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The 3′ − 5′ exonuclease of DNA polymerase I of *Escherichia coli*: contribution of each amino acid at the active site to the reaction

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We have used site-directed mutagenesis to change amino acid side chains that have been shown crystallographically to be in close proximity to a DNA 3′ terminus bound at the 3′−5′ exonuclease active site of Klenow fragment. Exonuclease assays of the resulting mutant proteins indicate that the largest effects on exonuclease activity result from mutations in a group of carboxylate side chains (Asp355, Asp424 and Asp501) anchoring two divalent metal ions that are essential for exonuclease activity. Another carboxylate (Glu357) within this cluster seems to be less important as a metal ligand, but may play a separate role in catalysis of the exonuclease reaction. A second group of residues (Leu361, Phe473 and Tyr497), located around the terminal base and ribose positions, plays a secondary role, ensuring correct positioning of the substrate in the active site and perhaps also facilitating melting of a duplex DNA substrate by interacting with the frayed 3′ terminus. The pH-dependence of the 3′−5′ exonuclease reaction is consistent with a mechanism in which nucleophilic attack on the terminal phosphodiester bond is initiated by a hydroxide ion coordinated to one of the enzyme-bound metal ions.

Key words: DNA polymerase I/Klenow fragment/metal ion catalysis/site-directed mutagenesis/3′−5′ exonuclease

Introduction

The Klenow fragment of *Escherichia coli* DNA polymerase I has two enzymatic activities, DNA polymerase and 3′−5′ editing exonuclease, which are located on separate structural domains of the molecule (reviewed by Joyce and Steitz, 1987). The Klenow fragment structure has been solved at high resolution (Ollis *et al.*, 1985), making it an excellent model for structure−function studies of a DNA synthesizing enzyme at the molecular level. In this paper we describe a detailed study of the 3′−5′ exonuclease active site. The value of Klenow fragment as a model system for such studies is reinforced by protein sequence comparisons, which have shown that most of the residues at the 3′−5′ exonuclease active site of DNA polymerase I are conserved among a large number of other prokaryotic and eukaryotic polymerases (Joyce *et al.*, 1986; Spicer *et al.*, 1988; Bernad *et al.*, 1989; Leavitt and Ito, 1989). Thus, structural and mechanistic information on the 3′−5′ exonuclease active site of Klenow fragment may well be applicable to other polymerases for which structural data are not available. Certainly, the results of mutagenesis experiments on ϕ29 and T7 DNA polymerases seem to validate this approach (Bernad *et al.*, 1989; Patel *et al.*, 1990).

Crystallographic studies have provided a wealth of information on the 3′−5′ exonuclease active site. The initial high-resolution studies identified the binding site for deoxynucleoside monophosphate (dNMP), the product (and an inhibitor) of the 3′−5′ exonuclease reaction (Que *et al.*, 1978; Ollis *et al.*, 1985). More recent studies on Klenow fragment complexes with single-stranded DNA oligonucleotides bound at the exonuclease active site confirmed that the dNMP complex did indeed provide a valid model for the position of the DNA primer terminus in the exonuclease site (Freemont *et al.*, 1988). Moreover, an analysis of the DNA binding site on the exonuclease domain indicated that the DNA was bound as a single strand, not as duplex. From these structural data, Steitz and his co-workers have proposed that an editing reaction on duplex DNA involves melting of ~4 bp so that the frayed 3′ terminus can be accommodated in the exonuclease active site while the duplex portion remains bound to the proposed DNA binding cleft on the large polymerase domain (Joyce and Steitz, 1987; Steitz *et al.*, 1987; Freemont *et al.*, 1988). Solution studies using chemically cross-linked oligonucleotides (Cowart *et al.*, 1989) subsequently confirmed the requirement for melting of the terminal 4 bp.

Examination of the complexes with dNMP or single-stranded DNA has indicated those amino acids whose side chains form the 3′−5′ exonuclease active site and are in close proximity to the substrate (Figure 1). A prominent feature of this site is the presence of two divalent metal ions (Derbyshire *et al.*, 1988). The phosphate oxygen atoms of dNMP interact with one metal ion (metal A) bound to the protein by the carboxylate groups of Asp355, Glu357 and Asp501. A second metal ion (metal B) lies adjacent to Asp424 only when dNMP is bound, and shares the Asp355 ligand with the metal A site (Beese and Steitz, 1989). Crystallographic studies indicate that the metal sites can be filled by Mg^{2+}, Mn^{2+} or Zn^{2+} (Freemont *et al.*, 1988); however the chemical identity of the two metal ions in a catalytically competent enzyme−substrate complex remains to be established, due to the difficulty of creating reaction conditions in which the divalent metal ion composition is unambiguously defined. Aside from the two metal ions, their carboxylate ligands and the side chain of Tyr497, the active site region is notably empty of side chains capable of catalyzing phosphodiester bond cleavage. Moreover, the metal ions provide the only positive charges available for interaction with the DNA phosphate at the 3′ terminus. Thus it seems likely that the two metal ions play a major role both in substrate binding and in catalysis of the exonuclease reaction, consistent with our earlier mutagenesis study which established, at a minimum, that metal A functions in binding the substrate and metal B in catalysis (Derbyshire *et al.*,

