Mutational and pH Studies of the 3′ → 5′ Exonuclease Activity of Bacteriophage T4 DNA Polymerase*

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The 3′ → 5′ exonuclease activity of proofreading DNA polymerases requires two divalent metal ions, metal ions A and B. Mutational studies of the 3′ → 5′ exonuclease active center of the bacteriophage T4 DNA polymerase indicate that residue Asp-324, which binds metal ion A, is the single most important residue for the hydrolysis reaction. In the absence of a nonenzymatic source of hydroxide ions, an alanine substitution for residue Asp-324 reduced exonuclease activity 10–100-fold more than alanine substitutions for the other metal-binding residues, Asp-112 and Asp-219. Thus, exonuclease activity is reduced 10^5-fold for the D324A-DNA polymerase compared with the wild-type enzyme, whereas decreases of 10^3- to 10^4-fold are detected for the D219A- and D112A/E114A-DNA polymerases, respectively. Our results are consistent with the proposal that a water molecule, coordinated by metal ion A, forms a metal-hydroxide ion that is oriented to attack the phosphodiester bond at the site of cleavage. Residues Glu-114 and Lys-299 may assist the reaction by lowering the $pK_a$ of the metal ion-A coordinated water molecule, whereas residue Tyr-320 may help to reorient the DNA from the binding conformation to the catalytically active conformation.

Many DNA polymerases have the ability to proofread newly replicated DNA by transferring the 3′-end of the primer-strand from the polymerase to the 3′ → 5′ exonuclease active center where the terminal nucleotide is removed. Mutant DNA polymerases that have reduced ability to carry out the transfer process (1–4) or are defective in the hydrolysis reaction (4–7) replicate DNA with more errors (1, 5–9). A two-metal ion mechanism for the 3′ → 5′ exonuclease activity of Escherichia coli DNA polymerase I (DNA pol I) has been proposed from structural and mutational studies (10–12). The two divalent metal ions, which may be Mg^{2+}, Mn^{2+}, or Zn^{2+} (10), are bound by conserved carbonylate residues in the exonuclease active centers of proofreading DNA polymerases (reviewed in Refs. 13 and 14). According to the model (Fig. 1), a water molecule, coordinated by metal ion A, forms an attacking hydroxide ion, which is positioned in-line with the target phosphodiester bond. Metal ion B is proposed to stabilize the leaving 3′-hydroxy group and to position the O-P-O bond angles in the transition state (12). The two metal ions are central to the model because amino acid substitutions that prevent binding of one or both metal ions reduce exonuclease activity several thousandfold. The conservation of metal ion binding residues in the exonuclease active centers of all proofreading DNA polymerases, and the severe reduction in exonuclease activity when the metal binding residues are replaced by non-carboxylate residues, suggest a common mechanism for the hydrolysis reaction catalyzed by DNA polymerases (13).

The two-metal ion mechanism may extend to other enzymes that catalyze phosphoryl transfer, for example, bacterial alkaline phosphatase, RNase H of the human immunodeficiency virus reverse transcriptase, single-stranded P1 nuclease, and phospholipase (reviewed in Ref. 15). As observed for E. coli DNA pol I, a distance of about 3.9 Å separates two essential metal ions in the active centers of these enzymes. These observations led Steitz and Steitz (15) to propose that RNA molecules involved in hydrolysis and splicing reactions may similarly position two divalent metal ions to carry out phosphoryl transfer reactions. A two-metal ion model for the hammerhead ribozyme mechanism is illustrated in Fig. 2, but catalysis does not require a metal hydroxide ion (16, 17), although single-metal-hydroxide-ion models have been proposed (reviewed in Ref. 18). Both one- and two-metal ion models for hammerhead ribozyme activity require the ribose 2′-OH group, which is not present in DNA (Fig. 2). Since DNA does not have the 2′-OH, different mechanisms of metal-assisted hydrolysis of RNA and DNA are predicted.

