

Characterization of the interaction of lambda exonuclease with the ends of DNA

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ABSTRACT

Lambda exonuclease processively degrades one strand of double-stranded DNA (dsDNA) in the 5'–3' direction. To understand the mechanism through which this enzyme generates high processivity we are analyzing the first step in the reaction, namely the interaction of lambda exonuclease with the ends of substrate DNA. Endonuclease mapping of lambda exonuclease bound to DNA has shown that the enzyme protects ~13–14 bp on dsDNA, and no nucleotides on the single-stranded tail of the DNA product. We have developed a rapid fluorescence-based assay using 2-aminopurine and measured the steady-state rate constants for different end-structures of DNA. The relative k_{cat} for 5' ends decreases in the order 5' recessed > blunt >> 5' overhang. However, k_{cat}/K_m remains relatively constant for these different structures suggesting they are all used equally efficiently as substrates. From these data we propose that a single-stranded 5' overhang end can bind non-productively to the enzyme and the non-hydrolyzed strand is required to aid in the proper alignment of the 5' end. We have also measured the length-dependence of the steady-state rate parameters and find that they are consistent with a high degree of processivity.

INTRODUCTION

Recombination in bacteriophage lambda requires the product of α gene which encodes the 24 kDa lambda exonuclease (ENTREZ Sequence ID: 119702) (1–4). This enzyme is a very highly processive (>3000 bp) 5' to 3' exodeoxyribonuclease that releases 5'-mononucleotides and the non-hydrolyzed complementary single-stranded DNA (ssDNA) strand as products (5,6). The ssDNA with a free 3'-hydroxy end is then thought to act with the lambda β protein to initiate recombination (7).

Recently the crystal structure of this enzyme has been solved (8) and it was found to be a homotrimer with a toroidal quaternary structure, similar to the two DNA replication processivity factors, PCNA and the β subunit of *Escherichia coli* DNA polymerase III (9). In the model of the complex of the exonuclease with its substrate DNA proposed from the enzyme

crystal structure, the double-stranded DNA (dsDNA) enters the exonuclease through the center of the toroid and the ssDNA product exits through the back of the tapered enzyme channel. This threading of the DNA through the enzyme is hypothesized to account for high processivity since the enzyme is limited in its dissociation because it is topologically linked to the DNA. This topology has also been suggested to explain why the exonuclease has little or no activity at nicks and gaps (8).

Our goal is to elucidate the mechanism by which lambda exonuclease recognizes and interacts with the ends of DNA and subsequently hydrolyzes DNA in a very processive manner. The substrate preferences of lambda exonuclease have been qualitatively examined on long lambda and T4 DNAs (1–7,10). It has a preference for a phosphorylated 5' end (6) and no activity at nicks and limited activity at gaps in DNA (2,11), consistent with a requirement for a free double-stranded end. There is a slight preference for this 5' end to be recessed, although 3' tails >100 bases inhibit the activity of this enzyme (5,10).

We wished to build and expand upon the previous investigations into the mechanism of lambda exonuclease by quantitating the substrate binding interactions and initiating an investigation of the enzymatic mechanism through a kinetic analysis of lambda exonuclease with well defined substrates. Here, we have begun by examining the substrate preferences of lambda exonuclease and its interaction with DNA using short defined oligodeoxyribonucleotides. Given the high processivity of the enzyme, short oligodeoxyribonucleotides should bias its activity in favor of events involved in initiation of hydrolysis relative to the very long substrates used in the initial characterization. We have also developed a rapid continuous spectrophotometric assay that allows us to more easily examine activity on a variety of DNA structures.

MATERIALS AND METHODS

Lambda exonuclease

Recombinant lambda exonuclease was purchased from Novagen. The enzyme was found to be >95% homogeneous based on SDS-polyacrylamide gel electrophoresis. A molar extinction coefficient of $\epsilon = 46\,560\text{ cm}^{-1}\text{M}^{-1}$ was calculated from the known sequence by the method of Mach *et al.* (12). The concentration of enzyme was determined spectrophotometrically to be $152\ \mu\text{M}_{(\text{monomer})}$. The concentration was also determined using the method of Gill and Von Hippel (13) which gave a concentration within 9% of the above value. We found that standard Bradford assays (14,15) overestimated the concentration

