The Action of DNA Ligase at Abasic Sites in DNA*

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Apurinic/apyrimidinic (AP) sites occur frequently in DNA as a result of spontaneous base loss or following removal of a damaged base by a DNA glycosylase. The action of many AP endonuclease enzymes at abasic sites in DNA leaves a 5′-deoxyribose phosphate (dRP) residue that must be removed during the base excision repair process. This 5′-dRP group may be removed by AP lyase enzymes that employ a β-elimination mechanism. This β-elimination reaction typically involves a transient Schiff base intermediate that can react with sodium borohydride to trap the DNA-enzyme complex. With the use of this assay as well as direct 5′-dRP group release assays, we show that T4 DNA ligase, a representative ATP-dependent DNA ligase, contains AP lyase activity. The AP lyase activity of T4 DNA ligase is inhibited in the presence of ATP, suggesting that the adenylated lysine residue is part of the active site for both the ligase and lyase activities. A model is proposed whereby the AP lyase activity of DNA ligase may contribute to the repair of abasic sites in DNA.

DNA repair pathways have evolved to process a wide range of chemically distinct lesions in DNA (1). One of the most common types of damage is spontaneous or enzymatic hydrolysis of the N-glycosidic bond between a DNA base and the sugar phosphate backbone generating an abasic site (AP site). AP sites are highly mutagenic and require rapid and efficient repair. AP sites are processed by a base excision repair pathway that is frequently initiated by the action of a class II AP endonuclease that cleaves the DNA backbone adjacent to the lesion to produce 3′-OH and 2′-deoxyribose 5′-phosphate termini (2). The latter residue, referred to as a 5′-dRP moiety, is relatively alkali labile and can be removed by AP lyases that facilitate β-elimination. Most enzymes demonstrated to have dRPase activity operate through this lyase mechanism, although some, such as the Escherichia coli recD protein, catalyze hydrolysis (3). The two classes of dRPase enzymes can be distinguished by the fact that the lyase mechanism frequently involves formation of a transient Schiff base intermediate in which an amino group on the enzyme is covalently bound to the DNA. This lyase mechanism, first proposed for E. coli endonuclease III (4), provides a simple method to identify a polypeptide with AP lyase activity because the Schiff base intermediate can be trapped in a stable form by reaction with a strong reducing agent, such as NaBH₄ or NaBH₃CN. Thus, with an appropriate radioactively labeled substrate, the label can be transferred to the AP lyase enzyme. This borohydride trapping has been documented for several repair enzymes (5–9) and, more recently, for DNA pol β (10, 11).

The combined action of AP endonuclease and AP lyase leaves a one-nucleotide gap that is filled by a DNA polymerase. The final step in repair involves DNA strand sealing by DNA ligase. The mechanism of DNA ligase involves covalent modification of the enzyme by adenylation, transfer of the AMP residue in a phosphoanhydride linkage to the 5′-phosphate of nicked DNA, followed by resealing of the DNA strand driven by the energy of AMP hydrolysis.

We recently characterized a mtDNA ligase as part of an effort to reconstitute repair of AP sites using mitochondrial enzymes (12). The size, template specificity, and immunological properties of the mtDNA ligase suggested that this was a form of DNA ligase III. In the course of this work we found that mtDNA ligase is active on a DNA substrate containing an AP site incised on the 5′ side by a class II AP endonuclease. Our observations prompted a detailed investigation of the action of T4 DNA ligase as a prototype for ATP-dependent DNA ligases at AP sites in DNA. In this paper we show that in the presence of ATP, T4 DNA ligase is able to reseal an incised AP site. In the absence of ATP, T4 DNA ligase acts as an AP lyase to facilitate a β-elimination reaction that leads to removal of the 5′-dRP residue. A model is presented whereby an intrinsic AP lyase activity in DNA ligase may facilitate repair of AP sites.