Structural studies of the Klenow fragment of DNA pol I suggest that a water molecule or hydroxide ion is bound to metal ion A (Ref. 12; Fig. 1); however, only one study has examined the pH dependence of the exonuclease reaction and correlation to metal ion $pK_a$ values (11). We present data here from pH and buffer studies of the exonuclease reaction catalyzed by wild-type and mutant bacteriophage T4 DNA polymerases that are consistent with formation of a metal hydroxide ion. Our studies focus on the ExoIII motif, which is conserved in the 3′ → 5′ exonuclease active centers of proofreading DNA polymerases (13, 14). The motif sequence is a tyrosine residue, followed by three amino acids, and then an aspartate residue, which is a ligand to metal ion A (5, 13). The conserved ExoIII residues in E. coli DNA pol I are Tyr-497 and Asp-501 (Fig. 1). The corresponding residues in T4 DNA polymerase are Tyr-320 and Asp-324 (5, 19, 20) and Tyr-323 and Asp-327 in the T4-like RB69 DNA polymerase (Refs. 21 and 22; Fig. 3). Note the structural similarities in the exonuclease active centers of the bacterial and phage enzymes (Ref. 22; compare Figs. 1 and 3). The other essential residues for the 3′ → 5′ exonuclease activity of E. coli DNA pol I are Asp-355, which may provide another ligand to metal ion A, and residue Asp-424, which provides a ligand to metal ion B (Fig. 1).
corresponding phage DNA polymerase residues are Asp-112 and Asp-219 in the T4 DNA polymerase and Asp-114 and Asp-222 in the RB69 DNA polymerase (Fig. 3). Functional similarities for the bacterial and phage DNA polymerases have also been demonstrated. For example, alanine substitutions for residue Asp-501 in E. coli DNA pol I and for residue Asp-324 in T4 DNA polymerase reduce 3′ to 5′ exonuclease activity by 3–4 orders of magnitude (5–7, 11, 20). The large reductions in exonuclease activity indicate that Asp-501 in E. coli DNA pol I and Asp-324 in T4 DNA polymerase are essential for catalysis. Smaller reductions in exonuclease activity are detected when phenylalanine is substituted for Tyr-497 in E. coli DNA pol I (11) and when phenylalanine or alanine are substituted for Tyr-320 in T4 DNA polymerase. The larger drop in residual exonuclease activity for the D324A-DNA polymerase in HEPES compared with Tris buffer indicates that residue Asp-324 can function partially by Tris buffer. We propose that Tris may interact with the T4 DNA polymerase in the exonuclease active center to produce a locally high concentration of hydroxide ions or, possibly, to act directly as a nucleophile. The implication of this proposal for the wild-type T4 DNA polymerase is that metal ion A, bound by residue Asp-324, normally provides this function by coordinating a water molecule to form an attacking metal-hydroxide ion, as proposed for E. coli DNA pol I (Ref. 12; Fig. 1).

For T4 DNA polymerase, residues Glu-114 and Lys-299 may assist formation of the attacking hydroxide ion at physiological pH. Additionally, T4 DNA polymerase residue Tyr-320 may assist orientation of the DNA substrate in the catalytically active conformation.

**EXPERIMENTAL PROCEDURES**

**Construction and Purification of T4 DNA Polymerase 3′ to 5′ Exonuclease Mutants**—Amino acid substitutions Y330F and D324A were engineered into the T4 DNA polymerase expression vector (23) using standard site-directed mutagenesis procedures (24). Mutations in the plasmid vector were verified by sequencing the entire DNA polymerase transcript (9). Wild-type and the Y320F/D324A-DNA polymerases were purified following published methods (25). The D324A-, D112A/E114A-, and D219A-DNA polymerases have been described (6). The enzyme preparations were judged pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining of overloaded lanes; only a single band was visible at the mobility predicted for the 103,572-dalton T4 DNA polymerase. Enzyme concentration was measured spectrophotometrically using the experimentally determined molar extinction coefficient at 280 nm of 1.49 × 10^4 M^−1 cm^−1 (5).

**DNA Polymerase Assay**—DNA polymerase activity was measured using a 60-μl volume reaction with 67 mM Tris-HCl (pH 8.8), 16.7 mM (NH₄)₂SO₄, 0.5 mM dithiothreitol, 6.7 mM MgCl₂, 167 μM guanine bovine serum albumin, 83 μM dNTPs (one labeled [3H]dNTP at about 100 cpm/μmol), and 833 μM “activated” DNA, expressed as nucleotide equivalents and prepared as described (6). The reactions were initiated by addition of wild-type or mutant T4 DNA polymerase to give a final concentration of 10 nM. A linear incorporation rate was observed for at least 30 min at 30 °C. Reactions were stopped by spotting 20 μl of the reaction mixture onto GF/A filters (Whatman) that were prespotted with 20 μl of 0.4 M EDTA. The filters were immersed in an ice-cold solution of freshly prepared 0.1 M sodium pyrophosphate and 7.5% trichloroacetic acid. The filters were washed under vacuum with cold water, dried, and counted in scintillation fluid.