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60LL          5' -TGCAGGCATG-CAAGCTTGGC-CTGGCCGTC-GTTTTACAAC-GTCGTGACTG-ATGTCCGTAG 3'
60L           5' CAGCACTGAC-TGCAGGCATG-CAAGCTTGGC-CTGGCCGTC-GTTTTACAAC-GTCGTGACTG 3'
50LR          5' CAGCACTGAC-TGCAGGCATG-CAAGCTTGGC-CTGGCCGTC-GTTTTACAAC 3'
50LL          5' CAAGCTTGGC-CTGGCCGTC-GTTTTACAAC-GTCGTGACTG-ATGTCCGTAG 3'
50L           5' TGCAGGCATG-CAAGCTTGGC-CTGGCCGTC-GTTTTACAAC-GTCGTGACTG 3'
40L           5' CAAGCTTGGC-CTGGCCGTC-GTTTTACAAC-GTCGTGACTG 3'
30L           5' ACTGGCCGTC-GTTTTACAAC-GTCGTGACTG 3'

50U7-2AP     3' ACGTCCGTAC-GTTCGQACCG-TGQCCGGCQG-CAAATGTTG-CQGCQCTGC 5'
50U           3' ACGTCCGTAC-GTTCGAACCG-TGACCCGGCAG-CAAAATGTTG-CAGCACTGAC 5'
60U           3' GTCGTGACTG-ACGTCCGTAC-GTTCGAACCG-TGACCCGGCAG-CAAAATGTTG-CAGCACTGAC 5'
60U7-2AP     3' GTCGTGACTG-ACGTCCGTAC-GTTCGQACCG-TGQCCGGCQG-CAAATGTTG-CQGCQCTGC 5'

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Figure 1. Oligodeoxyribonucleotide substrates. The sequences of the oligodeoxyribonucleotides used in this study are presented. The oligodeoxyribonucleotides are designated with a number indicating their length and an L or U for different complementary strands. Sequences with 2-aminopurine are designated with 2AP and the location of the 2-aminopurine within the sequence by the letter Q.

of this enzyme by ~30% relative to the spectrophotometrically determined value. All enzyme dilutions were prepared with dilution buffer (10 mM Tris-HCl pH 8.0 and 50% glycerol).

Oligodeoxyribonucleotides

Oligodeoxyribonucleotides used in this study were purchased from Operon (Fig. 1). Those oligodeoxyribonucleotides used for nuclease protection assays were further purified on preparative 12.5% acrylamide (29:1, acrylamide:bis-acrylamide) gels prepared with 8 M urea and 130 mM Tris-Borate pH 8.8, 2.5 mM EDTA (1× TBE). Hybridizations were performed in 50 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 100 mM NaCl by heating to 95°C for 5 min and slow cooling over 2 h to room temperature. Gel analysis indicated that <5% of the hybridized DNA was single-stranded. Given the low reported activity of this enzyme for ssDNA (10) we used the hybridized dsDNA without further purification.

Miscellaneous reagents

Polynucleotide kinase was purchased from New England Biolabs. DNase I and micrococcal nuclease (MCNase) and [α - 32 P]-dATP were obtained from AP Biotech. [γ - 32 P]ATP was from ICN. All chemicals were of reagent grade and were made up in 18 MOhm water. Solutions used in enzymatic assays were passed through 0.22 μ m filters before use.

DNase I protection

Oligodeoxyribonucleotide 60L (Fig. 1) was labeled at the 5' end with [γ - 32 P]ATP and polynucleotide kinase and the reactions were chased with 2 mM ATP to get quantitative phosphorylation of the ends. Labeled oligodeoxyribonucleotides were purified over Pharmacia G-50 Nick Spin columns. End-labeled 60L was then hybridized to oligodeoxyribonucleotide 60U that was phosphorylated at the 5' end with polynucleotide kinase and ATP to give a 60 nt blunt ended dsDNA. Hybridized DNA (15.3 nM) was incubated in 50 mM glycine/KOH pH 9.5, 50 μ g/ml bovine serum albumin with 150 nM_(monomer) lambda exonuclease for 15 min at 25°C in 96 μ l. Six microliters of 40 μ g/ml DNase I (50 mM CaCl₂, 2 mM MgCl₂ at 5:1) was added to bring the final concentrations to 2 μ g/ml DNase I, 0.5 mM CaCl₂, 20 μ M MgCl₂. The reactions were incubated at 25°C for 2.5 min and quenched with 25 μ l of 1% SDS, 0.5 M EDTA, 0.1 M sodium acetate, 0.2 mg/ml tRNA. Samples were phenol/chloroform extracted, chloroform extracted and ethanol precipitated. The final sample was brought up in 4 μ l of 95%

formamide, 20 mM EDTA with tracking dyes. Reactions products were heated to 100°C for 4 min, loaded on a 12.5% acrylamide (29:1, acrylamide:bis-acrylamide) sequencing gel with 8 M urea in 1× TBE. Gels were run at 90 W (constant power) and dried. The resolved products were visualized using a phosphor screen and the Molecular Dynamics Storm system.

