The Mitochondrial DNA Polymerase β from Crithidia fasciculata Has 5'-Deoxyribose Phosphate (dRP) Lyase Activity but Is Deficient in the Release of dRP*

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DNA polymerase β (pol β) has long been described as a nuclear enzyme involved in DNA repair. A pol β from the trypanosomatid parasite Crithidia fasciculata, however, is the first example of a mitochondrial enzyme of this type. The mammalian nuclear enzyme functions not only as a nucleotidyl transferase but also has a dRP lyase activity that cleaves 5'-deoxyribose phosphate (dRP) groups from DNA, thus contributing to two consecutive steps of the base excision repair pathway. We find that the mitochondrial pol β also has dRP lyase activity. Interestingly, the $K_m$ of this enzyme for a dRP-containing substrate is similar to that for the rat enzyme, but its $k_{cat}$ is very low. This difference is due to a deficiency of the mitochondrial enzyme in the release of dRP from the enzyme following its cleavage from the DNA.

Crithidia fasciculata is a parasitic protist belonging to the order Kinetoplastida. This order also includes Trypanosoma brucei, Trypanosoma cruzi, and Leishmania sp., all of which are important human pathogens. C. fasciculata is an ideal model organism for these pathogens, especially for biochemical studies. The kinetoplasts are characterized by an unusual mitochondrial DNA, called kinetoplast DNA or kDNA. This structure contains thousands of topologically interlocked DNA minicircles condensed in vivo into a disk-shaped structure situated within the matrix of the cell's single mitochondrion near the base of the flagellum. For a review of kDNA see Ref. 1.

The mechanism by which the kDNA network is replicated has been explored for many years (see Refs. 2 and 3 for reviews). Several laboratories, including ours, have purified a number of C. fasciculata proteins involved in this process. The characterization of these proteins, both in terms of their enzymatic activities and their intramitochondrial locations, has provided considerable insight into how replication of this network occurs. Briefly, minicircles are released from the network into the kinetoflagellar zone, the space between the kDNA disk and the mitochondrial membrane near the basal body of the flagellum (4). Here they encounter DNA primase (5), universal minicircle sequence binding protein (an origin binding protein) (6), and two pol I-like DNA polymerases (7). Because replication is unidirectional, the newly replicated minicircles include one sister with a single gap (the product of leading strand synthesis) and another with multiple gaps (the result of lagging strand synthesis). It is unclear whether replication is completed in the kinetoflagellar zone, but eventually newly replicated minicircles migrate to discrete sites flanking the kDNA disk, called the antipodal sites. At the antipodal sites, minicircles encounter SSE1 (8), an enzyme with RNase H activity that is believed to remove the RNA primers (9), and DNA polymerase β (pol β) (10), an enzyme thought to fill in many of the gaps. Once this processing is complete, the newly replicated minicircles are reattached to the periphery of the network by a topoisomerase II (11) also located at the antipodal sites (12). Ultimately, the remaining gaps are repaired and the doublesized network splits in two for segregation into daughter cells.

The C. fasciculata pol β, the first mitochondrial enzyme of its type (13), is small, non-processive, error-prone, and not likely to be the replicative polymerase (14). This protein has a short N-terminal mitochondrial targeting signal sequence resembling other targeting signals in C. fasciculata and related parasites (15), and this signal is cleaved upon import of the protein into the mitochondrion (16). Interestingly, the protein is only detectable in the antipodal sites while the kDNA is undergoing replication. During the remainder of the cell cycle, pol β is undetectable by immunofluorescence, although Western blot analysis on synchronized cultures indicates that protein level does not change (17). This finding raises the possibility that the pol β may have some role other than gap filling during replication.

