A recent study demonstrated that rat DNA polymerase β (β-pol) releases 5'-deoxyribose phosphate (dRP) termini from preincised apurinic/apyrimidinic DNA, a substrate generated during certain types of base excision repair. This catalytic activity resides within the amino-terminal, 8-kDa domain of β-pol and occurs via β-elimination as opposed to hydrolysis (Matsumoto, Y., and Kim, K. (1995) Science 269, 699–702). The latter finding suggested that the dRP excision reaction might proceed via an imine intermediate. In order to test this hypothesis, we attempted to trap β-pol on preincised apurinic/apyrimidinic DNA using NaBH₄ as the reducing agent. Both 8-kDa domain-DNA and intact β-pol-DNA complexes were detected and identified by autoradiography coupled to immunoblotting. Our results indicate that the chemical mechanism of the β-pol dRpase reaction does proceed through an imine enzyme-DNA intermediate and that the active site residue responsible for dRP release must therefore contain a primary amine.

Vertebrate DNA polymerase β (β-pol); a constitutively-expressed monomeric protein ranging from 39 to 45 kDa, has been implicated in DNA repair. Specifically, in vitro experiments indicate that β-pol can perform two of the five reactions involved in base excision repair: excision of a 5'-terminal dRP from a preincised AP site (1) and DNA synthesis to fill the single-nucleotide gap (2–5). The overall pathway for base excision repair that has been initiated by simple N-glycosylases (glycosylases lacking a concomitant AP lyase activity) is believed to proceed according to the following scheme. (i) A damage-specific DNA N-glycosylase creates an AP site by cleaving an N C glycosyl bond, thereby releasing a modified base; (ii) a class II AP endonuclease incises the DNA 5' to the AP site; (iii) β-pol or another dRpase excises the 5' dRP terminus to leave a single-nucleotide gap; (iv) β-pol or another polymerase fills in the single-nucleotide gap; and (v) DNA ligase seals the gap (6). 5'-Terminal dRP might eventually undergo an uncatalyzed β-elimination reaction to generate a single-nucleotide gap (half-life ~ 2 h) (7), but this reaction is probably too slow to be relied upon in vivo. Coordination of steps (iii) and (iv) by β-pol presumably would lead to more efficient DNA repair. Thus, reaction (iii) assures an adequate substrate for β-pol, an enzyme without an intrinsic 5'-exonuclease activity.

Proteolysis studies have revealed a two-domain structure for rat β-pol; independent digestions with four proteases resulted in the cleavage of the enzyme within a protease-sensitive region between residues 82 and 88 (6). Treatment with trypsin, for example, yielded an 8-kDa amino-terminal fragment (residues 3–75) and a 31-kDa carboxy-terminal fragment (residues 87–334). In addition to its recently discovered dRpase activity, the 8-kDa domain (residues 1–87) is known to bind single-stranded DNA with an affinity similar to that of the intact enzyme (8). The 31-kDa domain (residues 88–334) binds double-stranded DNA but not single-stranded DNA and retains <2% of the polymerase activity of the intact protein. Circular dichroism experiments revealed that the 8-kDa domain is essentially α-helical in nature (9), and the predominance of this secondary structural motif has since been confirmed by NMR (10) and x-ray crystallography (11, 12). The NH₂-terminal domain of β-pol comprises helix-1 (residues 15–26), a long loop (27–35), helix-2 (36–47), turn-1 (48–51) plus a segment of loop (52–55), helix-3 (56–61), turn-2 (62–65) plus a segment of loop (66–68), and helix-4 (69–78). Helix-1 and helix-2 lie antiparallel to each other as do helix-3 and helix-4, with the two pairs of antiparallel helices crossing at an 80° angle.

Matsumoto and Kim (1) found that β-pol releases 5'-terminal dRP via β-elimination, as opposed to hydrolysis. This finding suggested that the dRP excision reaction likely proceeds via an imine or Schiff base intermediate. Fischer et al. (13) first demonstrated in 1958 that an imine intermediate formed between a substrate and an enzymatic amino group can be trapped by reduction with sodium borohydride (NaBH₄). In subsequent years, this chemical technique helped to elucidate the reaction mechanisms of acetocacetal decarboxylase (14, 15) and aldolase (16). In both cases, the α-NH₂ group of a lysine was involved in the formation of the imine intermediate. More recently, several N-glycosylase/AP lyase DNA repair enzymes have been shown to proceed via Schiff base intermediates; NaBH₄ trapping has enabled the identification of bacteriophage T4 endonuclease V-DNA (17), Escherichia coli endonuclease III-DNA (18), E. coli Fpg-DNA (18, 19), and Micrococcus luteus UV endonuclease-DNA covalent complexes (20). This paper reports the NaBH₄-mediated conversion of transient 8-kDa domain and intact β-pol dRP excision intermediates (imines) to stable, covalent enzyme-DNA derivatives (2⁺ amines).