Micrococcal nuclease (MCNase) protection

Oligodeoxyribonucleotide 50U was labeled at the 5' end with [γ - 32 P]ATP and polynucleotide kinase and purified as for DNase I protection. The labeled 50mer was hybridized to oligodeoxyribonucleotide 30L that had been phosphorylated at the 5' end with polynucleotide kinase and ATP giving a one end blunt and the other having a 20 bp 5' phosphorylated recessed end. Hybridized DNA (9.6 nM) was incubated with 150 nM_(monomer) lambda exonuclease as for DNase I protection assays. One microliter of 1 ng/ml MCNase was added and the reaction was initiated by bringing the solution to 0.5 mM CaCl₂. Reactions were run, quenched and analyzed as for DNase I protection.

Exonuclease assays

Discontinuous 33 P based assay. Long DNA substrates (0.5–20 kb) were amplified from lambda DNA using PCR primers according to manufacturer's instructions (XL-gene amp kit, Perkin/Elmer). The addition of 100–150 μ Ci [α - 33 P]dATP to the PCR reaction resulted in internally labeled substrate. A series of 5'-phosphorylated 20mer primers (3' positions 21359, 5645, 2559, 1030 of lambda DNA) and a 5'-biotinylated 20mer primer (5' position 506 of lambda DNA) were used to create a series of DNA substrates with only one end active to exonuclease activity. These DNAs were assumed to have blunt ends or possibly a 1 bp overhang. Synthesized DNAs were purified by gel electrophoresis and quantitated spectrophotometrically. DNA substrates internally substituted with [33 P]dATP were assayed in 50 mM glycine/KOH, pH 9.5, 50 μ g/ml BSA, and 2 mM MgCl₂ at 25°C unless otherwise indicated. Activity of lambda exonuclease was assayed discontinuously by removing aliquots of reactions over time and assaying for the formation of acid soluble 33 P. The reactions were run in sufficient DNA excess to insure on average only one exonuclease molecule per substrate DNA.

Fluorescence based assay. Activity of lambda exonuclease was also assayed by the increase in fluorescence of 2-aminopurine upon its release from dsDNA into solution (16). Oligodeoxyribonucleotide substrates substituted with 2-aminopurine were

assayed in 50 mM glycine/KOH, pH 9.5, 50 μ g/ml BSA and 2 mM $MgCl_2$ at 25°C. Reactions were initiated by addition of enzyme to the appropriate concentration and activity was monitored on a Perkin/Elmer LS50B spectrofluorimeter with $\lambda_{excitation} = 310$ nm and $\lambda_{emission} = 365$ nm with both slits set at 10 nm. The excitation wavelength was moved slightly off peak to cut down on the background fluorescence from the BSA in the assay buffer. Initial rates were calculated from the initial slope of the progress curves and converted to 2-aminopurine concentration units using a standard curve of 2-aminopurine fluorescence measured under the same buffer and instrument conditions. As in the ^{33}P based assay, the reactions were run in sufficient DNA excess to insure on average only one exonuclease molecule per substrate DNA.

Calculation of rate constants. Rate constants were calculated from the data using direct fits of the Michaelis/Menten equation or linear fits of Hanes–Woolf plots using fitting programs in KaleidaGraph 3.0. In calculating the steady-state rate constants it was assumed that only the phosphorylated 5' end was active for hydrolysis given the measured 10-fold rate difference between 5' phosphate and 5'-hydroxy ends. Any titration of enzyme by binding to inactive DNA ends or residual ssDNA from the oligodeoxyribonucleotide hybridizations would be equivalent for the tested substrates and not affect the ratios of the rate constants. Non-phosphorylated substrate was assumed to have two active ends per DNA molecule. The enzyme preparations were assumed to be 100% active when calculating k_{cat} from measured V_{max} . We have preliminary measurements that our enzyme preparation is only 75% active to DNA binding (data not shown). Since all our measurements are done using the same enzyme preparation the relative rates will be the same, but the determined k_{cat} s should be considered minimal estimates.

RESULTS

Nuclease protection mapping

Modeling of substrate DNA with the crystal structure of lambda exonuclease suggested ~11 bp is enclosed by the enzyme (8). There are, however, no direct biochemical measurements of the extent of this interaction. To test this model we examined the maximal extent of coverage of lambda exonuclease on both the dsDNA substrate and the ssDNA product by nuclease protection.

Excess lambda exonuclease was bound to a blunt ended 60 bp DNA fragment and treated with DNase I under conditions that minimize exonuclease activity. The interaction of lambda exonuclease at both ends of the 60mer resulted in protection of the ends from digestion by DNase I (Fig. 2). This protection occurred whether or not the 5' end was phosphorylated (data not shown). The protected regions were approximately 13 nucleotides on the 3' end and 19 nucleotides on the 5' end of the labeled strand, which is consistent with a site size of 13–14 bp given the known occupancy of DNase I of 5 bp 5' to its cut site (17).