EXPERIMENTAL PROCEDURES

Materials—mtDNA ligase, mitochondrial AP endonuclease, and DNA ligase I were purified from Xenopus ovary tissue as described (12). T4 DNA ligase was obtained from Boehringer Mannheim. T7 DNA ligase was a gift from Dr. J. Dunn (Brookhaven National Laboratory). Variable quantities of both preparations of bacteriophage DNA ligase were subjected to SDS-PAGE analysis (13) in parallel with standard proteins of known concentration. The gels were stained with Coomassie Blue to confirm that both preparations were essentially homogeneous and to permit estimation of protein concentrations using densitometry of the stained gels. Radiochemicals were purchased from ICN Radiochemicals. Uracil DNA glycosylase (UDG) was obtained from Epicenter Technologies (HK-UNG). FPG protein was a gift from J. Tchou and A. P. Grollman (SUNY-Stony Brook). Sodium borohydride and sodium thioglycolate were obtained from Sigma-Aldrich. Other reagent grade chemicals were obtained from Sigma-Aldrich or Fisher. The Furos Q 4.6 × 50-mm column used for anion exchange HPLC was obtained from Perceptive Biosystems. Oligonucleotides were either synthesized by the phosphoramidite method at the SUNY-Stony Brook Oligonucleotide Synthesis Facility or were obtained from Operon. A continuous duplex oligonucleotide was prepared by annealing a 5′-32P kinase-labeled 32-mer (5′-CATGGGCGACATGUAUCAAGCATTGAGGCAAG) to a complementary oligonucleotide (5′-TCTTGGCTCAACGTGATCATGTCGCCCATCACT). Two nicked duplex oligonucleotides were prepared by annealing a 5′-32P kinase-labeled 17-mer (5′-UCAAAGTGTGGAGGCA) (referred to as U17) and either a nonradioactive 15-mer (5′-CATGGGGCGACATGUAUCAAGCATTGAGGCAAG) (referred to as U15) or a 12-mer (5′-GGGGGGCGCATGUAUCAAGCATTGAGGCAAG) (referred to as U12) to the same complementary strand described above. The 12-mer was used in the

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‡ The abbreviations used are: AP, apurinic/apyrimidinic; UDG, uracil DNA glycosylase; dRP, 2′-deoxyribose 5′-phosphate; FPG, formamidopyrimidine glycosylase; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

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from the initial labeled substrate. A Poros Q anion exchange column was used. Sodium thioglycolate was performed as described (5, 15), except that trichloroacetic acid, analyzed by SDS-polyacrylamide gel electrophoresis (13), and detected by autoradiography or PhosphorImager analysis (Molecular Dynamics). dRPase activity was measured as described (3), and HPLC analysis of products generated in the presence of sodium thioglycolate was performed as described (5, 15), except that a Puros Q anion exchange column was used.

RESULTS AND DISCUSSION

DNA Ligases Are Able to Reseal DNA Strands Nicked on the 5’ Side of an AP Site—Our laboratory has studied the base excision repair pathway with nuclear and mitochondrial protein fractions using templates containing precisely positioned single AP sites embedded in covalently closed circular DNA (12, 15). These sites are readily cleaved on the 5’ side by class II AP endonuclease to yield a 3’-OH terminus and a 5’-deoxyribose phosphate (dRP) residue. When templates bearing incised AP sites are incubated with DNA ligase in the presence of ATP, the DNA ligases are able to reseal the nicked strand (Fig. 1). This reaction has been reported for T4 DNA ligase (16) but not for eukaryotic DNA ligases. Because ligation of a 5’-dRP moiety directly reverses the action of AP endonuclease, it is counterproductive for repair. It is also a potential confounding factor in efforts to reconstitute repair reactions in vitro, because religation of an abasic site might not be differentiated from actual repair. In the experiment in Fig. 1, we used a substrate with a synthetic analogue of an abasic site, a tetrahydrofuran analogue that has been used extensively in repair studies (15, 17). This is an analogue of a reduced deoxyribose moiety and is not subject to β-elimination. Experiments presented below show that T4 DNA ligase can also reseal an authentic (nonreduced) AP site.