EXPERIMENTAL PROCEDURES

Materials—[γ-³²P]dATP (3000 Ci/mmol) was purchased from Amersham Corp., and NENSORB-20 columns were purchased from DuPont NEN. Terminal deoxynucleotidyltransferase and double-stranded pol(dI-dC) were obtained from Pharmacia Biotech Inc. New England Biolabs was the supplier for the acetylated BSA. UDG was obtained from Epicentre Technologies, and uracil-containing 49-mer oligonucleotide was ordered from Midland Research. Its complement was synthesized by the Sealy Center Reombinant DNA Laboratory. Human APE

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DNA Polymerase b Drase Reaction Mechanism

that was generously supplied by Dr. Phyllis Strauss (Northeastern University, Boston, MA) had been purified according to the method of Strauss et al. 2 Recombinant rat b-pol, a K72A mutant, and the 8-kDa domain were overexpressed in E. coli and purified as reported earlier (8, 22). A 32-amino acid peptide from the 8-kDa domain of rat b-pol (residues 27–58; NH2-KNVQAIHKNAYRAASiVIYHKGSAE-COOH) is sequenced on an Applied Biosystems PerkinElmer 430A machine by the Protein Chemistry Core Facility. Antipeptide antisera were generated in rabbits using standard immunization protocols (23). Polyclonal antibodies against recombinant b-pol were raised by immunization of rabbits, purified, and characterized as described previously (5). Prestained low and high molecular size marker kits can be purchased from Life Technologies, Inc. Nitrocellulose membrane and goat anti-rabbit IgG horseradish peroxidase conjugate were bought from Bio-Rad. NaBH4 was purchased from Fisher Scientific Co.

Preparation of Preincised AP DNA Substrate—Uracil-containing 49-mer oligonucleotide (5′-AGCTAATCATGGTCATAGCT-3′) was 32P-labeled on its 3′ end with terminal deoxynucleotidyltransferase and then annealed to its complement. Free [32P]-ddATP and transferase were separated from the duplexed DNA by passage of the mixture over a Nensorb-20 column as per the supplier’s instructions. Column fractions containing the substrate DNA were evaporated to dryness in a Savant SC110 SpeedVac and resuspended in UDG reaction buffer (70 mM Heps, pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.05% TritonX-100) before addition into the trapping reaction ([residual NaBH4 from preincubation of the 8-kDa domain with pre- or postimmune antiserum was removed against a 32-amino acid peptide for 20 min on ice). For (iii), a highly concentrated stock of 8-kDa domain was used so that the enzyme plus magnesium was 10-fold molar excess) to sequentially remove uracil from the DNA target DNA was chosen as a matter of convenience; human APE incises double-stranded DNA more efficiently than single-stranded DNA. Magnesium was included in the reaction buffer to ensure that the 8-kDa domain would be able to bind to double-stranded DNA with a gap of only one nucleotide (24, 1).

RESULTS AND DISCUSSION

Experimental Rationale—As outlined in the Introduction, the Drase reaction of b-pol is hypothesized to proceed via an imine intermediate. Accordingly, once the ω- or the ε-amino group of the active site residue has engaged in a nucleophilic attack on the sugar C-1′ of an AP nucleoside, it should be possible to trap the ω-pol-DNA intermediate by reduction with NaBH4. The covalent nature of the resultant complex can be tested by subjecting it to denaturing polyurea gel electrophoresis or SDS-PAGE. Although ω-pol can release DR from both single-stranded and double-stranded substrate, duplexed substrate was chosen as a matter of convenience; human APE incises double-stranded DNA more efficiently than single-stranded DNA. Magnesium was included in the reaction buffer to ensure that the 8-kDa domain would be able to bind to double-stranded DNA with a gap of only one nucleotide (24, 1).