Subsequent to cleavage, lambda exonuclease translocates along the dsDNA leaving a ssDNA product. To examine how much of the ssDNA tail is interacting with the exonuclease, we constructed a substrate having a 30 bp dsDNA region and a 20 base ssDNA tail. After binding of lambda exonuclease to

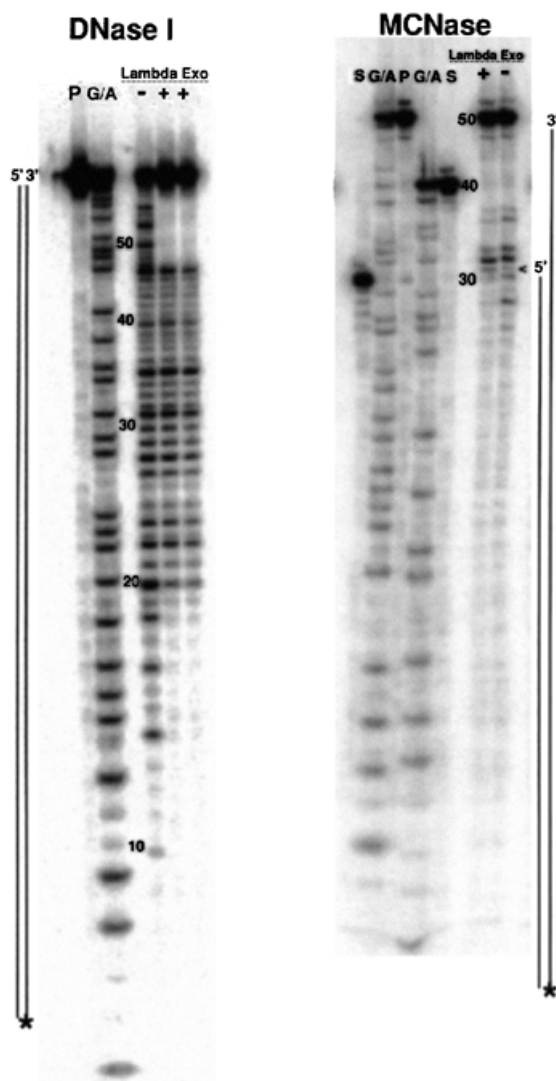


Figure 2. Nuclease protection. Complexes of lambda exonuclease and DNA were formed and then probed with either DNase I or MCNase as described in Materials and Methods. The structures of the probe DNAs relative to their sizes in the sequencing gel are indicated adjacent to each panel with the 5' labeled end indicated by an asterisk. An arrowhead marks the enhancement in MCNase cleavage seen at the double-stranded–single stranded DNA boundary. The sizes of the fragments are indicated at appropriate locations in the gel and are derived from the Maxam–Gilbert purine sequencing ladder. P, starting probe DNA; G/A, purine ladder produced from probe DNA by Maxam–Gilbert reaction. S, oligodeoxyribonucleotide 40L or 30L size standard. +/-, with or without addition of lambda exonuclease. The DNase I panel has two duplicate + exonuclease lanes. The two gel panels are not aligned with regard to fragment size. No DNase I cleavage was observed less than 10 bases from the 5' end of DNA.

the DNA, the exonuclease/DNA complex was probed with micrococcal nuclease (MCNase), which preferentially cleaves ssDNA.

MCNase digestion in the presence or absence of lambda exonuclease shows little difference in the protection of 3'-ssDNA tail exiting the enzyme (Fig. 2). There is, however, protection in the double-stranded region of the DNA from the low level of

MCNase cleavage in this region, indicating that lambda exonuclease is bound. A slight enhancement of cleavage at the ssDNA-dsDNA junction is also observed. This suggests that exonuclease may deform the DNA in some way at this point, but there seems to be no coverage of the 3' tail by lambda exonuclease.

We conclude that lambda exonuclease covers ~13–14 bp of the double-stranded substrate and has minimal interaction with the 3' ssDNA product. This information allows us to design DNA substrates of the appropriate length to investigate the effect of end modifications on activity.

Activity assays of lambda exonuclease

Traditional exonuclease assays have relied on radioactively labeled substrates prepared by enzymatic methods. Although these methods are quite sensitive, preparation of large amounts of substrate of a specific structure as well as the discontinuous nature of these assays make them slow and laborious. To rapidly examine a variety of conditions and substrate structures, we developed a continuous spectrophotometric assay using oligodeoxyribonucleotides containing the fluorescent nucleotide analog 2-aminopurine (2-AP) (Fig. 1). This analog exhibits an ~100-fold increase in fluorescence when it is free in solution relative to that in dsDNA (16), giving an excellent signal to noise ratio in a DNA hydrolysis assay.