In higher eukaryotes, pol β is found in the nucleus and involved primarily in base excision repair. Enzymes in this pathway recognize and correct damage such as misincorporated uracil and oxidized or alkylated bases. The damaged base is removed by a specific DNA glycosylase, leaving the sugar phosphate backbone intact and creating an abasic site (AP site). An AP endonuclease cleaves 5' of the AP site leaving a 3'-OH competent for nucleotide addition and a 5'-deoxyribose phosphate (dRP) moiety. The dRP group is removed by a dRP lyase, creating a single nucleotide gap that is filled in by a DNA polymerase. Finally, a DNA ligase seals the repaired strand. The mammalian nuclear pol β, in addition to its nucleotidyl transferase activity, catalyzes the dRP lyase reaction of the pathway (18). Thus, we were compelled to examine whether the C. fasciculata pol β also possesses this lyase activity, which might suggest a role for this protein in mitochondrial DNA repair.
Expression and Purification of Recombinant Proteins—For expression of the full-length rat nuclear pol β in Escherichia coli, we used the vector pRSETBpol (18). Expression and purification were performed as previously described (19) with the exception of the order of columns being DEAE-cellulose, phosphocellulose, SP-Sepharose (Amersham Biosciences), and then ssDNA-cellulose (Amersham Biosciences). The final fraction from ssDNA-cellulose dialyzed against buffer A (50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), and concentrated in a Vivaspin 6 concentration device (Sartorius, 10000 MWCO). This purification yielded 8.7 mg of protein. Glycerol was added to 50%, and the protein was stored at −20 °C. Fig. 1 (lane 1) shows the preparation analyzed by SDS-PAGE and stained with Coomassie Blue. We assayed for polymerase activity in a 25-μl reaction containing 150 mM Tris-HCl (pH 9.0), 5 mM MgCl2, 0.1 mg/ml bovine serum albumin, 100 μM [α-32P]dTTP (400 Ci/mmol), and a poly(dA): oligo(dT)19 substrate (where the (dT)19 primer was 5’-phosphorylated, dA nucleotide was in 2-fold molar excess over dT, and the concentration of the primer 3’-ends was 1 μM) for 10 min at 37 °C. Reaction mixtures were spotted onto DE81 filters (Whatman) that were then washed exhaustively in 0.5 M sodium phosphate (pH 7.1), washed in 80% ethanol, dried, and counted in a scintillation counter. The specific activity of this enzyme under these conditions was 1020 units/mg of protein where a unit is defined as the incorporation of 1 nmol of dTMP in 1 min. Although the buffer conditions are different, this value is similar to that previously published (20).

The rat coding sequence was excised from the vector using Ndel and Scal and replaced with the coding sequence for the C. fasciculata pol β lacking the first 487 amino acid mitochondrial targeting signal sequence (underlined in Fig. 2). After electroporation into E. coli BL21(DE3), expression of the untagged protein in a 1.5-liter culture was induced overnight at room temperature with 0.5 mM isopropyl-1-thio-

G-1-β-D-galactopyranoside. The cells were centrifuged, resuspended in 15 ml of buffer B (50 mM Hepes (pH 7.6), 5 mM EDTA, 25 mM KCl, 1 mM phenylmethylsulfonyl fluoride) supplemented with 200 μg/ml lysozyme (Sigma), incubated on ice for 30 min, and lysed using a Dounce homogenizer with a tight-fitting pestle. The lysate was centrifuged (30,000 g, 15 min, 4 °C), and the supernatant was loaded onto a 40-ml DEAE-cellulose column in buffer B. The column was washed with 100 ml of buffer B, and the combined wash and flowthrough fractions were applied to a 10-ml SP-Sepharose column equilibrated with buffer B. The column was washed with 50 ml of buffer B and eluted with a 100-ml gradient to 0.6 M KCl in buffer B. Fractions containing pol β (0.18–0.22 M KCl) were pooled, dialyzed into buffer B, and concentrated in a Vivaspin 6 concentration device (Sartorius, 10000 MWCO). This purification yielded 0.7 mg of protein. Glycerol was added to 50%, and the protein was stored at −20 °C. Fig. 1 (lane 2) shows the preparation analyzed by SDS-PAGE. The specific activity of the enzyme under these conditions was 0.27 units/mg of protein. The K72A mutation affects the rat coding sequence was excised from the vector using NdeI and ScalI and replaced with the coding sequence for the C. fasciculata pol β lacking the first 487 amino acid mitochondrial targeting signal sequence (underlined in Fig. 2). After electroporation into E. coli BL21(DE3), expression of the untagged protein in a 1.5-liter culture was induced overnight at room temperature with 0.5 mM isopropyl-1-thio-