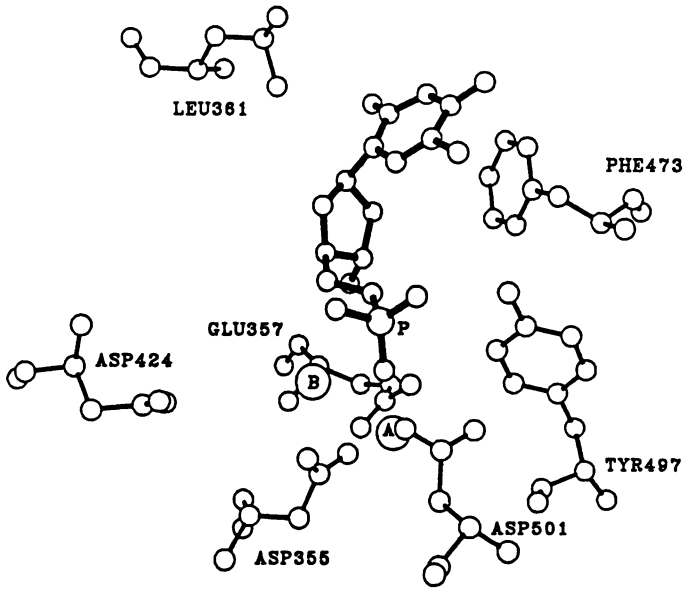

Fig. 1. The 3′−5′ exonuclease active site with the bound product molecule, deoxycytidine monophosphate (dCMP) (shown with thickened bonds). The side chains of the residues interacting with the metal ions and dCMP are shown. The two binding sites for divalent metal ions are labeled A and B. Reproduced, with permission, from Derbyshire *et al.* (1988) (© AAAS 1988); further details of the interactions within the active site are described in the accompanying paper (Beese and Steitz, 1991).

1988). The observation that the 3′−5′ exonuclease reaction takes place with inversion of stereochemical configuration at phosphorus (Gupta and Benkovic, 1984) places constraints on proposed reaction mechanisms, ruling out the possibility of covalent intermediates, and suggesting that the attacking water molecule must be situated close to Tyr497. Based on these considerations, Freemont *et al.* (1988) proposed that metal A polarizes, and is assisted by Tyr497 in orienting, the attacking nucleophile, while metal B stabilizes the pentacoordinate transition state or intermediate.

In this study we have made a series of mutant proteins in which each of the amino acid side chains at the exonuclease active site has been mutated in order to assess its contribution to the 3′−5′ exonuclease reaction. These experiments, together with studies on the wild-type enzyme, have allowed us to evaluate some of the ideas discussed above.

Results

Construction and purification of mutant derivatives of Klenow fragment

Using standard site-directed mutagenesis techniques (see Materials and methods) we mutated each of the amino acid side chains seen crystallographically to be in close contact with the DNA 3′ terminus at the 3′−5′ exonuclease active site (Figure 1). Mutations, constructed in appropriate M13 vectors, were subcloned into a high-level expression plasmid for protein overproduction. During these manipulations, it was important to prevent recombination between the plasmid-encoded mutant copy of the Klenow fragment coding sequence and the wild type chromosomal copy of the *polA* gene. For this reason, the plasmid clones were isolated and characterized in a *recA* background, and expression of the mutated proteins was induced in the *recA* host CJ376.

Another important consideration when studying potential exonuclease-defective derivatives of Klenow fragment was to reduce the contamination by cellular nucleases to acceptably low levels. As a partial solution to this problem, the overproduction host, CJ376, was deficient in exonuclease III. The need to remove cellular nucleases also placed very stringent requirements on the purification procedure, in contrast to our work on polymerase-defective mutants where a partial purification was sufficient (Polesky *et al.*, 1990). To this end, we developed a four-stage fractionation method using FPLC (see Materials and methods), which allowed us rapidly to purify substantial quantities of each protein to homogeneity (as judged by gel electrophoresis). Ammonium sulfate fractionation of a crude cell extract was used as the initial step to remove most of the soluble lipids before the FLPC columns. Chromatography on Mono Q then removed nucleic acids and gave some purification from other proteins. Phenyl Superose chromatography gave excellent purification from other proteins, yielding a Klenow fragment pool that was essentially free from cellular nucleases. The final Superose 12 column gave additional purification and buffer exchange. The exonuclease-deficient D424A mutant protein (Asp to Ala at residue 424), previously purified by conventional chromatographic techniques (Derbyshire *et al.*, 1988), was used to test the new purification scheme. The polymerase specific activity of D424A was the same after either purification procedure, confirming that the FPLC purification did not adversely affect enzyme activity. In either case, the exonuclease activity was essentially zero, indicating that the FPLC purification had been successful in removing endogenous nucleases.

Enzymatic activity of the mutant proteins

The mutant proteins were assayed for polymerase activity (Table I). In each case, the polymerase specific activity was within the range expected for wild type Klenow fragment, suggesting that the polymerase domain of Klenow fragment is unaffected by single amino acid changes in the exonuclease domain.

Each protein was assayed for 3′−5′ exonuclease activity on double-stranded DNA (Table I). The DNA substrate was

Table I. Enzymatic activity of wild type and mutant derivatives of Klenow fragment

Protein[a]	Polymerase sp. act. (10^{-4} × units/mg)	Ratio of exonuclease to polymerase activity[b]	
		Double-stranded DNA	Single-stranded DNA
Wild type	0.9 ± 0.2	100.0 ± 14.0	100.0 ± 31.0
D355A[c]	1.3 ± 0.1	0.0083 ± 0.001	
E357A	1.2 ± 0.2	0.18 ± 0.03	
L361A	0.9 ± 0.2	4.0 ± 1.4	37.0 ± 10.0
L361M	1.1 ± 0.2	8.3 ± 2.4	
D424A[c,d]	0.9 ± 0.2	0.0013 ± 0.0006	
D424E	1.1 ± 0.2	4.0 ± 1.1	8.3 ± 2.2
D424N[c]	1.2 ± 0.3	0.0025 ± 0.0003	
F473A[c]	1.0 ± 0.3	0.029 ± 0.008	
Y497F	1.1 ± 0.1	4.3 ± 1.2	1.6 ± 0.6
D501A[c]	1.2 ± 0.1	0.0075 ± 0.0003	
D501E	1.0 ± 0.1	0.56 ± 0.04	
D501N	1.0 ± 0.3	50.0 ± 16.0	

[a]Protein mutations are abbreviated using the following convention: the residue number, from the DNA polymerase I sequence (Joyce *et al.*, 1982), is preceded by the symbol (in the one-letter code) for the wild type amino acid and followed by the symbol for the mutant amino acid. Thus D424A denotes a mutation from Asp to Ala at position 424.