T4 DNA Ligase Has AP Lyase Activity—We previously showed Xenopus laevis mtDNA ligase can be labeled using a borohydride trapping procedure that is specific for AP lyase activities (12). To determine whether this is a general property of ATP-dependent DNA ligases, we tested T4 and T7 DNA ligases for the ability to react with AP sites in a borohydride trapping assay. This assay employed an oligonucleotide substrate designed to contain a specific U residue adjacent to a 5’-phospho-abasic site. This is an exact model of the reaction proceeds with elimination of the DNA from the C3’ position of deoxyribose, producing an enzyme-dRP intermediate in which the enzyme is joined to DNA. The AP lyase activity-dRP species can be reduced by NaBH₄ to generate the AP lyase activity.

Fig. 1. DNA ligases can reseal a nick adjacent to a 5’-phosphorylated abasic site. A 5’-end labeled oligonucleotide containing a single synthetic AP site (3-hydroxy-2-hydroxymethyltetrahydrofuran, designated F for furan) was ligated into a gapped heteroduplex, and the covalently closed circular DNA product was purified as described (15). PAGE-urea gel analysis of a Hinfl digest of this substrate confirmed that all substrate molecules were ligated with the tetrahydrofuran residue embedded in a 46-mer fragment, denoted as 46(F) (lane 1). Treatment with mitochondrial AP endonuclease led to cleavage on the 5’ side of the tetrahydrofuran residue, providing a 26-mer fragment following diagnostic Hinfl cleavage (lane 2). This fragment with a 5’-tetrahydrofuran residue is identified as F26. Samples of this mitochondrial AP endonuclease-incised substrate were incubated with X. laevis DNA ligase I, mtDNA ligase, T4 DNA ligase, or T7 DNA ligase (lanes 3–6). The products were deproteinized by organic extraction and cleaved with Hinfl endonuclease prior to electrophoresis. The radioactive species moving slightly slower than the F26 fragment, which is most apparent in lane 6, is an intermediate in the ligase reaction produced by transfer of AMP to the 5’ terminus at the nick.
cross-linking results are consistent with the hypothesis that T4 DNA ligase contains AP lyase activity.

In other experiments, we found that it was not necessary to present the AP site in the context of a nick in DNA, although this is the preferred substrate. Borohydride trapping was observed when the 15-mer oligonucleotide was omitted from the standard nicked substrate, leaving a 5'-AP site adjacent to single-stranded DNA. We also observed cross-linking to a free oligonucleotide with a 5'-dRP site generated by the action of UDG and labeled proteins were detected by following SDS-PAGE as described under "Experimental Procedures."
The critical question raised by our observations is whether the AP lyase activity associated with T4 DNA ligase plays a significant physiological role. A model for the action of T4 DNA ligase at AP sites is shown in Fig. 7. In living cells, AP sites are very rapidly incised by AP endonuclease to generate the sort of nicked AP substrate we have used in our reactions. The experiments reported here show that T4 DNA ligase can act as an AP lyase at these sites in the absence of ATP. Under these conditions, the deadenylated enzyme cannot seal the nick and instead facilitates \( \beta \)-elimination, leading to loss of the 5'-dRP residue. This produces the single nucleotide gap structure diagramed as species 5 in Fig. 7. This single base gap may be repaired by DNA polymerase and the conventional strand sealing action of conventional DNA ligase.

FIG. 4. The chemistry of AP lyase action accounts for DNA-enzyme and dRP-enzyme complexes following NaBH\(_4\) treatment. This scheme is based on those presented for other AP lyase enzymes (7, 19). The Schiff base reaction scheme requires attack by a free amino group of the AP lyase on the C1' residue of the deoxyribose. This reactive nitrogen is referred to as \( N \)-enz. Other functional groups within the enzyme may assist in the \( \beta \)-elimination reaction as indicated. Covalent intermediates in the Schiff base reaction scheme labeled 1 and 2 may be reduced by borohydride to yield stable species with the protein cross-linked to an oligonucleotide or to a dRP moiety, respectively.