G-1-β-D-galactopyranoside. The cells were centrifuged, resuspended in 15 ml of buffer B (50 mM Hepes (pH 7.6), 5 mM EDTA, 25 mM KCl, 1 mM phenylmethylsulfonyl fluoride) supplemented with 200 μg/ml lysozyme (Sigma), incubated on ice for 30 min, and lysed using a Dounce homogenizer with a tight-fitting pestle. The lysate was centrifuged (30,000 g, 15 min, 4 °C), and the supernatant was loaded onto a 40-ml DEAE-cellulose column in buffer B. The column was washed with 100 ml of buffer B, and the combined wash and flowthrough fractions were applied to a 10-ml SP-Sepharose column equilibrated with buffer B. The column was washed with 50 ml of buffer B and eluted with a 100-ml gradient to 0.6 M KCl in buffer B. Fractions containing pol β (0.18–0.22 M KCl) were pooled, dialyzed into buffer B, and concentrated in a Vivaspin 6 concentration device (Sartorius, 10000 MWCO). This purification yielded 0.7 mg of protein. Glycerol was added to 50%, and the protein was stored at −20 °C. Fig. 1 (lane 3) shows the preparation analyzed by SDS-PAGE. The specific activity of the enzyme under these conditions was 0.27 units/mg of protein. Although this value is significantly lower than that for the rat enzyme, it is comparable to that of the native protein purified from C. fasciculata (Fraction 10, see Ref. 13) on the same substrate (data not shown).

Site-directed Mutagenesis—To generate the K72A mutant of the C. fasciculata mitochondrial pol β, we performed PCR-based site-directed mutagenesis (21) using primers K72A(F) (5′-AGCTGTGAGCATCCGCACGACGAACT) and K72A(R) (5′-TCTCCGGCCGTCTTCAGCACGGTAGTATG) and K72A(E) (5′-CTGAAGGCGCCCGAGGAGATTATG) and K72A(C) (5′-TCCGCGCCGTCTTCAGCACGGTAGATG) to introduce the mutation. These primers introduced a new HaeIII restriction site, and clones were initially screened by HaeIII digestion. Positive clones were later verified by sequencing. The coding sequence was cloned into pRSET, the vector was electrocoverted into BL21(DE3) cells, and expression and purification were done as described for the wild-type protein above, except for one chromatography step. Between the SP-Sepharose column and the phosphocellulose column, the sample was applied to a 5-ml Affi-Gel Blue (Bio-Rad) column in buffer B. The column was eluted with a step gradient, and the mutant protein eluted between 0.4 and 0.6 M KCl in buffer B. This purification yielded 1.4 mg of protein, and circular dichroism spectroscopy verified that the mutation did not affect protein folding (data not shown). Fig. 1 (lane 3) shows the preparation analyzed by SDS-PAGE. The specific activity of this protein in the polymerase assay was 2.5 units/mg of protein. The K72A mutation affects $K_\text{m}$ and not $V_\text{max}$ (data not shown).

Oligonucleotides—For dRP lyase assays and Schiff base traps, a substrate consisting of B1 (5′-UAAGCAATTGATATCGATGCTAATTAGC), and the complementary B3 (5′-AGCTAGTTCGACATGCTAATTAGC) was used. For base excision repair assays, a substrate consisting of U1 (5′-CGTGGCCCGGCGCTGGT) and the complementary U2 (5′-GCCACGGCGCACGGCGGAC) was used.

Preparation of Substrate—To prepare substrate for dRP lyase assays, 5 nmol of B2U oligonucleotide (a 28-mer with a 5′ U residue) was 5′-labeled by 25 units of T4 polynucleotide kinase (New England BioLabs) in a reaction containing 0.7 μM [γ-32P]ATP (3000 Ci/mmol) under standard conditions. After incubation overnight at 37 °C, non-radioactive ATP (final concentration 0.625 mM) and another 25 units of enzyme were added, and the mixture was incubated at 37 °C for an additional 2 h. The enzyme was heat-inactivated, and the oligonucleotide was phenol:chloroform:isoamyl alcohol (25:24:1, v/v) extracted, and then purified over two successive G-25 spin columns (Amersham Biosciences). The 32P-labeled product was then ethanol-precipitated overnight at −20 °C, and resuspended in 40 μl of H2O. A small fraction of the final product and of the reaction mixture prior to phenol:chloroform:isoamyl alcohol extraction was run on a 20% polyacrylamide/7 M urea gel, and the final concentration of the product was determined by densitometry of the labeled bands using phosphorimaging (Fuji). Oligonucleotides B1 and B3 were then labeled to B2 to the same final concentration in 10 mM KCl. The mixture was heated to 95 °C for 3 min and then cooled slowly to room temperature to anneal. The resulting substrate was analyzed on a non-denaturing 20% polyacrylamide gel to verify that it was double-stranded.