3′ to 5′ Exonuclease Assays for T4 DNA Polymerase—Exonuclease activity was measured by two assay systems. One assay was similar to the exonuclease assay described above with 67 mM Tris-HCl (pH 8.8), 16.7 mM (NH₄)₂SO₄, 0.5 mM dithiothreitol, 6.7 mM MgCl₂, and 167 μM guanine bovine serum albumin, but the dNTPs were omitted and the DNA substrate was 80 μM alkali-denatured, ³²P-labeled E. coli DNA (100 cpm/μmol of nucleotide) (26). Wild-type T4 DNA polymerase was present at 10 nM, but the mutant DNA polymerases were assayed at 1 μM. Reactions were stopped by the addition of 0.44 ml of 1 mg/ml unlabeled single-stranded DNA, followed by 0.5 ml of ice-cold 15% trichloroacetic acid to precipitate the undegraded substrate. The solutions were chilled for 10 min and then centrifuged. A 200-μl sample of the supernatant, which contains the soluble DNMPs produced by the exonuclease reaction, was counted in 4.8 ml of scintillation fluid.

The second exonuclease assay used p(dT)₁₆ (Amersham Pharmacia Biotech), which was labeled at the 5′-end by using [γ-³²P]ATP and T4 polynucleotide kinase. The exonuclease reaction mixture contained 50 mM buffer described below), 1 mM dithiothreitol, 7 mM MgCl₂, 12.5 μM ³²P-labeled p(dT)₁₆, and 5 μM unlabeled p(dT)₁₆, to give a total DNA concentration approximately 10-fold higher than the Kis (app) for the p(dT)₁₆ substrate determined for the wild-type and D324A-DNA polymerases (20). Tris-HCl buffer was used in the pH range from 7.0 to 8.7 and glycine-NaOH buffer was used from pH 8.7 to 10.5. HEPES buffer, pH 8.0, was also used. Reactions were initiated by the addition of enzyme. Wild-type T4 DNA polymerase was assayed at 4 nM, the D324A-DNA polymerase at 2 μM, and the Y320F/D324A-DNA polymerase at 1 μM. Reactions were incubated at 37 °C.

Reaction products were separated by running samples in 15% or 20% polyacrylamide, 8 M urea denaturing gels. Exonuclease activity was measured spectrophotometrically using the experimentally determined molar extinction coefficient at 280 nm of 1.49 × 10^4 M^−1 cm^−1 (5).
Table I

| DNA polymerase     | Relative polymerase activity | Relative 3' → 5' exonuclease activity |
|--------------------|------------------------------|--------------------------------------|
| Wild-type          | 1.0                          | 1.0                                  |
| D324A              | 0.06                         | 2 × 10⁻⁵                             |
| Y320F/D324A        | 0.15                         | 1 × 10⁻⁴                             |

Hydrolysis Reaction Catalyzed by T4 DNA Polymerase

DNA polymerase and exonuclease assays are described under "Experimental Procedures." Both assays were done in buffer containing 67 mM Tris-HCl (pH 8.8), 16.7 mM (NH₄)₂SO₄, 0.5 mM dithiothreitol, 6.7 mM MgCl₂, and 167 μg/ml bovine serum albumin. For the DNA polymerase assay, 83 μM dNTPs (one [³²P]dNTP) and 833 μM (nucleotide equivalents) "activated" DNA were present. For the exonuclease activity, dNTPs were omitted and the DNA substrate was alkali-denatured, ³²P-labeled E. coli DNA.

determined by measuring the populations of degradation products with a PhosphorImager (Molecular Dynamics). The gels were analyzed by the method of Cheng and Kuchta (27), which considers full-length and all partially degraded oligomers as potential substrates. Band intensities were quantitated with the ImageQuant software supplied by Molecular Dynamics (Sunnyvale, CA). The mole fraction of each species was multiplied by the number of excision events required to generate that species. For the degradation of 5' ³²P-labeled p(dT)₁₆, the molar quantity of substrate degraded is given by \[ \text{mol of DNA} \times \text{(fraction 14-mer)} \times 1 + \text{(fraction 15-mer)} \times 2 + \ldots + (\text{mol of DNA}) \times (\text{mol of DNA substrate in the assay}). \]

3' → 5' Exonuclease Assay for the Klenov Fragment of E. coli DNA pol 1—Klenov fragment 3' → 5' exonuclease activity was examined under the assay conditions described above with the 5' ³²P-end-labeled p(dT)₁₆ substrate. The DNA substrate was present at 20 nm and Klenov fragment (Amersham Pharmacia Biotech) was at 75 nm.