[b]Relative to wild type (defined as 100). A value of 0.001 represents the lower limit of the assay using double-stranded DNA. A value of 0.4 is the lower limit of the single-stranded DNA assay.

[c]As indicated in the text, exonuclease assays of these proteins were not necessarily under V_{max} conditions; the exonuclease activities therefore represent relative activities under the assay conditions described in Materials and methods.

[d]From Derbyshire *et al.* (1988).

a heterogeneous mixture of 3′ end-labeled restriction fragments. The concentration of DNA 3′ termini in the assay (~300 nM) was shown to be saturating for wild type and for those mutant proteins having at least moderate levels of exonuclease activity. Thus, for these proteins, the values in Table I reflect the relative k_{cat} values. The remaining mutant proteins (D355A, D424A, D424N, F473A and D501A) had very low exonuclease activity, making it necessary to add large amounts of enzyme in order to obtain a detectable signal in the exonuclease assays. As a consequence, we could not establish unambiguously whether the assays were under V_{max} conditions. Thus, the extremely low exonuclease activities of these mutant proteins may indicate defects both in substrate binding and in catalysis.

From Table I it can be seen that the mutations which had the most severe effect on exonuclease activity were those that remove ligands to the divalent metal ions. The D424A, D355A and D501A mutations all reduced the exonuclease activity by at least four orders of magnitude. Moreover, the activity could not be boosted by increasing the Mg^{2+} concentration in the assay from 6 to 33 mM, implying that there was no significant change in occupancy of the metal sites in the mutant proteins over this concentration range. The E357A mutant protein had 20- to 100-fold more exonuclease activity than mutant proteins that had lost the other metal ligands (D424A, D355A and D501A). The ratio of the 3′–5′ exonuclease activity of the E357A mutant protein to that of wild type Klenow fragment was constant over a range of concentrations of Mg^{2+}, with both enzymes having optimal activity at 6–10 mM $MgCl_2$ (Figure 2).

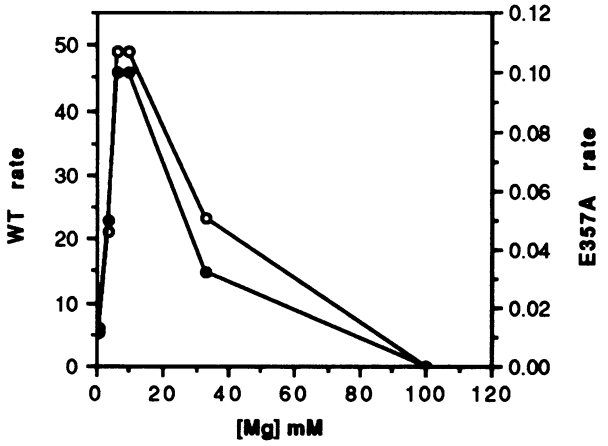

Fig. 2. 3′–5′ exonuclease activity of wild type Klenow fragment and the E357A mutant derivative as a function of the Mg^{2+} concentration. The substrate was double-stranded DNA labeled with ^{32}P at the 3′ end. The rates shown for each are expressed in the same arbitrary units. ○, Wild type; ●, E357A.

Three mutant proteins (L361A, D424E and Y497F) were also assayed for 3′–5′ exonuclease activity on single-stranded DNA. Because this assay was less sensitive than the double-stranded DNA assay, we could not assay proteins whose activity was $< 4 \times 10^{-3}$ of the wild type level. The most satisfactory substrate for the single-stranded DNA assay was a ^{32}P-labeled DNA homopolymer, made by using terminal deoxynucleotidyl transferase and an [α-^{32}P]dNTP to extend an octanucleotide. A particular advantage of this substrate was that the product from one round of exonuclease digestion could serve as substrate in subsequent rounds, so that the reaction rate remained constant for quite large extents of reaction (Figure 3). The relative reaction rates (Table I) reflect the k_{cat} values since the concentration of the DNA substrate was shown to be saturating in each case. Relative to wild type Klenow fragment, the D424E and Y497F proteins showed small, but significant, differences in activity on single-stranded versus double-stranded DNA. For the L361A mutant protein, the difference between the two assays was much greater, with the activity on single-stranded DNA 10-fold higher than on double-stranded DNA, when compared with wild type Klenow fragment. In fact, the activity of the L361A protein on single-stranded DNA was only slightly less than that of wild type.

Kinetic analysis of wild type Klenow fragment

Previous work (Kuchta *et al.*, 1988) established the kinetic constants for the 3′–5′ exonuclease reaction of wild type Klenow fragment using duplex DNA substrates. The reaction was found to be very slow, with a k_{cat} of $\sim 10^{-3}$ s^{-1}. The K_m value was too low to be measured accurately and was estimated to be in the nanomolar range, implying very tight substrate binding. We have now determined the kinetic constants for wild type Klenow fragment on single-stranded DNA, using poly(dT) as a substrate (Figure 4). The values for k_{cat} (0.09 s^{-1}) and K_m (5.6×10^{-7} M) imply weaker substrate binding but a more rapid reaction than with duplex DNA. Using an analogous poly(dA) substrate, k_{cat} was essentially the same (0.12 s^{-1}, data not shown).

