 60% solution of methanol. The eluted oligonucleotide was evaporated to dryness and resuspended in a 50% (v/v) isopropanol solution which was then made 1% in triethylamine, so that the concentration of oligonucleotide was 30 μM. Samples (20 μl) of the enzymatically cleaved and pure 5′-phosphorylated HP1 (30 μM) were submitted to electrospray mass spectrometry in negative ion mode on a Micromass Platform instrument.

dp(CGCTGTCGAACACACGCTTGCGTGTGTTC), calculated mol. wt 8938.74, found 8936.1; dp(CGCTGTCG), calculated mol. wt 2479.57, found 2480.0; dp(AACACACGCTTGCGTGTGTTC), calculated mol. wt 6475.17, found 6480.0.

Determination of Michaelis–Menten parameters for wild-type and mutant proteins

Separate reaction mixtures of appropriate concentrations of ^{32}P-labelled substrate HP1 in 25 mM potassium glycinate, pH 9.3, 10 mM $MgCl_2$, 50 mM KCl and 0.2 mg/ml bovine serum albumin (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 potassium glycinate, pH 9.3, 10 mM $MgCl_2$, 50 mM KCl and 0.2 mg/ml BSA and kept on ice until use. Reactions were initiated by addition of enzyme (final volume 50 or 10 µl, 25 mM potassium glycinate, pH 9.3, 10 mM $MgCl_2$, 50 mM KCl and 0.2 mg/ml BSA) and brief vortexing. The final concentrations of the substrates were 0.001–1 µM for the wild-type enzyme, 1–100 µM for K83A and K196A and 0.1–10 µM for K215A. The concentration of wild-type enzyme was varied from 3 to 30 pM and that of K83A from 20 to 100 nM, K196A from 3 to 30 nM and K215A from 12 to 120 pM. The progress of the reactions was followed by taking 5 or 1 µl aliquots at five appropriate time intervals. The individual samples were quenched by immediate addition to 10 µl of denaturing 'stop' mix (95% deionised formamide, 10 mM EDTA, 0.05% xylene cyanol FF and 0.05% bromophenol blue) and analysed by 20% denaturing PAGE followed by phosphorimaging.

Initial rates of the cleavage reaction at the various substrate concentrations were determined. This allowed the kinetic parameters to be calculated by non-linear regression fitting the data to the Michaelis–Menten equation (equation **1**)

$$v/[E] = (k_{cat}[S])/(K_m + [S]) \quad \mathbf{1}$$

where ν is the initial rate, [S] is substrate concentration and [E] is total enzyme concentration.

The changes in the apparent binding energies for the mutant enzymes were calculated from equation **2**.

$$\Delta\Delta G_{app} = -RT \ln[(k_{cat}/K_{m\ (wild\text{-}type)})/(k_{cat}/K_{m\ (mutant)})] \quad \mathbf{2}$$

Computational methods

Molecular grahics (Fig. 5) were produced using Molw PDB Viewer v.1.3 software (Molecular Images Software, San Diego, CA).

RESULTS AND DISCUSSION

Development of the single cleavage assay

Treatment of the radiolabelled flap structure shown in Figure 1a with T5 5′→3′ exonuclease results in the formation of labelled 19mer, 21mer and 14mer oligonucleotide products along with exonucleolytic products of ~3 and 5 nt in length (3). The major oligomer products are the 19mer and 21mer which are 1 nt distal or proximal to the single-stranded/double-stranded DNA junction as shown previously with FEN-1 (2). Exhaustive treatment of the flap structure with the enzyme results in the formation of only exonucleolytic products. Previous experiments have revealed that single-stranded DNA cleavage is slower than the structure-specific DNA hydrolysis (5).