dRP Lyase Assays—The substrate consisting of oligonucleotides B1, 32P-labeled B2U, and B3 was pre-treated with E. coli uracil N-glycosylase (New England BioLabs) at 37 °C in a reaction containing 50 mM Hepes (pH 8.0), 1 mM EDTA, and 1 mM DTT. Lyase assays (10 μl) contained 50 mM Hepes (pH 7.6) and 5 mM MgCl2 with oligonucleotide and enzyme concentrations as indicated in the figure legends. Where necessary, enzymes were diluted into 50 mM Hepes (pH 7.6) immediately before addition to the reaction. Reaction components were mixed on ice and then incubated at 25 °C for 10 min, except reactions for kinetic determinations, which were incubated at 37 °C for 10 min. Reactions were quenched with 1 μl of 2% SDS, and then the products were reduced by addition of NaBH4 to a final concentration of 45 μM. After 20 min at 25 °C, 1 μl of the reaction was spotted onto a 10-cm PEI-cellulose thin layer chromatography plate that was then developed with 1.0 M formic acid. The plates were dried and exposed to x-ray film. Based on the autoradiograms, spots corresponding to the free dRP derivative and the origin were cut out and counted in a scintillation counter.

Schiff Base Traps—Schiff base traps were similar to dRP lyase assays, except that following addition of NaBH4 the products were resolved by SDS-PAGE (10% gel). Gels were stained with Coomassie Blue to verify equal protein loading, dried onto Whatman 3MM paper, and autoradiographed.
Base Excision Repair Assays—These assays were carried out essentially as described previously (22) in a 10-μl reaction containing 50 mM Hepes (pH 7.6), 1 mM EDTA, 1 mM DTT, 100 μg/ml bovine serum albumin, 4 mM ATP, 55 mM α-32P-ATP (3000 Ci/mmol), and 1.5 μM uracil-containing oligonucleotide (U1/A2). E. coli uracil N-glycosylase (0.025 unit) was added, and reactions were preincubated at 37 °C for 10 min. E. coli AP endonuclease IV (0.025 unit, Trevigen), pol β (150 nm), and T4 DNA ligase (0.025 unit, Roche Molecular Biochemicals) were added, and reactions were incubated on ice for 10 min. Finally, MgCl2, which is required for both AP endonuclease and DNA polymerase activity, was added, and the reactions were incubated at 37 °C for 15 min. Reactions were diluted 1:100 into formamide gel loading buffer, boiled for 3 min, and placed on ice for 5 min. A small amount (4 μl) of each mixture was loaded onto a 20-cm 20% polyacrylamide/7 M urea gel and run for 2 h at 600 V at room temperature. Gels were wrapped and frozen at −80 °C, and the products were analyzed by autoradiography.

RESULTS

Conservation of Residues Critical for dRP Lyase Activity—The rat nuclear pol β can carry out the dRP lyase reaction, and the N-terminal 8 kDa of the protein is both necessary and sufficient for this step (18). Site-directed mutagenesis experiments have identified amino acid residues critical for this activity. Lysines at positions 35, 60, and 68 (Fig. 2, asterisks) are important in the binding of ssDNA, and in addition, Lys-35 is essential for recognizing the 5′-phosphate group (25). Lysine 72 (Fig. 2, arrowhead) is the catalytic residue that forms the Schiff base with the substrate and facilitates β-elimination of the downstream DNA (23–25). Finally, tyrosine 39 (Fig. 2, filled circle) is important for the dRP lyase activity (25), and it has been suggested to promote proton transfer to the aldehyde upon attack of the catalytic lysine residue (26).

Alignment of the 8-kDa domain of the rat nuclear enzyme with the corresponding portion of the C. fasciculata mitochondrial pol β sequence (Fig. 2) shows that all of the essential amino acid residues mentioned above are conserved. This observation supports the hypothesis that the mitochondrial pol β may possess dRP lyase activity.