RESULTS

DNA Polymerase Activity of Mutant T4 DNA Polymerases with Reduced 3' → 5' Exonuclease Activity—Ten-fold and greater reductions in polymerase activity were detected for the T4 D112A/E114A-, D219A-, and D324A-DNA polymerases in previous studies (6). The reduced polymerase activity observed for the T4 DNA polymerase exonuclease mutants indicates interdependence between polymerase and exonuclease activities as explained in Ref. 6. We confirmed our previous observation that DNA polymerase activity for the D324A-DNA polymerase is reduced (Table I). A similar or larger decrease in polymerase activity was predicted for the doubly mutant Y320F/D324A-DNA polymerase because the Y320F substitution also reduces polymerase activity (20). Surprisingly, the doubly mutant Y320F/D324A-DNA polymerase was more active in DNA polymerase assays than the singly mutant D324A-DNA polymerase. A possible explanation for this observation is presented under "Discussion."

3' → 5' Exonuclease Activity of Phage T4 D324A- and Y320F/D324A-DNA Polymers—An alanine substitution for residue Asp-324 reduced T4 DNA polymerase 3' → 5' exonuclease activity 4 orders of magnitude (Table I), as expected since the role of Asp-324 is in binding one of the two essential metal ions in the exonuclease active center (Fig. 3). The low level of exonuclease activity is due to residual rather than contaminating exonuclease activity, as shown by neutralizing antibody studies (20). An equal or larger reduction in exonuclease activity was expected for the doubly mutant Y320F/D324A-DNA polymerase, because the Y320F substitution alone reduces exonuclease activity about 100-fold (20). The Y320F/D324A-DNA polymerase, however, was 5-fold more active than the D324A-DNA polymerase (Table I). Thus, both higher polymerase and 3' → 5' exonuclease activities were detected for the doubly mutant Y320F/D324A-DNA polymerase compared with the singly mutant D324A-DNA polymerase. These observations suggest a pivotal function for residue Tyr-320. A model for Tyr-320 function is presented under "Discussion."

Effect of pH on the 3' → 5' Exonuclease Reaction Activity of Wild-type and Mutant T4 DNA Polymerases—pH titration of the 3' → 5' exonuclease activity was done using 5' ³²P-end-labeled p(dT)₁₆ as the DNA substrate (see "Experimental Procedures"). Exonucleolytic degradation of the labeled DNA was followed by gel electrophoresis of the partially degradedDNAs and by quantitation of the band intensities. Degradation of the 5' ³²P-end-labeled p(dT)₁₆ substrate by the wild-type T4 DNA polymerase is shown in Fig. 4. The use of single-stranded DNA substrates avoids the possibility of a pH effect on strand separation. The reactions were done at Vₘₐₓ conditions with the concentration of p(dT)₁₆ at 72 μM, which is approximately 10-fold higher than the Kₘ(app) (20). The high concentration of DNA ensures a constant population of enzyme-DNA complexes.

A high level of T4 DNA polymerase exonuclease activity was observed over the entire pH range examined, from pH 7.0 to 10.2, but the profile is complex, which suggests that the ionization states of several amino acid residues affect activity (Fig. 4). The pH titration profile for the exonuclease activity of T4 DNA polymerase differs from the pH titration reported for Klenov fragment in which a 45-fold stimulation in activity was observed at pH 10.2 compared with pH 7.5 (11).

The residual exonuclease activity detected for the mutant T4 D324A- and Y320F/D324A-DNA polymerases was also exam-
Hydrolysis Reaction Catalyzed by T4 DNA Polymerase

Fig. 4. Degradation of $^{32}$P-labeled p(dT)$_{16}$ by the $3' \to 5'$ exo-
quenecle activity of wild-type T4 DNA polymerase as a function of pH. Wild-type T4 DNA polymerase was present at 4 nM, and $^{32}$P-
labeled p(dT)$_{16}$ was at 72 μM. Panel A, lane 1 contains undegraded $[^{32}P]p(dT)_{16}$; lanes 2–5 contain degradation products from 1-min re-
actions at pH 7.0, 7.4, 7.9, and 8.7 in Tris-HCl buffer; lanes 6–10 contain de-
gradation products from 1-min reactions at pH 8.7, 9.2, 9.5, 9.9, and 10.2 in Gly-NaOH buffer. Panel B, quanti-
tation of the extent of degradation was done as described under “Ex-
perimental Procedures.” Samples were analyzed after 30 s (black symbols) and 1 min (gray symbols) of reaction. Tris-HCl buffer from pH 7.0 to 8.7 is indicated by squares ($\square$), and Gly-NaOH buffer is indicated by triangles ($\triangle$). Degradation rates are normalized to picomoles of substrate degraded per 20 pmol of enzyme in order to allow ready comparison of the wild-type T4 DNA polymerase to the exo-nuclease-deficient T4 DNA polymerases. The pH values in panel B are positioned below the corresponding gel lanes in panel A.