FIG. 5. Release of an acid soluble product from a 5'-\( ^{32}P \)-labeled abasic site by T4 DNA ligase. 15 pmol of 5'-\( ^{32}P \)-labeled U17 oligonucleotide pretreated with UDG was incubated with 100 ng of T4 DNA ligase in 40 mM Hepes buffer, pH 7.5, for varied periods of time at 30 °C (A), for 30 min at varied temperature (B), or in the presence of increasing concentrations of ATP (C). dRP release was measured as the generation of a radioactive product soluble in the presence of cold TCA and activated charcoal (3). The percentage of dRP removed was determined relative to the total alkali-labile cpm. The maximal amount of label solubilized in parallel reactions without enzyme represented 4% of the total available substrate.

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It is also important to consider the action of T4 DNA ligase at incised AP sites in the presence of ATP, because a large fraction of DNA ligase may exist in the adenylated state in vivo. Our results suggest that the adenylated T4 DNA ligase
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Fig. 7. Model for the action of DNA ligase at AP sites. A, the proposed action of DNA ligase at an incised AP site is illustrated in the presence or the absence of ATP. DNA ligase is shown as a C-shaped enzyme with a prominent groove (21). See "Results and Discussion" for details. B, a substrate containing an internal AP site was prepared by UDG treatment of a duplex oligonucleotide with a single U residue 15 nucleotides from the 32P-labeled 5'-end as diagrammed at the bottom. The oligonucleotide was incubated for 45 min at 25 °C in 40 mM Hepes, pH 7.5, either without additional enzyme (lane 1) or with either E. coli FPG protein (lane 2) or T4 DNA ligase (lane 3). Reactions were treated for 15 min with 0.1 M NaBH4, and products were subjected to autoradiography. Markers consisted of kinase-labeled oligonucleotides identified by size on the left. The 17-mer marker is the intact U17 oligonucleotide, and the 12-mer marker is a 5'-labeled sample of the same nonradioactive 12-mer used to prepare the substrate. The diagrams at the bottom of B and C show the oligonucleotides used in these experiments with the top strands oriented 5' to 3'.

This suggests that T4 DNA ligase is able to promote 3'-elimination. Instead, when a T4 DNA ligase molecule that is activated by adenylation binds this nicked substrate, it seals the nick to regenerate an internal AP site, as shown in Fig. 1 and diagramed as species 3 in Fig. 7A. The second product of the ligation reaction is a "disarmed" DNA ligase molecule that is no longer adenylated but is still in contact with the AP site. To test whether T4 DNA ligase is able to incise DNA on the 3' side of an internal AP site (i.e., without prior action of an AP endonuclease), we performed the experiment in Fig. 7B. This experiment shows that T4 DNA ligase is clearly capable of strand incision to yield a product with a slightly slower gel mobility than that produced by the well characterized AP lyase of FPG protein. These results suggest that T4 DNA ligase is able to promote 3'-elimination but unlike FPG protein does not efficiently promote 5'-elimination. This sort of incision reaction was not observed in Fig. 1 because that experiment employed a reduced AP site analogue. These results suggest that when T4 DNA ligase seals a nick generated by class II AP endonuclease, it may recognize the product as a mistake and employ its lyase activity to reopen the DNA. To test this prediction, we performed the experiment shown in Fig. 7C. In this experiment, T4 DNA ligase was incubated with a 17-mer oligonucleotide containing a 5'-32P-dRP residue adjacent to a nonradioactive 12-mer. DNA ligase was able to ligate the 12-mer to the 5'-dRP-17 mer to generate a 29-mer with an internal 32P-dRP residue (lane 3 of Fig. 7C). A limited extent of ligation was observed without the addition of exogenous ATP (lane 2), presumably because a fraction of the T4 DNA ligase is purified in an adenylated form. The more efficient ligation in the presence of ATP was followed by incision on the 3' side of the AP site to produce a labeled 12-mer with a 3'-dRP residue. Thus, the label transfer experiment in Fig. 7C confirms the model for the action of T4 DNA ligase at an AP site in the presence of ATP. The ring open 3'-dRP residue produced by AP lyase cannot be rejoined by DNA ligase due to the 2'-3'-double bond, but the 3'-dRP group would be susceptible to release by class II AP endonuclease. Taken together, these experiments suggest that the role of T4 DNA ligase in base excision repair may not be limited to the final step of strand closure.

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