With the putative threading behaviour of the oligonucleotide substrate in mind, we chose to investigate the potential of 5′-overhanging hairpins as single cleavage substrates for T5 5′→3′ exonuclease. The rationale behind our choice of substrate was that, with a duplex region stabilised by the presence of a hairpin loop, extensive threading of the substrate through the helical arch would be impaired. Furthermore, by minimisation of the length of the overhang, the fastest cleavage event would be restricted to the proximal site. The small 5′-single-stranded product of this reaction would be much more resistant to exonucleolytic reaction than the endonucleolytic structure-specific reaction of the substrate. In addition, the 3′-hairpin product would be resistant to further hydrolysis by inhibition of the threading mechanism. Our initial attempts with a shorter hairpin 19mer substrate demonstrated this to be completely resistant to exonuclease action of any kind. The 29mer 5′-overhanging hairpin oligomer shown in Figure 1b proved to be a substrate for the enzyme, producing a single labelled product from a 5′-^{32}P-labelled substrate (Fig. 2).

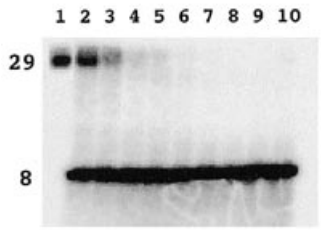

Figure 2. A phosphor image of a time course of T5 5′→3′ exonuclease catalysed hydrolysis of 5′-^{32}P-labelled HP1. The following conditions were utilised for this experiment: 25 mM CHES, pH 9.3, 50 mM KCl, 10 mM $MgCl_2$, 1 mM ^{32}P-labelled HP1, 60 nM enzyme, 37°C. Lane 1 is a control lane with no enzyme present. Lanes 2–10, time points were taken at the following time intervals 10 and 20 s and 1, 2, 5, 10, 17, 25 and 40 min.

To establish the site of T5 5′→3′ exonuclease catalysed hydrolysis of HP1 the products of the reaction were subjected to analysis by electrospray ionisation mass spectrometry. The molecular weight of the products indicated that cleavage had occurred 1 nt distal to the junction between double-stranded and single-stranded DNA to yield products of 2480.0 and 6480.0 Da, respectively. This mass spectral result clearly demonstrates that the hydrolysis reaction catalysed by T5 5′→3′ exonuclease undergoes scission of a 3′-oxygen phosphorus bond to produce products terminating in a 3′-hydroxyl group and a 5′-phosphate, respectively.

In experiments employing 5′-radiolabelled substrate, only the 5′-product of the reaction is observed. In order to assess whether the 3′-hairpin product would undergo further reactions which would complicate kinetic analysis we examined the 3′-product of the reaction using toluidine blue staining (data not shown). This experiment was not conducted by 3′-terminal labelling as this would result in the addition of an extra nucleotide which we considered may change the properties of the substrate in the reaction. Very small amounts of further products are observed after prolonged exposure to the enzyme at high concentration but these appear only after complete conversion to the initial 8mer and 21mer products. The identity of the product of the second reaction has been studied by mass spectrometry. The second reaction releases a single nucleotide from the 5′-terminus of the 21mer product generating a 20mer product (data not shown). A crude estimate of the rate of the secondary reaction has been obtained from HPLC and toluidine blue dye staining experiments as at least 1000 times slower than the primary reaction measured in our assay. Thus, during determination of initial rates of the first

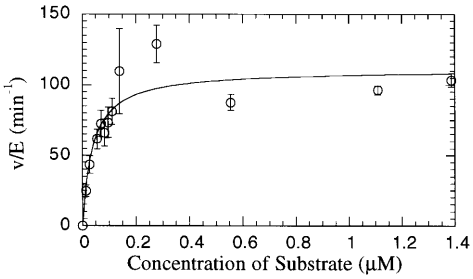

Figure 3. A plot of normalised initial rate of reaction against substrate concentrations for the wild-type enzyme. The data has been fitted to the Michaelis–Menten equation with an R factor of 0.92. Each data point is the mean of at least three independent experiments. Error bars represent the standard errors in these experiments. Full details of experimental conditions are given under Materials and Methods.

structure-specific enzyme catalysed hydrolysis, any secondary reactions are negligible and can be ignored.