Fig. 5. Degradation of $^{32}$P-labeled p(dT)$_{16}$ by the D324A-DNA polymerase as a function of pH. The D324A-DNA polymerase was present at 2 μM, and $^{32}$P-labeled p(dT)$_{16}$ was at 72 μM. Panel A, lane 1 contains undegraded $[^{32}P]p(dT)_{16}$; lanes 2–5 contain degradation products from 10-min reactions at pH 7.0, 7.4, 7.9, and 8.7 in Tris-HCl buffer; lanes 6–10 contain degradation products from 10-min reactions at pH 8.7, 9.2, 9.5, 9.9, and 10.2 in Gly-NaOH buffer. Panel B, quanti-
tation of the extent of degradation was done as described under “Ex-
perimental Procedures.” Samples were analyzed after 10 min (black symbols) and 20 min (gray symbols) of reaction. Tris-HCl buffer from pH 7.0 to 8.7 is indicated by squares ($\square$), and Gly-NaOH buffer is indicated by triangles ($\triangle$). Degradation rates are normalized to picomoles of substrate degraded per 20 pmol of enzyme in order to allow ready comparison of the wild-type T4 DNA polymerase to the exo-nuclease-deficient T4 DNA polymerases. The pH values in panel B are positioned below the corresponding gel lanes in panel A.

Effect of Buffer on the Residual Exonuclease Activity of the
D324A-, D219A-, and D112A/E114A-DNA Polymerases—The
residual $3' \to 5'$ exonuclease activity of the mutant enzymes may be due to a low amount of fortuitous binding of metal ions in the exo-nuclease active center, despite the absence of the metal-binding aspartate ligands. Another possibility is that reaction components, such as the buffer, may participate in the exo-nuclease reaction. Tris, for example, has a potentially reac-
tive primary amine, and Tris may also act as a nucleophile. Undesirable side effects of Tris have been described (28). To in-
vestigate the possibility of a buffer effect, the exo-nuclease

activity of the mutant DNA polymerases was measured in the
presence of another buffer, HEPES (Fig. 7). The exo-nuclease
activity of the D324A-DNA polymerase was reduced more than
10-fold in HEPES compared with Tris buffer, but only a 2-fold
reduction was observed for the D219A- and D112A/E114A-
DNA polymerases (Table III). The effect of Tris on the exo-
nuclease activity of the D324A-DNA polymerases was propor-
tional to the concentration of Tris buffer present; about twice as
much activity was detected with 50 mM Tris compared with 25 mM Tris (Fig. 7).

Effect of pH on the $3' \to 5'$ Exonuclease Reaction Catalyzed by
Klenow Fragment—Because the $3' \to 5'$ exonuclease activity of
T4 DNA polymerase was reduced at pH 10.2, rather than being
strongly stimulated as reported for Klenow fragment (11), the
pH dependence of the exonuclease reaction catalyzed by Kle-
now fragment was re-examined. Surprisingly, instead of the
expected 45-fold stimulation in 3$'$ to 5$'$ exonuclease activity at
pH 10.2 (11), exonuclease activity was gradually reduced from
pH 8.7 to pH 10.2 (Fig. 8). The $k_{cat}$ at pH 10.2 in our assay was
about 0.07 s$^{-1}$, which is similar to the 0.09 s$^{-1}$ rate reported for
Klenow fragment with a poly(dT) substrate at pH 7.5 (11). Differ-
ces in the reaction conditions between our studies and
those of Derbys rifle et al. (11) may account for the absence of a
strong pH dependence at pH 10.2 for the Klenow fragment in
our reactions. Alkaline conditions in general, however, are
expected to reduce exonuclease activity because Mg$^{2+}$ is less soluble at higher pH. Thus, exonuclease activity, which requires divalent metal ions, is expected to decline as the concentration of Mg$^{2+}$ ions is depleted.