Incubation of a double-stranded 50mer containing 2AP with lambda exonuclease results in a continuous linear increase in fluorescence consistent with release of the 2AP from the DNA (Fig. 3A). The rate of the hydrolysis is linear with respect to lambda exonuclease concentration (Fig. 3B), indicating no rate limiting exonuclease assembly occurs over the concentration range examined. These assays can now be used to directly measure steady-state kinetic parameters.

Our goal is to understand how lambda exonuclease initiates processive hydrolysis. The first question we address is the quantitative effect of the structure of the 5' end on the activity (Fig. 1, Table 1A). We first revisited the effect of a 5'-phosphorylated end on hydrolysis. Blunt-ended 50mer substrates were prepared plus or minus a 5' phosphate. Absence of the 5'-phosphate is manifest in a 10-fold decrease in activity which is expressed entirely in k_{cat} (Fig 4A and B, Table 1A). These results are entirely consistent with previous observations of the effect of phosphorylation of the 5' end on measured rates (6), which were performed in DNA saturation relative to the K_m s we have determined.

A series of oligodeoxyribonucleotide substrates were prepared containing either blunt 10 bp 5' overhang or 10 bp 5' recessed ends. Because of the previous report of the inhibition of lambda exonuclease by stretches of ssDNA (10), we designed the ends to be short (10 bp) and devoid of obvious secondary structure. Each substrate contained only one 5' phosphorylated end to bias the enzyme activity to that end. The steady-state rate constants measured for each of these substrates demonstrate that both k_{cat} and K_m decrease slightly from 5' recessed to blunt and then ~3–5-fold to 5' overhang (Table 1A). The values for k_{cat}/K_m , however, are relatively constant at 1.57 ± 0.22 , 1.57 ± 0.59 and $0.90 \pm 0.50 \text{ nM}^{-1}\text{s}^{-1}$ for blunt, 5' overhang and 5' recessed ends respectively.

When the structure at the 5' phosphorylated end is held constant (as blunt) there is no effect on the steady-state kinetics on altering the overhang structure of the 3' end (Table 1A).

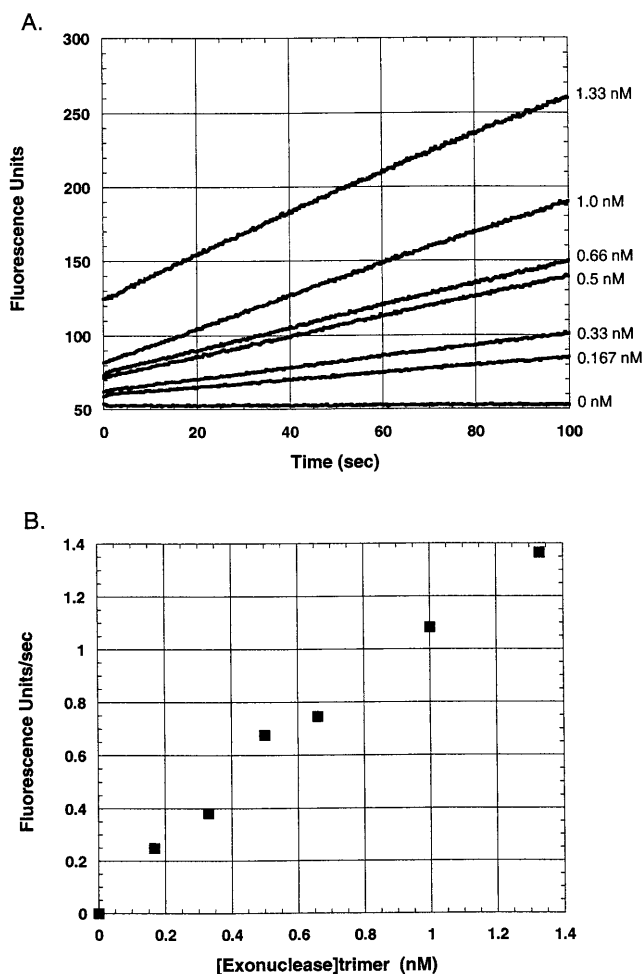








Figure 3. Spectrophotometric assay for nuclease activity. (A) Traces of raw data from an activity measurement are presented. Increasing amounts of lambda exonuclease were incubated with 100 nM of ds50mer, 5'-P-50U7AP:50LL as in Materials and Methods. Fluorescence is measured as arbitrary instrument units. The concentration of exonuclease is indicated on the right of each time course. (B) Slopes of the time course in (A) are plotted against exonuclease concentration.