Recently the characterisation of a 68mer 5′-overhanging hairpin substrate for the 5′→3′ exonuclease domain of *Escherichia coli* DNA polymerase I has been reported by Joyce and co-workers (16). In agreement with our experiments, this substrate is only cleaved once by the enzyme. However, the action of the 5′→3′ exonuclease domain of DNA polymerase I on flap type substrates results in only one cleavage reaction between the first 2 bp of the duplex region whereas reaction at the distal site does not take place with this enzyme (16).

The initial rates of reaction of the T5 5′→3′ exonuclease catalysed reaction, at substrate concentrations around the K_m, were determined using ^{32}P-labelled HP1 substrate, followed by separation by denaturing gel electrophoresis and quantitation by phosphorimaging. The concentration of substrate was determined by allowing for its hyperchromicity (40%). The conditions of pH and $MgCl_2$ and KCl concentrations used here have previously been determined to be optimum for the reaction (17). The results of this analysis are shown (Fig. 3). Each data point has been determined in at least three independent experiments and the mean value is plotted with the standard error shown. Following non-linear regression fitting to the Michaelis–Menten equation this yielded catalytic parameters of 0.04 μM for the Michaelis constant and 110 min^{-1} for the turnover number. The curve fits with a R factor of 0.92. The errors in the determination of these values of 10% in k_{cat} and 35% in K_m are similar to other assays of enzyme catalysed DNA and RNA cleavage which employ 5′-^{32}P-labelled substrates (18,19).

Characterisation of *h*FEN-1 using a flow cytometry assay based on a flap structure has determined the k_{cat} of this enzyme to be 6.6 min^{-1} (12). It has been suggested that a magnesium-dependent conformational change may be the rate limiting step in the reaction of the human enzyme (12). The maximal rate of the 5′→3′ exonuclease domain of *E.coli* DNA polymerase I has been determined under conditions of enzyme excess to be 5 min^{-1} with a flap-type structure and 0.035 min^{-1} with a 5′-overhanging hairpin substrate (16). These previously determined turnover numbers for related enzymes are some 15- to 3000-fold slower than that observed here.

The upper limit of the first order rate constant for water catalysed hydrolysis of dimethylphosphate at 25°C is $10^{-15}s^{-1}$ (20). A similar rate is predicted by considering the rate constant for the hydroxide ion catalysed hydrolysis of dimethylphosphate at pH 9.3 (21). Allowing for the difference in temperature and assuming our turnover number represents the rate of the chemical step in the reaction pathway, then T5 5′→3′ exonuclease enhances the rate of cleavage of a specific phosphodiester bond in HP1 by at least a factor of 10^{15}. If chemistry is not rate limiting then this figure would underestimate the actual extent of rate enhancement achieved by the enzyme.

Analysis of mutant proteins

The catalytic parameters (Fig. 4 and Table 1) of three mutants of T5 5′→3′ exonuclease in which lysine is converted to alanine have been determined using the HP1 substrate. In a model of DNA bound to T5 5′→3′ exonuclease (Fig. 5), these three lysine residues are predicted to contact the DNA substrate (5). Lys83, positioned in the helical arch region close to metal site 1, is strictly conserved throughout all the prokaryotic 5′→3′ exonuclease sequences (22). The structure of the K83A mutant has been solved by X-ray crystallography and demonstrated to be very similar to wild-type protein (10). Lys215 is conserved in all eubacterial 5′-nucleases and in T5 exonuclease (22). In contrast, K196 is less well conserved and is positioned between the two metal sites (22). The most dramatic effects were observed with the mutant enzyme K83A which displayed a 700-fold increased K_m and a 1000-fold reduced k_{cat} with respect to the wild-type protein. A more moderate increase of the Michaelis constant of 75-fold was observed with K196A, whilst the k_{cat} of this enzyme was reduced by ~30-fold. The most modest changes were observed with K215A where K_m is increased by 20-fold but the turnover number is only reduced 3-fold with respect to the wild-type enzyme. The data generated using mutant enzymes fits to the Michaelis–Menten equation (equation **1**) with R factors ranging from 0.90 to 0.98.