**Fig. 6. Degradation of $^{32}$P-labeled pdT$_{16}$ by the Y320F/D324A-DNA polymerase as a function of pH.** The Y320F/D324A-DNA polymerase was present at 1 μM, and $^{32}$P-labeled pdT$_{16}$ was at 72 μM. Panel A, lane 1 contains undegraded $[^{32}$P]pdT$_{16}$; lanes 2–5 contain degradation products from 10-min reactions at pH 7.0, 7.4, 7.9, and 8.7 in Tris-HCl buffer; lanes 6–10 contain degradation products from 10-min reactions at pH 8.7, 9.2, 9.5, 9.9, and 10.2 in Gly-NaOH buffer. Panel B, quantitation of the extent of degradation was done as described under "Experimental Procedures." Samples were analyzed after 10 min (black symbols) and 20 min (gray symbols) of reaction. Tris-HCl buffer from pH 7.0 to 8.7 is indicated by squares (■), and Gly-NaOH buffer is indicated by triangles (▲). Degradation rates are normalized to picomoles of substrate degraded per 20 pmol of enzyme in order to allow ready comparison of the wild-type T4 DNA polymerase to the exonuclease-deficient T4 DNA polymerases. The pH values in panel B are positioned below the corresponding gel lanes in panel A.

**TABLE II**

3′ → 5′ exonuclease activities for phage T4 wild-type and mutant D324A- and Y320F/D324A-DNA polymerases determined by digestion of pdT$_{16}$

| DNA polymerase | pH | $k_{cat}$  $s^{-1}$ |
|----------------|----|-----------------|
| Wild-type      | 7.9| 200             |
| D324A          | 9.5| $1 \times 10^{-2}$ |
| Y320F/D324A    | 8.7| $1 \times 10^{-1}$ |

**DISCUSSION**

The hydrolysis reaction catalyzed by bacteriophage T4 DNA polymerase—The hydrolysis reaction in the multistep T4 DNA polymerase exonucleolytic-proofreading pathway was studied by using mutant DNA polymerases with amino acid substitutions for conserved residues in the exonuclease active center. The EoloI motif residues, Tyr-320 and Asp-324, were changed to Y320F and D324A. The EoloI and EoloII motif resi-
TABLE IV

| DNA polymerase | Metal ion A | Metal ion B |
|---------------|-------------|-------------|
| D112A/E114A   | +/−         | +/−         |
| D219A         | +           | +/−         |
| D324A         | +/−         | −           |

Hydrolysis Reaction Catalyzed by T4 DNA Polymerase

The metal binding activities of the D112A/E114A-, D219A-, and D324A-DNA polymerases are adapted from the proposals by Sattar et al. (20), which are based on structural comparisons between the exonuclease active center of the T4 DNA polymerase and E. coli DNA pol I, and are supported by equilibrium Mg2+ binding studies by Beechem et al. (31). The “+” symbol indicates that the metal site is expected to be occupied with a divalent metal ion. The “−” symbol indicates that the metal site is expected to be vacant. The “+/−” symbol indicates that the metal site may retain some ability to bind a metal ion and partial or transient occupancy is expected.

Dues, Asp-112/Glu-114 and Asp-219, were changed to D112A/E114A and D219A. Mutant DNA polymerases with alanine substitutions for Asp-112, Asp-219, or Asp-324 have 103- to 105-fold less exonuclease activity than the wild-type level (Refs. 5–7 and 20; Tables II and III). Studies of the residual exonuclease activity detected for the mutant DNA polymerases have revealed insights into the metal ion-dependent step(s) in the hydrolysis reaction.

Analyses of experiments with the D112A/E114A-, D219A-, D324A-, and Y320F/D324A-DNA polymerases assume that only metal ion binding and not protein structure is changed by the amino acid substitutions. Structural alterations were not detected for E. coli DNA pol I mutants with analogous substitutions (29). For T4 DNA polymerase mutants, a fluorescence assay provides information on the structural integrity of the exonuclease active center. A fluorescent pre-exonuclease complex is formed with the wild-type T4 DNA polymerase and DNA labeled with 2-aminopurine at the 3′-terminal position of the primer strand (2–4, 30). Formation of the fluorescent pre-exonuclease complex is also observed for the D324A- and Y320F/D324A-DNA polymerases, and formation is observed with the same efficiency as detected for the wild-type enzyme (30). Thus, the D324A substitution does not appear to impede DNA binding in the exonuclease active center. The D112A/E114A and D219A substitutions also do not affect the rate for forming fluorescent pre-exonuclease complexes, but the steady-state level of the fluorescent complex is reduced (30). The higher residual exonuclease activities of the D112A/E114A- and D219A-DNA polymerases compared with the D324A-DNA polymerase (Table III), however, indicate that the differences detected by fluorescence intensity measurements may reflect only subtle changes in structure.