Given the observed differences in the 5' end preference, this indicates that there is not a significant effect of enzymatic activity at the non-phosphorylated end. This also suggests that at steady-state the dissociation from any residual non-hydrolyzed DNA is kinetically invisible with these substrates.

Given the measured high processivity of λ exo we tested the effect of length on the steady-state kinetics. Substrates were synthesized from 0.5–20 kb by PCR. Over the 400-fold increase in size, k_{cat} increases roughly 2-fold from 2.2 to 5.3 s^{-1} while the value for K_m decreases over 93-fold from 1.5 nM to 16 pM (Fig. 4C, Table 1B). This approximately 200-fold increase in k_{cat}/K_m with increasing length of substrate reflects the previously described extremely long processivity of lambda exonuclease.

Table 1. Steady-state rate constants for lambda exonuclease

A. 5' End dependence:				
Structure	DNA	K_m (nM)	k_{cat} (s ⁻¹)	n
<u>5' OH blunt</u>				
	5'OH-50U7-2AP : 50L	1.8 ± 0.4	0.29 ± 0.02	3
<u>5' P blunt</u>				
	5'P-50U7-2AP : 50L	1.4 ± 0.15	2.2 ± 0.17	4
	5'P-50U7-2AP : 60L	1.5 ± 0.05	2.2 ± 0.13	2
	5'P-60U7-2AP : 50L	1.5 ± 0.25	2.7 ± 0.77	2
<u>5' P recessed (10 bases)</u>				
	5'P-50U7-2AP : 50LL	2.3 ± 0.84	3.6 ± 0.33	3
<u>5' P overhang (10 bases)</u>				
	5'P-50U7-2AP : 50LR	0.51 ± 0.27	0.46 ± 0.07	3
B. Length Dependence:				
DNA (bp)	K_m (pM)	k_{cat} (s ⁻¹)	n	
21359	16 ± 4.8	5.3 ± 1.7	3	
5645	21 ± 9.9	6.7 ± 2.6	3	
2559	34 ± 15	6.3 ± 0.85	4	
506	63 ± 5	4.5 ± 0.68	3	

The steady-state rate constants for lambda exonuclease on a variety of substrates are presented. (A) 5' end dependence. A schematic of each structure and its component oligodeoxynucleotides are described. K_m and k_{cat} are given as mean ± SD, where n is the number of replicants. (B) Length dependence. Length given is based on blunt ended PCR product. Other labels as in (A).

DISCUSSION

We determined that lambda exonuclease specifically protects ~13–14 bp at the end of its dsDNA substrate and little if any of the ssDNA product from endonuclease digestion. The strength of the interaction is indicated by the saturation of the footprint at the nanomolar concentrations of both nuclease and DNA. This is consistent with the nanomolar K_m determined in the steady-state kinetic analysis and previous measurements of the interactions of lambda exonuclease with DNA (2,11).

The extent of protection supports the molecular modeling which predicted ~11 bp of the substrate DNA protected as it threads through λ exo and consequently that this threading is responsible for properties of lambda exonuclease such as the high processivity and inactivity at nicked DNA (8). The protection assays, however, were performed under conditions of very low lambda exonuclease activity (low Mg²⁺ and high Ca²⁺ concentrations). Even though a specific region of the end of the DNA is protected, it is possible that the observed protection patterns represent an inactive complex. Further biochemical and structural analyses, therefore, are required to rigorously determine the actual path of the DNA through the enzyme to confirm this initial model.

One consequence of this model is that there is an ~15 Å distance between the DNA and the proposed location of the active site. There must therefore be a significant motion of either the DNA substrate (for example by melting out of the dsDNA), of the

protein, or of some combination of both relative to the proposed fit of the DNA in the exonuclease crystal structure for catalysis to occur. The consequences of such a motion may be visible either at steady-state or in pre-steady state kinetics.

To examine the steady-state kinetics of lambda exonuclease in a rapid and sensitive manner we developed a spectrophotometric assay based on 2-aminopurine fluorescence. This analog has been used to monitor insertion and excision kinetics of DNA polymerases and their nucleolytic proofreading activity (18,19), RNA polymerases (20) and the action of helicases (21), and we have extended its use to the examination of lambda exonuclease activity. 2-AP substituted oligodeoxyribonucleotides have been shown by NMR to be in native B-form (22) and show normal cleavage by *EcoRI* endonuclease (23), suggesting that this analog should be utilized normally by exonuclease. We have not seen any large effects on the activity of lambda exonuclease using 2-AP relative to radioactively labeled DNA (data not shown), although we must directly test this to rigorously quantitate any potential effects. We find that this simple sensitive assay is highly reproducible and rapid and therefore will be well suited for use for comparison between substrate structures and the analysis of the properties of exonucleases in general and is applicable to future use in rapid reaction single turnover kinetics.