Table 1. The catalytic parameters of wild-type and mutant T5 5′→3′ exonucleases

Enzyme	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M (μM^{-1} min^{-1})	$\Delta\Delta G_{app}$ ($kJmol^{-1}$)
Wild Type	110±10	0.04±0.01	$2.8*10^3$	-
K83A	0.1±0.01	28±4	$3.5*10^{-3}$	+ 34
K196A	3.5 ± 0.2	2.8±0.8	1.3	+ 19.5
K215A	38 ± 3	0.76±0.02	50	+ 10

Experimental conditions are described in Material and Methods.

The dissociation constants for the lysine mutants binding to a pseudo-Y DNA structure (the flap structure minus the adjacent strand) have been previously determined using a gel retardation assay in the absence of magnesium co-factor to prevent cleavage (10). Interestingly, attempts to develop a gel retardation assay with the substrate HP1 used in kinetic studies were not successful and no band shift was obtained under the conditions required to obtain retardation of the pseudo-Y structure. Our K_m determinations are by necessity carried out in the presence of magnesium ions. The contribution that magnesium ions make to substrate binding is unknown although in the case of *h*FEN-1 one metal ion has been proposed to be involved in substrate binding (13). In the gel retardation assay (10), K83A and K196A both increased the dissociation constant of the pseudo-Y structure by a factor of 40-fold whilst K215A changes this value by 10-fold with respect to wild-type enzyme. The effects observed on the K_m in the HP1

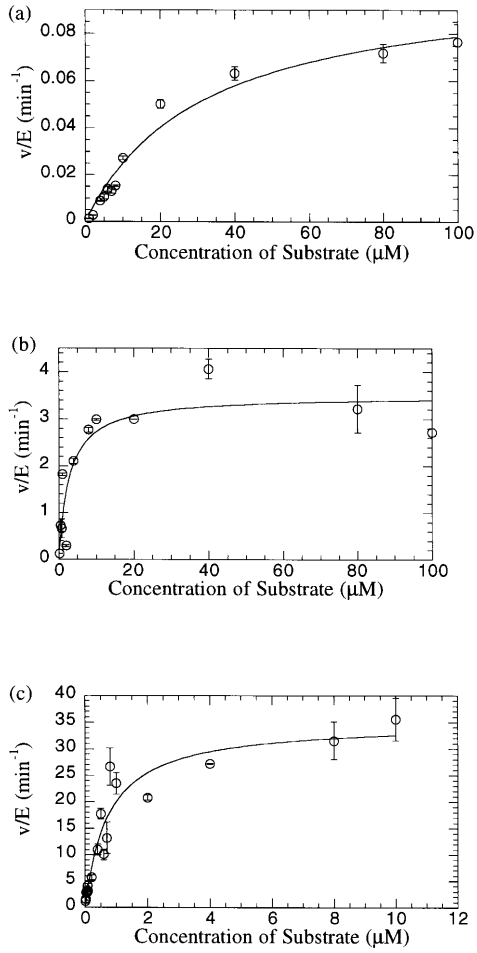

Figure 4. Plots of normalised initial rate of reaction against substrate concentrations for the mutant enzymes. Each data point is the mean of at least three independent experiments. Error bars represent the standard errors in these experiments. Full details of experimental conditions are given under Materials and Methods. (**a**) K83A. The data have been fitted to the Michaelis–Menten equation (equation **1**) with an R factor of 0.98. (**b**) K196A. The data have been fitted to the Michaelis–Menten equation (equation **1**) with an R factor of 0.90. (**c**) K215A. The data have been fitted to the Michaelis–Menten equation (equation **1**) with an R factor of 0.96.