NaBH4-mediated Trapping of the Intact ω-pol-DNA and 8-kDa Domain-DNA Covalent Complexes—Upon exposure to NaBH4 but not NaCl, b-pol (M, 39,000) was expected to be bound covalently to the 3′-labeled 28-mer (M, 9,000) produced by sequential UDG and APE treatments of 3′-labeled 49-mer containing uracil at position 21. Indeed, polyurea gel electrophoresis revealed a slow-migrating band (Fig. 1A, lane 4), presumably the covalent b-pol-DNA complex. In order to determine the molecular mass of the presumed intact b-pol-DNA complex and to identify it via immunoblotting, a more extensive version of the same experiment was carried out using SDS-PAGE. Fig. 1 (B and C) shows the autoradiograph and the corresponding immunoblot from SDS-PAGE trapping experiments on intact b-pol, respectively. As predicted, the most prominent product band on the autoradiograph migrated with an apparent molecular mass of 48 kDa (39,000 + 9,000 = 48,000). This complex, highlighted by asterisks, was easily identified by its immunoreactive and radioactive properties. Minor bands at 62 and 67 kDa on the autoradiograph also had the same mobility as the covalent complex of (b-pol + 28-mer + 49-mer) (calculated M, 64,000) and (b-pol + 28-mer + 49-mer + 20-mer) (calculated M, 71,000). They would not have been observed in Fig. 1A because polyurea electrophoresis completely denatures DNA. Unlabeled 20-mer would have been produced along with the labeled 28-mer during the APE preincision reaction. Secondary enzyme-DNA complexes and b-pol breakdown products were not observed when less b-pol was introduced into the reactions (data not shown), and heat inactivation of b-pol completely eliminated the formation of covalent complexes (lane 5). Finally, there was no evidence to indicate that any of the protein-DNA complexes were derived in whole or in part from APE (M,

2 P. R. Strauss, W. A. Beard, T. A. Patterson, and S. H. Wilson submitted for publication.
NaBH₄ trapping occurred in a substrate-dependent manner. The position of the primary dRpases (RecJ, M₄, panel C, b, 4Xless, 5 b) did survive polyurea gel electrophoresis (Fig. 2) and possessed a mobility in between that of the 28-mer substrate.

Analogous to intact β-pol, 8-kDa domain was expected to be bound covalently to the 3'-labeled 28-mer (Mₛ = 9,000) by NaBH₄ reduction of an imine intermediate. A single product did survive polyurea gel electrophoresis (Fig. 2A, lane 4) and possessed a mobility in between that of the 28-mer substrate and the intact β-pol product seen in Fig. 1A (Figs. 1A and 2A were derived from the same gel). Fig. 2 (B and C) shows the autoradiograph and the corresponding immunoblot from trapping experiments on the 8-kDa domain of β-pol, respectively. The primary product band on the autoradiograph migrated with an apparent molecular mass of 17 kDa (8,000 + 9,000 = 17,000). This product, highlighted by asterisks, was both immunoreactive and radioactive. Again, a protein doublet appeared on the autoradiograph, but this time it accounted for 50% of the complexed protein. The protein pair was not detected on the immunoblot shown in panel C but was seen on other occasions. With observed molecular masses of 30 and 37 kDa, the secondary bands probably corresponded to complexes of (8-kDa domain + 28-mer + 49-mer) (calculated Mₛ = 33,000) and (8-kDa domain + 28-mer + 49-mer + 20-mer) (calculated Mₛ = 40,000). Importantly, as demonstrated in lanes 7 and 8, NaBH₄ trapping occurred in a substrate-dependent manner. Pre-exposure of the 8-kDa domain to NaBH₄ failed to significantly reduce the enzyme's ability to become trapped to substrate upon re-exposure to NaBH₄.