Using the poly(dA) substrate we determined the effect of pH on V_{max} for wild type Klenow fragment. The advantage

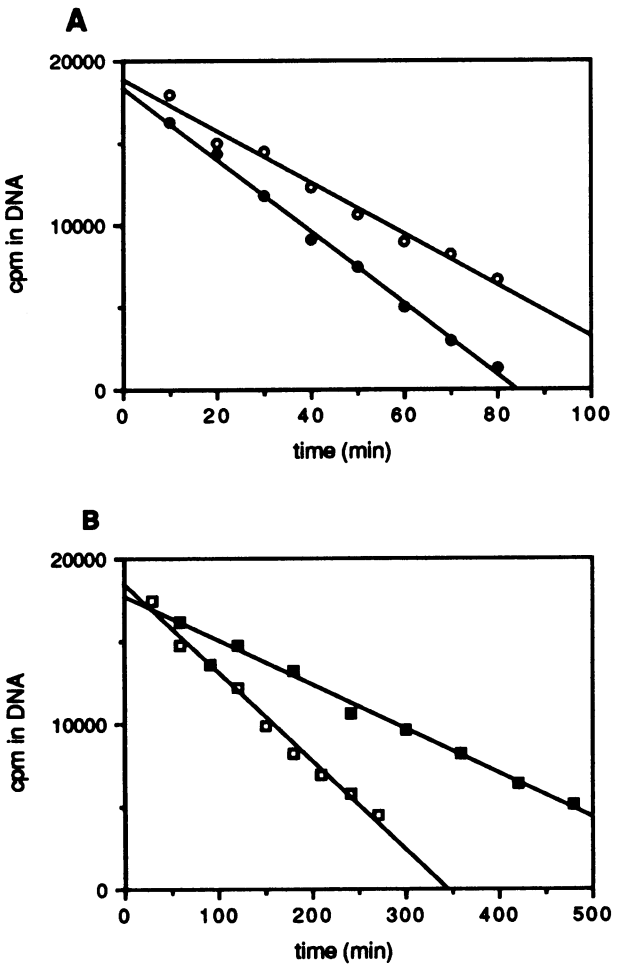

Fig. 3. 3′−5′ exonuclease activity of wild type and mutant derivatives of Klenow fragment. The loss of radioactivity from the single-stranded substrate poly(dA) (average length 34) was plotted as a function of time. **Panel A** shows wild type Klenow fragment assayed at 6.7×10^{-7} M protein (●) and the L361A mutant protein assayed at 8.7×10^{-7} M (○). **Panel B** shows the activity of the mutant proteins D424E, assayed at 1.4×10^{-6} M (□), and Y497F, assayed at 2.7×10^{-6} M (■).

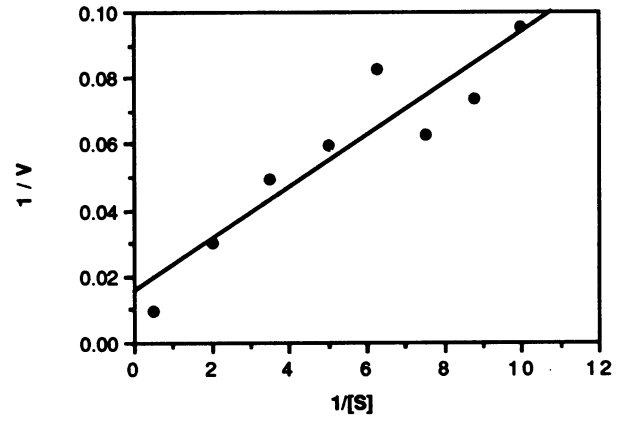

Fig. 4. Lineweaver−Burk plot to determine k_{cat} and K_m for the hydrolysis of single-stranded DNA by wild type Klenow fragment. The substrate was poly(dT) of average length 31. The substrate concentration [S] is expressed in μM, and the rate, V, as fmol product released per second per 10 μl reaction mix.

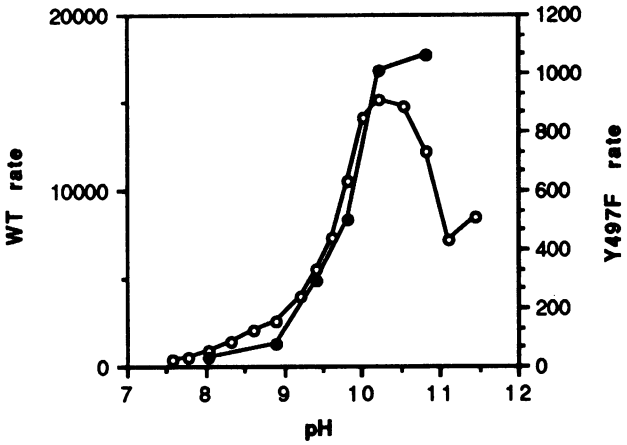

Fig. 5. 3′−5′ exonuclease activity of wild type Klenow fragment and the Y497F mutant derivative as a function of pH. The substrate was poly(dA), of average length 34. The rates shown for each are expressed in the same arbitrary units. ○, Wild type; ●, Y497F.

of using a single-stranded DNA substrate is that there are no additional complications due to melting of the substrate, as was the case in previous determinations using duplex DNA (Lehman and Richardson, 1964). As shown in Figure 5, the rate of the 3′−5′ exonuclease reaction is 45-fold greater at pH 10.2 than at pH 7.5. The pH-dependence curve shows a point of inflexion at a pH of ~9.8, implying that the reaction is dependent on deprotonation of a functional group having a pK_a ~9.8. The same result was obtained with poly(dT) as substrate (data not shown), eliminating the possibility that ionization of the nucleotide bases of the DNA substrate was responsible for the observed pH-dependence. Since a tyrosine hydroxyl group has a pK_a in the appropriate range, we repeated the experiment with the Y497F mutant protein (Figure 5). The pH-dependence of V_{max} for Y497F gave the same apparent pK_a as for wild type Klenow fragment, with the rates being 62-fold lower for the mutant protein. It is unclear whether the difference between the two curves at very high pH is significant. If it is, it may indicate that the H-bond donor potential of the Tyr hydroxyl is important, as proposed by Beese and Steitz (1990).