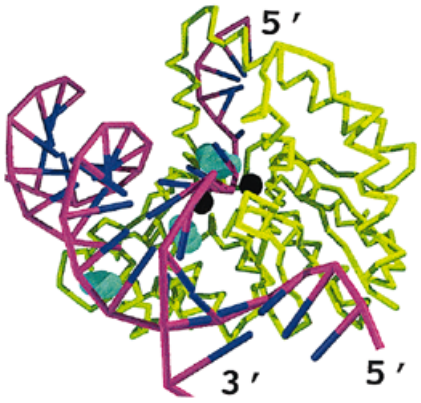

Figure 5. Schematic diagram showing the positions of the lysine residues mutated in this study (space filling in cyan, K83, 196 and 215 from top to bottom). The backbone C_α residues of T5 exonuclease (PDB code 1EXN) are shown as a pale green tube. A DNA flap structure modelled into the active site (5) is shown by the pink and blue ladders. The position of the bound divalent metal ions is shown by the black spheres.

assay are generally larger than those observed on K_d in gel retardation and interestingly this catalytic parameter differentiates between the K83A and K196A mutant by an order of magnitude. One possible explanation of this is that the mutation K83A also perturbs metal ion binding.

The catalytic activity of the lysine mutants has been characterised earlier using spectrophotometric detection of acid-soluble nucleotides from high molecular weight DNA (10). In this non-specific cleavage assay the rate of K83A is reduced ~200-fold, whilst that of K196A is reduced by a factor of almost 20 and K215 has an activity similar to wild-type. The effects observed here with K196A and K215A closely parallel those obtained in the spectrophotometric assay. In contrast, we observe a much larger decrease in k_{cat} than suggested by the activity assay for the K83A mutant. The spectrophotometric assay is susceptible to quite large errors with low activity mutants due to the heterogeneous nature of the substrate. It therefore overestimates the nucleolytic activity of very disabled mutants such as K83A.

In Table 1 we also show the k_{cat}/K_m ratios for the mutant 5′→3′ exonucleases. From this apparent second order rate constant it is possible to calculate (equation **2**) the energetic penalty ($\Delta\Delta G_{app}$) paid upon amino acid mutation (23). Removal of a hydrogen bond leaving an uncharged partner generally incurs an energetic penalty of 2–7.5 kJ/mol whilst a penalty of 12–25 kJ/mol occurs if a charged partner is left (23). The intermediate value of 10 kJ/mol observed for K215A could either be explained by a strong hydrogen bond to an uncharged partner or a weak hydrogen bond to a charged partner. This could take the form of a hydrogen bond between the ε-amino function of lysine to a phosphate group or a hydrogen bond interaction with another part of the DNA molecule. This binding energy is used to stabilise the ground state enzyme–substrate complex. In the case of K196A, the $\Delta\Delta G_{app}$ observed is entirely consistent with a hydrogen bond to a charged partner, namely a phosphate group of the DNA. The magnitude of this interaction is realised in both the ground state of the substrate and more modestly in the transition state.

The large energetic penalty paid upon mutation of Lys83 cannot be explained by conventional hydrogen bonding interactions. Lys83 appears to contribute significantly both to ground state binding of the substrate and the catalytic reaction as attested to by dramatic changes in both steady-state parameters. The magnitude of the effect of the K83A mutation on K_m is suggestive of an electrostatic interaction between the protonated ε-amino function and a phosphate group. Although there is no X-ray structure of any of the exonucleases in the presence of DNA, the location of K83 close to the helical arch and between the two metal sites makes it tempting to speculate that it may contact the scissile phosphodiester bond. The full potential of this interaction could be realised to lower the energy of the transition state by neutralising the negative charge on the penta-coordinate transition state or intermediate or alternatively by forming a short strong hydrogen bond. A similar role is ascribed to a lysine residue in RNase A and in *Eco*RV restriction endonuclease (24–26). Alternatively, as well

as contributing to ground state binding of the substrate, K83 could participate directly in the chemical mechanism as a general base/acid as has been suggested recently (10).

In conclusion, we have developed a single cleavage assay for T5 5′→3′ exonucleases and characterised the products of this reaction. This assay has allowed us to quantify the effect of mutations on the catalytic parameters of the enzyme. All the lysine residues studied here contribute to ground state binding of the enzyme and substrate. Lys83 also plays a significant role in the catalytic reaction. The single cleavage assay will be useful in further mechanistic studies on 5′-nucleases and their mutants.

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