Metal ion occupancy in the exonuclease active center of the mutant T4 DNA polymerases has been predicted by comparisons to structural studies of Klenow mutants with alanine substitutions for metal binding residues (20). The metal ion B site is predicted to be vacant in the D219A-DNA polymerase, and the metal ion A site is predicted to be vacant in the D324A-DNA polymerase (Table IV). Since residue Asp-112 may provide ligands to both metal ion A and B, little or only partial metal ion occupancy is predicted at either site. This metal-binding pattern is supported by equilibrium Mg2+ binding studies (31). Two classes of Mg2+ binding sites were detected: a high affinity site, Ka about 5 μM for the wild-type T4 DNA polymerase, and a low affinity site, Kb about 2 mM. Mg2+ binding in the high affinity metal binding site, but not the low affinity site, is affected by alanine substitutions for residues Asp-112, Asp-219, and Asp-324 (31). The Mg2+ dissociation constants determined for the D219A- and D324A-DNA polymerases are similar at 11 and 27 μM, respectively, but apparent 10-fold weaker binding, Ka = 225 μM, is detected for the D112A/E114A-DNA polymerase. These data are consistent with a single metal ion bound in the exonuclease active centers of the D219A- and D324A-DNA polymerases and a severe reduction in binding of both metal ions by the D112A/E114A-DNA polymerase.

The first step in the proposed two-metal ion model for the DNA polymerase hydrolysis reaction is formation of an attacking metal hydroxide ion by metal ion A (Fig. 1). If residues that bind metal ion A are missing, then any residual exonuclease activity is expected to derive from hydroxide ions produced by interactions between water molecules and between water molecules and the buffer. The contribution of buffer to the residual exonuclease activity was assessed by using HEPES in place of the potentially reactive and interactive Tris (Fig. 7, Table III). The residual exonuclease activity of the D324A-DNA polymerase was reduced more than 10-fold in HEPES compared with Tris buffer, and 2-fold reductions were detected for the D112A/E114A- and D219A-DNA polymerases. The stronger buffer effect observed for the D324A-DNA polymerase indicates a role for residue Asp-324 and metal ion A in producing hydroxide ions.

The role of residue Asp-324 and metal ion A in producing hydroxide ions is supported by studies of the residual exonuclease activity of the D219A- and D112A/E114A-DNA polymerases. The highest level of exonuclease activity was observed for the D219A-DNA polymerase (Table III). This mutant is predicted to retain metal ion binding in site A and, thus, according to the model, still has the potential to generate an attacking metal-hydroxide ion. If production of a metal-hydroxide ion is rate-limiting, then this mutant is expected to have higher activity, which is observed.

Residue Asp-112 is also predicted to provide a ligand to metal ion A, but some metal binding in site A may be possible in the D112A/E114A-DNA polymerase due to residue Asp-324 (Table IV). No metal A binding is predicted, however, for the D112A/D324A-DNA polymerase, which has lost both carboxylate ligands to metal ion A. In keeping with this proposal, 10-fold less residual activity is detected for the D112A/D324A-DNA polymerase compared with the D112A/D219A- or D219A/D324A-DNA polymerase (20). The D112A/D219A- and D219A/D324A-DNA polymerases also have the same amount of residual exonuclease as the singly mutant D112A- and D324A-DNA polymerases. Thus, the largest reduction in hydrolysis activity is observed only when both ligands to metal ion A, Asp-112 and Asp-324, are removed. These experiments (20) were done in Tris buffer; hence, a larger reduction in residual exonuclease activity is predicted for the D112A/D324A-DNA polymerase in HEPES buffer. The proposed partial occupancy of metal site A caused by the D112A substitution differs from structural studies of the D355A/E357A-Klenow fragment in which no metal binding was observed in metal sites A or B (Fig. 1; Ref. 28). Structural studies, however, may not have sufficient sensitivity to detect partial or transient metal ion binding.

Additional residues in the exonuclease active center of the T4 DNA polymerase appear to assist the hydrolysis reaction. A comparison to carboxypeptidase A, a zinc metalloenzyme, is informative (32). A bell-shaped pH-rate profile is observed for the peptide bond cleaving reaction catalyzed by carboxypeptidase A with inflection points at pH 6.1 and 9.0, which suggests the importance of both acidic and basic catalysis. Hydrogen bonding between a zinc-coordinated water molecule and a nearby glutamate residue in the active center is proposed to reduce the pK of the coordinated water from pH 10 to 9 (31, 32).
The model for formation of a metal hydroxide ion by T4 DNA polymerase requires both oxygens of Asp-324 (Fig. 9). Mutational analysis indicates that both oxygens are required for exonuclease activity of the T4 DNA polymerase (20), but not for E. coli DNA pol I (11). DNA pol I also lacks a lysine residue that corresponds to T4 Lys-299 or RB69 Lys-302 (22). These differences could provide part of the explanation for why the T4 DNA polymerase has about 1000-fold more exonuclease activity than DNA pol I (33). The proposed role for Lys-299 in the T4 DNA polymerase to increase the electronegativity of the metal-binding carboxylate oxygen of residue Asp-324 is also consistent with Asp-324 being the single most important residue in the hydrolysis reaction catalyzed by the T4 DNA polymerase.