Discussion

We have generated a collection of mutant derivatives of Klenow fragment and have examined their 3′−5′ exonuclease activity in order to investigate the role of the amino acid side chains at the exonuclease active site. Mutations were introduced into all of the side chains shown in Figure 1. These account for all but one of the close contacts between the exonuclease region of Klenow fragment and the 3′-terminal residue of the DNA substrate. The remaining contact, which is not amenable to this type of study, is the interaction between the backbone amide of Thr358 and the 3′ hydroxyl of the substrate (Ollis *et al.*, 1985). The majority of the side chains in Figure 1 are highly conserved in the sequences of a large number of polymerases from prokaryotic, eukaryotic and viral sources (Table II, Bernad *et al.*, 1989). At each position the wild type functional group was eliminated by replacing the wild type residue with alanine or, in the case of Tyr497, with phenylalanine. Additional mutations were made at Asp424 (a metal B ligand) and Asp501 (a metal A ligand) to examine in more detail the role of these ligands to the divalent metal ions. At these

Table II. Conservation of amino acid side chains at the 3′−5′ exonuclease active site of DNA polymerase I

Residue[a]	Occurrence in 19 polymerase sequences[b]
Asp355	15 Asp
Glu357	19 Glu
Leu361	11 Leu, 1 Val, 1 Met
Asp424	15 Asp, 4 Glu
Tyr497	15 Tyr, 4 Phe
Asp501	16 Asp, 3 Glu

[a]In the DNA polymerase I sequence.
[b]From the data of Bernad *et al.* (1989). Occurrences of the identical or a chemically similar residue are noted. The side chain of Phe473 does not appear to be conserved except, as previously noted, in the DNA polymerase III (ε) sequence (Joyce *et al.*, 1986).

two positions Asp was replaced by Glu to assess the importance of ligand geometry, and by Asn to assess the importance of charge. At Leu361, an additional mutation to Met was made to increase the similarity to the ε subunit of *E.coli* DNA polymerase III, such that the active site residues are identical in the two enzymes (Joyce *et al.*, 1986; Bernad *et al.*, 1989). The data of Maki and Kornberg (1987) suggest that ε (as a part of core enzyme) is a more active exonuclease than Klenow fragment. However, the L361M mutation caused a 12-fold decrease in the 3′−5′ exonuclease activity, suggesting that residues other than those contacting the 3′-terminal residue are responsible for the difference in activity between the two enzymes.

An important assumption, in a mutagenesis study of this type, is that changes in protein structure due to the mutations are confined to the position of the altered amino acid. Crystallographic studies of D424A and of the double mutant D355A,E357A showed this to be the case for these mutant proteins (Derbyshire *et al.*, 1988). It is therefore probable that our assumption will also be correct for the more conservative D424N mutation and for the single D355A and E357A mutations. The following lines of circumstantial evidence argue against any of the other mutations in this study causing a substantial rearrangement of the overall Klenow fragment structure. All of the mutant proteins had wild type levels of polymerase activity. None of the mutations resulted in a decreased yield from the overproducer plasmid, which might indicate an unstable protein structure (Parsell and Sauer, 1989), and none of the mutations gave a protein having solubility or chromatographic behavior different from that of wild type Klenow fragment. However, we cannot rule out the possibility that these single amino acid substitutions lead to more subtle structural changes within the exonuclease active site region. Crystallographic studies of the mutant proteins (currently in progress) will not only address this issue but will also be able to detect changes in the binding of substrate and/or metal ions at the active site, as in the previous study (Derbyshire *et al.*, 1988).

Exonuclease assays of the mutant Klenow fragment derivatives confirmed our earlier conclusion that the metal ions at the 3′−5′ exonuclease active site are crucial for activity. Just as in our previous mutagenesis study of the metal B ligand, Asp424, we have now shown that replacement by alanine of the metal A ligand, Asp501, or the shared ligand, Asp355, both cause a large decrease in exonuclease activity, which we attribute to the loss of binding of the relevant metal ion. Details of the metal−ligand interactions are revealed by the more conservative asparagine substitutions at positions 424 and 501. It appears that both carboxylate oxygens of Asp424 are required for binding metal B since the D424N mutation reduced exonuclease activity almost to background levels. By contrast, only a single oxygen of Asp501 appears to be involved in binding metal A since the D501N mutant, which retains one oxygen, had virtually wild type exonuclease activity. Although the mutant data suggest that the second oxygen of the Asp501 side chain is not involved in any critical interaction, this flexibility is not reflected in the protein sequence comparisons (Bernad *et al.*, 1989), which show that a carboxylate group is invariably found at this position (Table II). The only possible exception is the DNA polymerase III (ε) sequence, where an alternative alignment gives Asn in the corresponding position (Joyce *et al.*, 1986). In considering the results for the D501N protein, we were concerned therefore that a metal-catalyzed deamidation of the Asn side chain could have regenerated the wild type protein. However, mass spectrometric analysis of the appropriate tryptic peptide indicated that the purified D501N protein did indeed contain Asn at position 501 (see Materials and methods).

The effect of glutamate substitutions at positions 424 and 501 was more difficult to interpret, but presumably reflects differences in the metal−ligand geometry in the two cases. The glutamate substitution was better tolerated at position 424 where it seems likely that the direction of the metal−oxygen bonds would be very similar with either Asp or Glu as the side chain (Figure 1). In considering the interaction of metal B with the side chain at position 424 and the DNA substrate, it is important to bear in mind that the crystallographic data are, of necessity, derived from situations where the exonuclease activity has been suppressed, either by omission of divalent metals, by use of the D424A mutant, or by the binding of the product, dNMP, instead of the DNA substrate. Thus the geometry of the metal B site in a catalytically competent enzyme−substrate complex may differ in subtle, but important, ways from that seen in the complexes that can be analyzed crystallographically.