As illustrated by the amounts of free versus complexed β-pol or 8-kDa domain that were transferred to the Western blots (compare arrows versus asterisks on panel C in Figs. 1 and 2), only a small fraction of either protein was trapped. Although autoradiography was adequate to detect β-pol-DNA complexes when substrate was incubated with as little as a 20-fold molar excess of β-pol, immunodetection became extremely difficult (data not shown). PhosphorImager/ImageQuant analysis of the polyurea gel shown in Fig. 1A revealed that NaBH₄ mediated the incorporation of 0.9 ng of the intact β-pol (23 fmol, approximately 0.04% of the protein) into enzyme-DNA complexes. In Fig. 1B, the 49-kDa bands in lanes 4 and 7 contained 0.5 ng (13 fmol) and 0.6 ng (15 fmol) of intact β-pol, respectively. Overall, 0.09% of the β-pol protein in lane 4 or 0.04% of the β-pol protein in lane 7 was trapped in the form of a 49-, 62-, or 67-kDa complex. In Fig. 2A, 0.1 ng of the 8-kDa domain (18 fmol, approximately 0.03% of the protein) was incorporated into enzyme-DNA complexes. More clearly than Fig. 1B, Fig. 2B demonstrates quantitative trapping of β-pol protein: 0.02 ng (2.3 fmol) and 0.07 ng (8.8 fmol) of the 16-kDa product were trapped with 150- and 600-fold molar excesses of enzyme over substrate, respectively. Overall, 0.03% of the 8-kDa domain protein in lanes 4 or 7 was trapped in the form of a 16-, 30-, or 67-kDa complex.
NaBH₄-mediated trapping of the 8-kDa domain-DNA covalent complexes. Panel A, preincised AP DNA (1.6 ng = 95 fmol) was incubated with enzyme dilution buffer (lanes 1 and 2) or a 600X molar excess (480 ng = 56 pmol) of 8-kDa domain (lanes 3 and 4). Seconds after control buffer or 8-kDa domain was pipetted into the reaction mixtures, trapping was initiated by the addition of NaCl (lanes 1 and 3) or NaBH₄ (lanes 2 and 4) to a final concentration of 150 mM. Incubations were terminated after 60 min at 37°C by the addition of an equal volume of formamide loading buffer. The samples were denatured, loaded onto a 10% polyacrylamide gel containing 8 M urea, and subjected to electrophoresis. Oligonucleotide sizing markers ranging from 8 to 32 bases were run in the outer lanes of the gel, and their migration distances are marked to the left of panel A. The asterisk to the left of panel A denotes the position of the 8-kDa domain-DNA complex, and the arrow to the left of panel A points to 28-mer substrate. Panels B and C, preincised AP DNA (1.6 ng = 95 fmol) was incubated with enzyme dilution buffer (lanes 1 and 2), a 150X molar excess (120 ng = 14 pmol) of active (lanes 3 and 4) or heat-inactivated (lane 5) 8-kDa domain, or a 600X molar excess (480 ng = 56 pmol) of active (lanes 6 and 7) or NaBH₄-pretreated (lane 8) 8-kDa domain. Seconds after control buffer or 8-kDa domain was pipetted into the reaction mixtures, trapping was initiated by the addition of NaCl (lanes 1, 3, and 6) or NaBH₄ (lanes 2, 4, 5, 7, and 8) to a final concentration of 150 mM. Incubations were terminated after 60 min at 37°C by the addition of an equal volume of 4% SDS loading buffer. The samples were denatured, loaded onto a 10% SDS-PAGE gel, and subjected to electrophoresis. Prestained low molecular size markers were run in the outer lanes of the gel, and their migration distances are marked between panels A and B: ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), β-lactamase (18.4 kDa), lysozyme (14.3 kDa), and bovine trypsin inhibitor (6.2 kDa). The direct autoradiograph of the gel (panel B) and the exposure of the probed immunoblot (panel C) were representative of those from 3 other independent experiments. The asterisks to the left of panel B and to the right of panel C denote the position of the primary 8-kDa domain-DNA complex, and the arrow to the right of panel C points at uncomplexed 8-kDa domain.

37-kDa complex. A comprehensive comparison of the quantitative results from Figs. 1 and 2 suggests that intact β-pol was trapped somewhat more readily than the 8-kDa domain.

Experimental Design Considerations—Reproducible trapping of the β-pol-DNA and 8-kDa domain-DNA complexes was accomplished by rigorous adherence to the protocols outlined under “Experimental Procedures.” Pilot studies established that our substrate preparation achieved complete conversion of the duplex 49-mer to the intended product. When preincised AP DNA was run on 10% polyacrylamide gels containing 8 M urea, it co-migrated as expected with a 28-mer oligonucleotide that our substrate preparation achieved complete conversion of the substrate prior to the addition of 8-kDa domain and NaBH₄ to the reaction mixture. However, this treatment did not interfere with trapping. In (ii), 8-kDa domain was preincubated with pre- or postimmune antiserum before it was added into the trapping reaction. Preincubation of the enzyme with post- versus preimmune antiserum reduced the level of 8-kDa domain-DNA trapping by 1%. In (iii), trapping reactions were started.
by the addition of varying amounts of peptide in place of β-pol and either NaBH₄ or NaCl. A covalent complex was not seen at the predicted 12-kDa position. Presented with these generally negative results, it appears unlikely that the active site reside for the β-pol dRP excision reaction resides within helix-2 or its adjacent loop segments.

Speculation on the Identity of the Active Site Residue Responsible for dRP Excision—In summary, this report has shown that the dRPase reaction of rat β-pol proceeds via an imine intermediate. This intermediate arises when the nitrogen atom of a primary amine forms a bond with the C-1' atom of an AP nucleoside. Our results would tend to support the recent finding that the dRPase reaction of rat β-pol proceeds via an imine intermediate. This intermediate arises when the nitrogen atom of a primary amine forms a bond with the C-1' atom of an AP nucleoside. Our results would tend to support the recent finding that the dRPase reaction of rat β-pol proceeds via an imine intermediate. This intermediate arises when the nitrogen atom of a primary amine forms a bond with the C-1' atom of an AP nucleoside.

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