Residue Tyr-320 in T4 DNA Polymerase Affects DNA Binding in the Exonuclease Active Center—

Residues Tyr-320 in T4 DNA polymerase and Tyr-497 in Klenow fragment appear to play secondary roles in the exonuclease reaction since alanine or phenylalanine substitutions for these residues reduce exonuclease activity less than alanine substitutions for any of the conserved aspartate residues required for metal binding (11, 20). Structural studies of the T4-like RB69 DNA polymerase suggest that the phenolic side chain of the conserved tyrosine is in position in the transition state to orient the attacking water molecule or hydroxide ion (Fig. 1; Refs. 12 and 22). Thus, we anticipated that the doubly mutant Y320F/D324A-T4 DNA polymerase would have similar or less exonuclease activity than the singly mutant D324A-DNA polymerase. Surprisingly, the 3′→5′ exonuclease activity of the Y320F/D324A-DNA polymerase was 10-fold more active than the exonuclease activity of the D324A-DNA polymerase (Table II, Figs. 5 and 6).

The phenolic group of the conserved tyrosine could reduce activity by inhibiting catalysis or by reducing DNA binding in the exonuclease active center. Experiments that measured partitioning of DNA between the polymerase and exonuclease active centers of Klenow fragment found that Tyr-497, which corresponds to residue Tyr-320 in the T4 DNA polymerase, affects DNA binding in the exonuclease active center (35). Increased partitioning of DNA to the exonuclease active center was observed for the Y497A/D424A-Klenow mutant compared with the D424A-Klenow mutant. To reconcile the seemingly contradictory observations that an alanine substitution for Tyr-497 in Klenow fragment can both reduce exonuclease activity, but increase partitioning of DNA to the exonuclease active center, Lam et al. (35) suggest that Tyr-497 may affect different binding conformations in the exonuclease active center. Thus, residue Tyr-497 in Klenow may interfere with the initial binding mode, but may assist in reorienting the DNA substrate to a catalytically optimal conformation. The T4 D324A- and Y320F/D324A-DNA polymerases, however, form the fluorescent pre-exonuclease complex with apparent equal efficiency.2 Thus, residue Tyr-320 must affect a reaction step that follows formation of the pre-exonuclease complex, but before hydrolysis in order to explain the higher exonuclease activity of the T4 Y320F/D324A-DNA polymerase compared with the D324A-DNA polymerase.

Interdependence of Polymerase and 3′→5′ Exonuclease Activities of T4 DNA Polymerase—Unlike Klenow fragment, many amino acid substitutions in the T4 DNA polymerase that reduce exonuclease activity also reduce polymerase activity (6, 20). Although the reductions in polymerase activity are small compared with the reductions in exonuclease activity (Table I), the reduced polymerase activity demonstrates the interdependence between reactions catalyzed in the polymerase and exonuclease active centers. Such interdependence is expected for a DNA polymerase, like T4 DNA polymerase, which alternates between polymerase and exonuclease activities without dissociating from the DNA primer-template (36). One explanation for the decrease in polymerase activity detected for the exonuc-

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**Fig. 9. Proposed two-metal ion enzymatic mechanism for the hydrolysis reaction catalyzed by T4 DNA polymerase.** The model presented for T4 DNA polymerase is adapted from the model proposed by Beese and Steitz (12) for E. coli DNA pol I (see Fig. 1). A key feature of the hydrolysis reaction by the bacterial and phage enzymes is a metal-hydroxide ion, formed by metal ion A, which is oriented to attack the phosphodiester bond at the site of cleavage. An important difference between the bacterial and phage enzymes is the augmented role of residue Asp-324 in the T4 DNA polymerase compared with residue Asp-501 in E. coli DNA pol I.
cleave-deficient mutants is that reduced ability to bind DNA in
the exonuclease active center may result in dissociation. Amino
acid substitutions that can restore processivity by assisting
DNA binding, as suggested for the Y320F substitution, can
increase both the polymerase and exonuclease activities of
the D324A-DNA polymerase (Table 1).

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