Our data suggest that Glu357 should be placed in a separate category from the other carboxylate side chains at the exonuclease active site. The E357A mutant protein had at least 20-fold higher exonuclease activity than the D355A or the D501A proteins, suggesting that Glu357 is less important in binding metal A than Asp501 or Asp355. We hypothesized that, whereas other mutations in the metal A ligands may effectively eliminate metal A binding, the E357A mutation may merely have weakened binding such that the metal A site was only partially occupied under the assay conditions (6 mM Mg^{2+}). However, this appears not to be the case since the E357A protein shows the same Mg^{2+} dependence as wild type, suggesting that there is no change in the occupancy of the metal A site over the range of Mg^{2+} concentrations tested. Thus, the metal A site in the E357A protein must be either completely filled or completely empty under our assay conditions. We favor the idea that the metal site is completely filled (and, therefore, that Glu357 may contribute little, if anything, to metal binding) for two reasons. One is the higher exonuclease activity of the E357A protein, compared with the D355A and D501A mutant proteins. The second reason is our ability to achieve a

saturating concentration of DNA in assays of the E357A mutant protein, implying that substrate binding is not seriously impaired, as would have been expected if the metal A site were empty. If we are indeed correct in our inference that the E357A mutation does not affect metal binding under our assay conditions, then the decrease in exonuclease activity caused by this mutation would indicate that Glu357 plays a separate role in the exonuclease reaction. For example, the interaction between Glu357 and the 3′ hydroxyl at the primer terminus (Beese and Steitz, 1989) may be important for positioning the substrate correctly within the active site. In addition, the refined active site structure indicates a potentially important interaction of this side chain with a water molecule (Beese and Steitz, 1991). A pivotal role for Glu357 within the active site is suggested by the protein sequence comparisons (Table II), which indicate that this residue is invariant in the 19 available sequences.

Whereas alanine substitutions at ligands to the two divalent metal ions reduced the exonuclease activity almost to the assay background (except for the E357A mutation), similar mutations in the other active site residues (Leu361, Phe473 and Tyr497) had a more modest effect. Since the corresponding mutant proteins all had measurable exonuclease activity, these side chains are clearly not essential for catalysis. The position of these residues, in close proximity to the bound substrate molecule, suggests that they may be important in facilitating catalysis by appropriately positioning the substrate at the active site. In the crystal structure of Klenow fragment complexed with single-stranded DNA, the side chain of Leu361 is inserted between the penultimate and terminal bases, Phe473 interacts with the terminal base, and Tyr497 is H-bonded to the phosphodiester bond that is to be cleaved (Freemont *et al.*, 1988). Quantitatively, the interaction with Phe473 may be the most important since the F473A mutation had a greater effect on exonuclease activity than L361A and Y497F. (However, it should be noted that the low exonuclease activity of the F473A mutant protein may result from defects both in substrate binding and in catalysis since the assays were not necessarily carried out under conditions of saturating DNA. The L361A and Y497F proteins, on the other hand, were assayed under V_{max} conditions.)

Wild type Klenow fragment and those mutant proteins having sufficient levels of exonuclease activity (L361A, D424E and Y497F) were assayed on a single-stranded DNA substrate as well as the duplex substrate. The hydrolysis of single-stranded DNA by wild type Klenow fragment was ~100 times faster than the hydrolysis of double-stranded DNA, but the K_m for single-stranded DNA was at least 100-fold greater. A plausible explanation for the K_m difference is that a double-stranded DNA substrate makes greater use of contacts with the proposed DNA-binding cleft on the polymerase domain of Klenow fragment. This explanation is compatible with the suggestion of Catalano *et al.* (1990) that the exonuclease reaction with duplex DNA may follow a three-step pathway involving initial binding of the substrate to the polymerase domain, followed by a rate-limiting translocation and melting to place the unpaired 3′ terminus at the exonuclease active site in readiness for the catalytic step. For a single-stranded substrate the slow translocation step would be eliminated so that the higher k_{cat} reflects the rate of the chemical step. The more complex reaction pathway for duplex DNA is also consistent with the considerable differences between the pH-dependence curves for the two substrates (Figure 5; cf. Lehman and Richardson, 1964).

When the activities of mutant proteins on single-stranded and double-stranded DNA were compared (Table I), the L361A protein was the only mutant protein tested that gave substantially different results in the two assays. The L361A mutation had a much more severe effect on the degradation of a duplex DNA substrate than on the degradation of single-stranded DNA, suggesting that the interaction between Leu361 and the nucleotide bases of the substrate is important in promoting the binding of the frayed 3′ terminus to the exonuclease active site.

Freemont *et al.* (1988) have discussed the possibility that the attacking nucleophile in the 3′−5′ exonuclease reaction may be a hydroxide ion whose formation is facilitated by coordination to the divalent metal ion in the A site. Our pH-dependence experiments are consistent with this hypothesis since the observed pK_a of ~9.8 is in the appropriate range for ionization of a water molecule coordinated to a divalent metal ion (Baes and Mesmer, 1976). Thus the first stage of the reaction would be analogous to the mechanisms proposed for the hydrolytic reactions carried out by alkaline phosphatase, a zinc enzyme, and RNase P, which uses Mg^{2+} as a cofactor (Sowadski *et al.*, 1985; Guerrier-Takada *et al.*, 1986). Although the identity of the preferred metal ion for occupancy of the A site has not been established with certainty by our experiments, it is interesting to note that the arrangement of ligands to this site conforms to the pattern noted by Vallee and Auld (1990) for catalytic zinc ions, with two ligands (Asp355 and Glu357 in this case) separated by a short spacer and the third ligand (Asp501) at a much greater distance on the primary sequence.