We determined the relative activity of lambda exonuclease on several structures. The previous studies of lambda exonuclease were performed on long viral DNA or random *E.coli* DNA substrates at saturation relative to our K_m determinations (1,6). We can, however, roughly estimate a k_{cat} if we assume that the observed rates are saturated. For example we calculated the turnover number of lambda exonuclease on lambda DNA to be ~4 s⁻¹ [from fig. 1 in (6)] which agrees well with our data.

Previous reports of the relative preferences of types of 5' overhangs were given as 5' recessed ≥ blunt >> 5' overhang, (4,6) however no concentrations of substrate were reported. Under substrate saturation conditions, our data agree well with the reported qualitative preference. Determination of the steady-state rate constants, however, demonstrate that although there is a decrease in k_{cat} between these substrates, which would manifest itself as a drop in rate under substrate saturation conditions, there is also a concomitant drop in K_m . The k_{cat}/K_m , which can be taken as an approximate measurement of enzyme efficiency, is not significantly different for all the structures. In addition, if we make the simplifying assumption that k_{cat}/K_m is roughly equivalent to the second order rate constant for association of DNA and enzyme for these substrates, the observed k_{cat}/K_m is closed to diffusion controlled. Consistent with these data we have observed a rapid association of DNA and lambda exonuclease in preliminary gel mobility shift assays using these substrates (data not shown). These data demonstrate that DNAs with simple 5' overhangs can serve as an efficient substrate for this enzyme. This may bear on the action of lambda exonuclease *in vivo* where a natural substrate is lambda DNA which has 5' overhangs.

The 5' overhangs at the ends of lambda DNA have been suggested to be the cause of an observed lag in the digestion of lambda DNA by lambda exonuclease (6,10). There is, however, no demonstrable lag in the hydrolysis of our 5' overhang substrates; therefore, the previously observed lag cannot be due to the 5' overhang *per se*. It has been suggested that lambda