Schiff Base Trap of the Mitochondrial Pol β—The dRP lyase reaction proceeds through multiple steps (Fig. 3). Briefly, the 5′-dRP group exists in equilibrium with the ring-open aldehyde form of the sugar. This aldehyde undergoes nucleophilic attack by the catalytic lysine residue (Lys-72) of the protein (Step 1), resulting in formation of a Schiff base and yielding a covalent

![Diagram of dRP Lyase Activity](image)

**Fig. 2.** Residues important for dRP lyase activity are conserved. The N-terminal 8-kDa domains of the rat and C. fasciculata pol β enzymes were aligned with the corresponding portion of the mitochondrial pol β from C. fasciculata. The asterisks indicate lysine residues important for DNA binding in the mammalian enzyme. The filled circle indicates the tyrosine residue essential for dRP lyase activity. The arrowhead indicates the lysine implicated in formation of the Schiff base. Identical residues are highlighted in gray. Glutamate residues suggested to contribute a carboxyl group as a general acid in the rat enzyme are boxed. The mitochondrial signal sequence for the C. fasciculata enzyme, which is cleaved upon import into the mitochondrion (16), is underlined.

![Diagram of dRP Lyase Activity](image)

**Fig. 3.** Mechanism of dRP removal. This figure was adapted from Ref. 33. See text for description of the reaction mechanism.

![Diagram of dRP Lyase Activity](image)

**Fig. 4.** The mitochondrial pol β can be trapped as a Schiff base. 32P-Labeled dRP lyase substrate (10 pmol, lanes 1–7; 12.5 pmol, lanes 8 and 9) was incubated with no pol β (lanes 1 and 3), or 5 pmol of mitochondrial pol β (lanes 2, 4–6, and 8) or rat nuclear pol β (lane 7) for 10 min before NaBH4 was added and products were resolved by SDS-PAGE (10% gel). Uracil N-glycosylase was omitted (lane 4), substrate was treated with NaBH4 prior to addition of pol β (lane 5), the mitochondrial pol β was heat-inactivated (50 °C, 5 min) prior to incubation (lane 6), or the substrate was incubated with 5 pmol of K72A mutant protein (lane 9). The autoradiograph is shown in the top panels, and the corresponding Coomassie Blue-stained gel is shown in the bottom panels.
enzyme-DNA complex (E-DNA). A proton at the 2' position is abstracted (Step 2), possibly by a carboxyl group on the protein, and a subsequent β-elimination reaction leads to release of the 3'-DNA and generates an enzyme-dRP (E-dRP) complex. Finally, a hydrolysis step (Step 3), again possibly facilitated by a carboxyl group on the protein, releases an unsaturated dRP derivative and regenerates the protein for another round of catalysis.

To assess whether the C. fasciculata mitochondrial pol β has dRP lyase activity, we first examined whether Schiff base intermediates could be trapped by addition of NaBH₄. We incubated pol β with a 32P-labeled dRP-containing substrate, and after reduction, we observed a single radiolabeled band corresponding in size to the mitochondrial protein and representing the enzyme-dRP (E-dRP) intermediate (Fig. 4, lane 2). At longer exposures, the enzyme-DNA (E-DNA) complex can also be detected (see below, see Fig. 6B), but at steady state the bulk of the protein exists as the E-dRP intermediate. This band was absent when pol β was omitted (lane 1), if the uracil was not removed (lane 4), or if the substrate was reduced with NaBH₄ prior to addition of the pol β (lane 5). Heat inactivation of the mitochondrial pol β (50 °C, 5 min) prior to addition (lane 6) also abolished trapping, indicating that an appropriately folded protein is required. The rat nuclear protein was included (lane 7) as a positive control.

We also examined Schiff base formation in a mutant C. fasciculata mitochondrial pol β (K72A) in which the catalytic lysine at position 72 was replaced with an alanine. This mutant protein is deficient in forming the Schiff base intermediate (Fig. 4, lane 9), as has been found in similar studies with the mammalian protein (23, 25). The labeling of the K72A mutant is decreased 92% relative to that of the wild-type protein (lane 8) as determined by phosphorimaging. This decrease may be an underestimate given that there appears to be more Coomassie Blue-stained protein in the K72A lane. As mentioned earlier, the dRP lyase active site contains several lysine residues, and the residual labeling of mutant protein may be due to fortuitous nucleophilic attack from one of these other residues. To ensure that decreased labeling was not due to the protein's decreased ability to bind the substrate, we conducted a trapping experiment with the mutant protein in a reaction with 3-fold higher substrate concentration. There was no significant increase in the trapping of the mutant protein, and furthermore, PEI-cellulose of reaction products indicated that free unsaturated dRP was not generated above background levels for the K72A mutant (data not shown).