If the proposed mechanism is indeed correct, to what extent do side chains within the active site act to orient the attacking nucleophile or to assist in deprotonation of the metal-bound water molecule to form the hydroxide ion? Three residues, Glu357, Tyr497 and Asp501, could be considered as candidates for one or both of these functions based on their location on the correct side of the terminal phosphodiester bond. The results of the current study place constraints on the roles that can be proposed for these side chains. Any mechanistic description must take account of our observations that mutation of Tyr497 to Phe has almost no effect on the pH-dependence of the reaction, and that replacement of Asp501 with Asn, which cannot function as a general base, causes only a 2-fold decrease in exonuclease activity. The data therefore eliminate Tyr497 and Asp501 (but not Glu357) from a role in deprotonation, but any of the three could assist in orienting the nucleophile.

Materials and methods

Materials

Oligonucleotides for site-directed mutagenesis were synthesized on a Biosearch 8600 DNA synthesizer. Ultrapure dNTP solutions, terminal deoxynucleotidyl transferase, pdA_8 and pdT_8 were purchased from Pharmacia LKB Biotechnology Inc. [α-^{32}P]dATP and [α-^{32}P]dTTP were from Amersham. Restriction enzymes were purchased from New England Biolabs or Boehringer-Mannheim and were used as recommended by the suppliers.

General techniques

Standard cloning techniques were used (Maniatis *et al.*, 1982). DNA sequences were determined by the dideoxynucleotide chain-termination procedure (Biggin *et al.*, 1983). Protein concentrations were determined

by the Bradford colorimetric assay (Bradford, 1976), using the reagent supplied by BioRad. Homogeneous wild type Klenow fragment of accurately determined concentration, was used as a standard. SDS–PAGE was carried out according to Laemmli (1970).

Site-directed mutagenesis

Synthetic oligonucleotides containing the desired mutations were used as primers on uracil-containing M13 templates (Kunkel *et al.*, 1987) containing cloned regions of the *polA* gene. The templates for mutagenesis at positions 355, 357 and 361 were derived from the overproducer plasmid, pCJ122 (see below), and contained DNA from the *Ava*I site 150 bp upstream of the Klenow fragment translational start to the *Xho*I site at position 1205 in the *polA* sequence (Joyce *et al.*, 1982) inserted into the *Hin*cII site of M13mp18 or mp19. The templates for the remaining mutagenesis experiments were derived from the pCJ55 Klenow fragment overproducer plasmid (Joyce and Grindley, 1983) and contained DNA from the *Bam*HI site immediately upstream of the Klenow fragment translational start to the unique *Sac*I site (position 1675), inserted between the *Bam*HI and *Sac*I sites of M13mp11. After characterization in M13 the mutations were subcloned into an overproducer plasmid. Because each fragment that was to be subcloned had to be sequenced in its entirety to check that there were no additional mutations, the cloning strategy was designed to minimize the size of fragment being transferred.

Plasmid construction

Each mutation was introduced into the overproducer plasmid, pCJ122 (Polesky *et al.*, 1990), which encodes wild type Klenow fragment under the control of the λ P_L promoter. Mutations at positions 355, 357 and 361 were subcloned on a 302 bp fragment extending from the *Bst*XI site 70 bp upstream of the Klenow fragment translational start to the *Xho*I site (position 1205). The remaining mutations were subcloned on a 470 bp *Xho*I–*Sac*I fragment (1205–1675 in the *polA* sequence). The cloning procedure was facilitated by the use of deletion derivatives of pCJ122 in which the appropriate pair of unique sites (*Bst*XI and *Xho*I, or *Xho*I and *Sac*I) were joined by a short (~10 bp) adaptor containing a *Bam*HI site. The primary advantage of this cloning strategy was that there was no danger of recovering wild type information instead of the desired mutation since the corresponding region was absent from the recipient plasmid. Additional advantages were that the cloning efficiency could be increased by digestion of the ligation mixture with *Bam*HI to reduce the background of starting plasmid, and that plasmids containing the desired mutations could easily be differentiated from the starting plasmid by the increase in size of an appropriate restriction fragment.

Growth and induction of bacteria

Plasmids for overproduction were introduced into the host CJ376, a *recA* derivative of the Δ*xthA* strain BW9109 containing a plasmid encoding a temperature-sensitive λ repressor gene (Polesky *et al.*, 1990). Heat induction of Klenow fragment expression was carried out as described previously (Joyce and Grindley, 1983).

Purification of the wild type and mutant derivatives of the Klenow fragment

Wild type Klenow fragment was purified as described previously (Joyce and Grindley, 1983; Derbyshire *et al.*, 1988). The mutant proteins were purified by FPLC (Pharmacia-LKB). A crude cell extract was obtained as described previously (Joyce and Grindley, 1983). Solid ammonium sulfate was added to 50% saturation (29.1 g per 100 ml extract). The precipitate was discarded and solid ammonium sulfate was added to 85% saturation (a further 23.0 g per 100 ml). An amount of ammonium sulfate precipitate equivalent to 0.5 g original cells was dissolved in 10 ml of 50 mM Tris–HCl, pH 7.5, 1 mM DTT (TD buffer) and dialyzed against this buffer. The dialyzed protein was filtered through a Millipore 0.22 μm filter unit and applied to a Mono Q HR 5/5 column equilibrated with TD buffer. The column was washed with 5 ml of this buffer and then the Klenow fragment was eluted with a 30 ml linear gradient of 0–0.5 M NaCl in TD buffer at 1 ml/min. Klenow fragment elutes as a large peak at 140–200 mM NaCl. The peak fractions were pooled (typically 4 ml in total) and dialyzed against TD buffer containing 1.7 M ammonium sulfate. The dialyzed protein was applied to a phenyl Superose HR 5/5 column equilibrated with TD buffer containing 1.7 M ammonium sulfate. The column was washed with 5 ml of this buffer and then the Klenow fragment was eluted with a 30 ml reverse ammonium sulfate gradient (1.7–0 M in TD buffer) at 0.5 ml/min. Klenow fragment elutes at 850–935 mM ammonium sulfate. The peak fractions were pooled (typically 3.5 ml in total) and, assuming the pool to be 15% saturated, solid ammonium sulfate was added to 85% saturation (46.0 g per 100 ml). The ammonium sulfate precipitate was dissolved in 150–200 μl of 100 mM Tris–HCl, pH 7.5, 1 mM DTT. This was clarified by centrifugation and 200 μl was applied to a Superose 12 HR 10/30 column equilibrated in the same buffer. The column was developed with 30 ml of this buffer at 0.5 ml/min. The Klenow fragment eluted after ~14 ml. Peak fractions (typically 1.6 mg/ml) were diluted with an equal volume of glycerol and stored at −20°C.