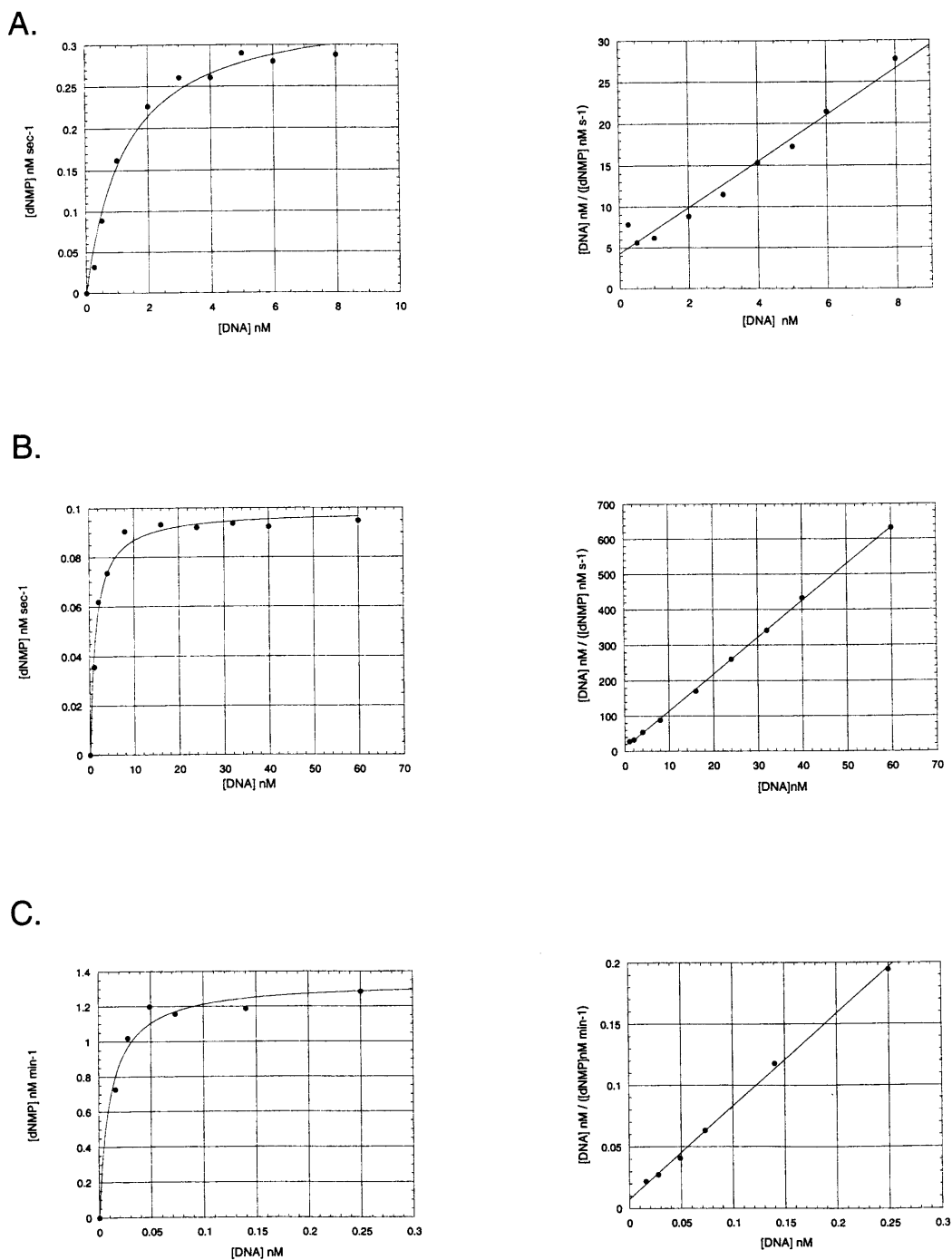


Figure 4. Steady-state rate determinations for lambda exonuclease. Presented are three examples of the steady-state data obtained with different substrates and fitted to both the Michaelis/Menten equation (left) and Hanes–Woolf plots (right). (A) 5' phosphorylated, blunt ended substrate 5'P-50U7-2AP:50L assayed with 0.17 nM_(trimer) lambda exonuclease. (B) 5' hydroxy substrate, 5'OH-50U7-2AP:50L assayed with 0.33 nM_(trimer) lambda exonuclease, (C) 5645 bp substrate assayed with 0.005 nM_(trimer) lambda exonuclease.

exonuclease has difficulty passing through regions of secondary structure in ssDNA (10) and therefore it is possible that a specific structure in the 5' overhang of lambda DNA may cause this lag. We can find no obvious hairpins or other secondary structures in the 12 bp overhangs of lambda DNA. This

suggests that this lag is caused by another attribute of the mechanism of lambda exonuclease on this substrate such as its reported high non-specific DNA affinity (10).

The observation of a proportional decrease in k_{cat} and K_m is characteristic of non-productive binding of the substrate to an

enzyme (24). We suggested that based on the crystal structure there must be some motion to bring the 5' end of the DNA to the enzyme active site. The notion of this motion is supported by our steady state kinetic analysis. We speculate that if a 5' ssDNA overhang has more conformational freedom than a 5' blunt or recessed end, the 5' ssDNA may bind in a kinetically inactive manner. Adoption of these non-productive binding modes would lead to the observed kinetic behavior, and suggests the requirement of the non-hydrolyzed strand of DNA to assist in properly positioning the 5' end of the DNA for catalysis.

The steady-state kinetics of lambda exonuclease were also examined with long DNAs which will decrease the relative contributions of the association and dissociation to the observed kinetics. This effect is especially manifest in the length dependence of the apparent K_m , which decreases 100-fold as the DNA length increases from 50 bp to 20 kb. Such a decrease is expected if K_m can be thought of as the overall dissociation constant for all enzyme/substrate complexes. Then, as the length of the DNA increases, it is less likely the enzyme will dissociate from the DNA, decreasing the apparent K_m .

A qualitatively similar effect has been observed for the very processive 3'-5' ssDNA specific exonuclease, *E.coli* exonuclease I (exo I) (25). As the length of the DNA substrate increased, there was a concomitant linear decrease in apparent K_m of exo I for DNA, while k_{cat} stayed constant. With the rate constant for hydrolysis by exo I independent of polymer size, these results fit a model where there are no interactions with DNA distant from the active site, for example a linear diffusion mechanism to search for the end of the DNA (25).

The effect of length on the steady-state kinetics of lambda exonuclease, however, is quantitatively different. Unlike exo I, there is a small increase in k_{cat} with length. The K_m does not change linearly with length, as with exo I, but decreases hyperbolically. This suggests a fundamental mechanistic difference between these two enzymes. Contributing to this difference could be the influence of having potentially three active sites per holoenzyme, or any non-specific interaction with DNA during the catalytic cycle. Derivation of the steady-state rate equations would include terms for binding of exonuclease and substrate, bringing the DNA to the active site, hydrolysis, dNMP release and partitioning between translocation and dissociation. Given the complex nature of the reaction it is not possible to assign k_{cat} and K_m to any specific steps at this time. Although steady-state kinetics can give some insight into a reaction mechanism,

a more thorough understanding requires using a combination of equilibrium assays and pre-steady state kinetic methods to distinguish and further characterize the elemental steps of the lambda exonuclease reaction.

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