### Mitochondrial Pol β in Base Excision Repair

—We next examined whether the dRP lyase activity of the mitochondrial pol β was sufficient to allow the completion of a round of base excision repair. As a substrate we used a double-stranded 18-bp oligonucleotide (U1/U2) containing a U:A mismatch at position 3 (A) or 5 (B) for various times at 25 °C.

| Enzyme | $K_m$ (μM) | $k_{cat}$ (min⁻¹) | $k_{cat}/K_m$ (min⁻¹) |
|--------|-------------|-------------------|-----------------------|
| C. fasciculata mitochondrial pol β | 0.45 | 0.11 | 0.24 |
| Rat nuclear pol β | 0.92 | 4.12 | 4.48 |

Resolution of the E-DNA and E-dRP complexes by 10% SDS-PAGE is shown in the top panels. Minor species migrating faster than E-DNA could correspond to E-DNA complexes missing oligonucleotide components of the DNA substrate. Separation of free unsaturated dRP on PEI-cellulose thin layer chromatography is shown in the bottom panels. The origin contains unreacted substrate as well as the E-DNA and E-dRP intermediates. The B2U oligonucleotide preparation contained a small amount of contaminating oligonucleotide that did not contain a uracil at the 5'-end, accounting for the radioactivity that remains in the origin even after 10 min. A control reaction lacking pol β was incubated for 10 min at 25 °C prior to NaBH₄ addition and resolved on PEI-cellulose (C).
10. We incubated this substrate with *E. coli* uracil N-glycosylase, followed by addition of *E. coli* AP endonuclease IV, mitochondrial pol β, and T4 DNA ligase in a reaction containing [α-³²P]dTMP. When all components were included in the mixture, pol β incorporated the [³²P]dTMP into a 10-nucleotide intermediate, which was then ligated into a full-length 18-nucleotide product, indicating that the dRP had been removed (Fig. 5, lane 1). There was no incorporation if uracil N-glycosylase (lane 2), AP endonuclease (lane 3), or pol β (lane 4) were omitted, and when ligase was omitted (lane 5), the 10-nucleotide intermediate accumulated and was not converted to the full-length product.

To show that spontaneous cleavage of the dRP or a contaminant in any of the other proteins added could not account for the incorporation of [α-³²P]dTMP into the full-length ligated product, we conducted the same experiment using the K72A mutant. This mutant is active in nucleotide addition but unable to cleave dRP groups efficiently. As expected, the reaction containing the K72A mutant accumulated the 10-nucleotide intermediate (Fig 5, lane 6) and generated very little full-length product. This result contrasts with that involving the wild-type protein (lane 1), which efficiently converted most of the 10-nucleotide intermediate to a full-length product.

**Kinetics of dRP Lyase Activity**—Using PEI-cellulose thin layer chromatography to follow the generation of free ³²P-labeled unsaturated dRP, we measured the kinetic parameters of the mitochondrial pol β, comparing it to the rat nuclear enzyme (Table I). The *Kₘ* values determined were similar for both enzymes, on the order of ~1 μM, and are in reasonable agreement with the *Kₘ* of 0.5 μM reported for the rat enzyme on a similar substrate (27). Unexpectedly, the *kₗ* values differed markedly between the two enzymes. Although the rat nuclear enzyme was able to liberate 4.12 unsaturated dRP residues per minute, a value consistent with that previously observed (27), the C. fasciculata mitochondrial protein was ~40-fold less active. The catalytic efficiency, *kₗ/Kₘ*, is about 20-fold higher for the rat nuclear enzyme.

**Schiff Base Trap Time Course**—As described above, the dRP lyase reaction has multiple steps (Fig. 3). We took advantage of the fact that both Schiff base-containing intermediates and the unsaturated dRP product can be stabilized by reduction with NaBH₄. E-DNA and E-dRP intermediates can be separated by SDS-PAGE, and free unsaturated dRP can be resolved by PEI-cellulose thin layer chromatography.