Polymerase assay

The wild type and mutant derivatives of Klenow fragment were assayed by the standard DNA polymerase assay (Setlow, 1974), with poly[d(A-T)] as the template. One unit of polymerase activity is defined as the amount of enzyme catalyzing the incorporation of 3.3 nmol of dNTP into trichloroacetic acid-insoluble material in 10 min at 37°C.

Exonuclease assays on double-stranded DNA

3′–5′ exonuclease activity on double-stranded DNA was measured as previously described (Freemont *et al.*, 1986; Derbyshire *et al.*, 1988). The substrate was a heterogeneous mixture of 3′ end-labeled DNA fragments. The standard reaction (20 μl) contained ~3×10^{-7} M DNA 3′ termini in 6 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol and 50 mM NaCl. This was shown to be saturating substrate for all of the proteins except for D501A, D424A, D424N, D355A and F473A as the rates measured at 3×10^{-7} and 9×10^{-7} M were identical (data not shown).

Exonuclease assays on single-stranded DNA

The substrate for 3′–5′ exonuclease assays on single-stranded DNA was synthesized by incubating pdA_8 (typically 80 nmol) with 4 μmol [α-^{32}P]dATP (5–10 μCi/μmol) in a 100 μl reaction containing 100 mM Tris–HCl, pH 7.5, 100 μg/ml bovine serum albumin, 1 mM DTT, 10 mM $MgCl_2$ and 80 units of terminal deoxynucleotidyl transferase for 16 h at 37°C. Unincorporated dATP was removed by gel filtration on a 1 ml Biogel P4 column. The average chain length was determined by fractionation of a sample of the substrate on a 10% polyacrylamide–urea sequencing gel followed by densitometric scanning and integration of all peaks visible on the resulting autoradiograph. To compare the activitities of mutant derivatives of Klenow fragment, the standard assay reaction (20 μl) contained ~1×10^{-4} M DNA 3′ termini in 50 mM Tris–HCl, pH 7.5, 8 mM $MgCl_2$. This was shown to be saturating substrate for each enzyme assayed because the rates were identical with both 1×10^{-4} and 3×10^{-4} M DNA (data not shown). Reactions were initiated by addition of enzyme and were incubated at 37°C. At intervals, 2 μl samples were removed and quenched in 53 μl of 30 mM EDTA. The quenched reaction solutions were assayed for loss of [α-^{32}P]dAMP from the single-stranded DNA by applying 50 μl to a 2.5 cm diameter DE81 filter (Whatman), which was processed as described by Bryant *et al.* (1983).

Measurement of k_{cat} and K_m for wild type Klenow fragment was carried out using poly(dT), made as described above for poly(dA), except that the sp. act. of the labeled nucleotide was higher because lower concentrations of the poly(dT) substrate were to be used. The reaction mix (100 μl) contained 7.4×10^{-9} M Klenow fragment and poly(dT) (1×10^{-7} to 2×10^{-6} M) in 6 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 50 mM NaCl and 100 μg/ml bovine serum albumin. Samples (5 μl) were removed at intervals during incubation at 37°C and processed as described above. Initial rates, determined by a least-squares analysis, were used to generate a Lineweaver–Burk double-reciprocal plot, from which k_{cat} and K_m were calculated.

The pH-dependence of the 3′–5′ exonuclease activity of wild type and the Y497F mutant Klenow fragment was measured using the poly(dA) substrate described above. The standard assay reaction (10 μl) contained ~5×10^{-4} M substrate in 50 mM buffer and 8 mM $MgCl_2$. Tris–HCl was used in the pH range 7.5 to 8.9, glycine–NaOH from pH 8.9 to 10.8, and CAPS from pH 10.8 to 11.4. Wild type Klenow fragment was assayed at 1.1×10^{-6} M and Y497F at 2.7×10^{-6} M.

Peptide analysis

Wild type Klenow fragment and the D501N mutant protein (5 nmol of each) were digested with trypsin and the resulting peptides were separated by HPLC on a Vydac C_{18} reversed-phase column in potassium phosphate buffer, pH 6.0, as described by Stone *et al.* (1989). Comparison of the chromatograms identified a new peak in the profile from the mutant protein as well as a peak that was substantially decreased in intensity. The fractions corresponding to these two peaks were isolated from the two digests and characterized by mass spectrometry, demonstrating that the mutant protein did indeed contain Asn at position 501, and that the corresponding Asp-containing peptide was completely absent.

The peptide mapping was carried out at the Yale University School of Medicine Protein and Nucleic Acid Chemistry Facility. Mass spectra were

recorded at the Mass Spectrometry Facility of the Yale Comprehensive Cancer Center with the technical assistance of Drs W.McMurray and G.Giordano.

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