To identify the step in this pathway in which the mitochondrial pol β was deficient, we incubated the dRP lyase substrate with enzyme for various times prior to the addition of NaBH₄. We used excess protein so that only a single turnover could occur. With the rat nuclear pol β, we found that the initial attack was very fast, because even at time zero when protein and NaBH₄ were added simultaneously, a significant amount of E-DNA complex had already formed (Fig. 6A, upper panel). By 0.5 min, most of the radioactivity had proceeded quickly through the E-dRP intermediate and appeared as the free unsaturated dRP derivative (Fig. 6A, lower panel). The pattern for the C. fasciculata mitochondrial pol β differed significantly. First, the initial attack was slower, as indicated by the lower levels of E-DNA at early time points. The most striking difference, however, was the accumulation of the E-dRP complex, suggesting that the hydrolysis step to release dRP from the enzyme was impaired (Fig. 6B, upper panel). This conclusion was supported by the observation that the level of free unsaturated dRP rose much more slowly for the mitochondrial enzyme than for the rat nuclear enzyme (Fig. 6B, lower panel). The native mitochondrial enzyme exhibited a similar pattern (data not shown). Fig. 6C shows a control demonstrating that the dRP lyase reaction can occur at basal levels in the absence of a specific enzyme activity. Basic pH or the inclusion of polyamines or basic proteins such as histones facilitate this spontaneous non-enzymatic cleavage (28).

**DISCUSSION**

The pol β from *C. fasciculata* is the first example of an enzyme of this type found in the mitochondrion. Its localization to the antipodal sites during kDNA replication suggests a role in this process. This class of polymerases is known to fill small gaps efficiently (29), making them suitable to fill gaps between the numerous Okazaki fragments that are generated during minicircle replication. However, the well-characterized role of nuclear pol βs in DNA repair led us to speculate that the mitochondrial enzyme may also play a similar role. There has been growing evidence that mitochondrial DNA in mammalian cells is repaired via base excision repair, and this pathway is thought to utilize DNA polymerase γ (pol γ) for both dRP lyase and polymerase activity (22). For recent reviews on mitochondrial DNA repair see Refs. 30–32. It is not unreasonable that a pathway for base excision repair exists in the mitochondrion of *C. fasciculata* given the large amount of DNA, the importance of its integrity to parasite survival, and its proximity to the free-radical-generating electron transport chain. To investigate this possibility, we assayed the mitochondrial pol β for dRP lyase activity.

As expected from the sequence alignments showing that residues important for dRP lyase activity are conserved, mitochondrial pol β does indeed have dRP lyase activity. However, the mitochondrial protein is much less active in this capacity than its rat nuclear homolog, and most of this deficiency is in the conversion of the E-dRP intermediate to free enzyme and the unsaturated dRP product. Previous studies based on structural data have suggested both glutamate 26 and glutamate 71 (Fig. 2, boxes) are potential candidates for supplying a carboxyl group to facilitate this hydrolysis reaction (33). Notably, neither of these residues is conserved in the *C. fasciculata* enzyme. It is interesting that a similar pattern of E-dRP accumulation is observed for pol γ from *Xenopus laevis* (34), although a homologous dRP lyase domain has not been reported for that enzyme.

What do these data reveal about the involvement in the *C. fasciculata* pol β in mitochondrial DNA repair? It has been reported for the mammalian enzyme that nucleotide addition occurs prior to dRP removal, thus making the dRP lyase reaction the rate-limiting step of the entire base excision repair pathway (35). This means that once cleavage of the dRP occurs (Step 2 of Fig. 3), the DNA can continue through the repair process, regardless of the state of the polymerase. Based on the purification data (13), we believe this protein to be abundant, and perhaps the sheer number of pol β molecules compensates for its poor catalytic abilities, both as a dRP lyase and as a polymerase. An overabundance of protein molecules in the cell would then be sufficient to meet the repair needs of kDNA. In addition, if protein is rapidly turned over, the problem of the accumulation of pol β as an E-dRP complex is avoided.

There are other possibilities as well. Perhaps the mitochondrial pol β has an accessory factor that facilitates the regeneration of the protein following dRP cleavage. Or maybe the cell can alleviate the need for pol β to have an efficient dRP lyase activity. This could include having other mitochondrial proteins with dRP lyase activity or funneling the base excision repair pathway through the alternative branch that involves AP lyase enzymes. These enzymes, through a similar β-elimination reaction, cleave 3' to the AP site leaving an unsaturated dRP derivative on the 3'-end of the DNA that can be removed by an AP endonuclease (36). Alternatively, perhaps the mitochondrial pol β has evolved to specifically recognize a substrate.
we have yet to identify. Studies to further elucidate the role of this enzyme in the mitochondrion of C. fasciculata and other kinetoplastid organisms are ongoing.

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