Enzymatic Release of 5′-Terminal Deoxyribose Phosphate Residues from Damaged DNA in Human Cells

Allan Price* and Tomas Lindahl*

Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts, EN6 3LD, U.K.

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ABSTRACT: Activities that catalyze or promote the release of 5′-terminal deoxyribose phosphate residues from DNA abasic sites previously incised by an AP endonuclease have been identified in soluble extracts of several human cell lines and calf thymus. Such excision of base-free sugar phosphate residues from apurinic/apyrimidinic sites is expected to be obligatory prior to repair by gap filling and ligation. The most efficient excision function is due to a DNA deoxyribophosphodiesterase similar to the protein found in *Escherichia coli*. The human enzyme has been partially purified and freed from detectable exonuclease activity. This DNA deoxyribophosphodiesterase is a Mg^{2+}-requiring hydrolytic enzyme with an apparent molecular mass of approximately 47 kDa and is located in the cell nucleus. By comparison, the major nuclear 5′→3′ exonuclease, DNase IV, is unable to catalyze the release of 5′-terminal deoxyribose phosphate residues as free sugar phosphates but can liberate them at a slow rate as part of small oligonucleotides. Nonenzymatic removal of 5′-terminal deoxyribose phosphate from DNA by β-elimination promoted by polyamines and basic proteins is a very slow mechanism of release compared to enzymatic hydrolysis. We conclude that a DNA deoxyribophosphodiesterase acts at an intermediate stage between an AP endonuclease and a DNA polymerase during DNA repair at apurinic/apyrimidinc sites in mammalian cells, but several alternative routes also exist for the excision of deoxyribose phosphate residues.

The major pathways of DNA excision repair appear to be highly conserved between microorganisms such as *Escherichia coli* and higher eukaryotes. Bulky lesions, e.g., pyrimidine dimers, are excised as part of large oligonucleotides in ATP-dependent reactions (LaBelle & Linn, 1982; Sancar & Rupp, 1983; Caron et al., 1985; Wood et al., 1988). In contrast, many base lesions causing little distortion of the double helix can be removed by DNA glycosylases, which initiate very short patch excision–repair. The latter enzymes show strong evolutionary conservation (Olsen et al., 1989; O'Connor & Laval, 1990). Spontaneous DNA depurination by nonenzymatic hydrolysis and the action of DNA glycosylases specific for deaminated or alkylated base residues or certain mismatches result in apurinic/apyrimidinic (AP)[1] sites occurring as common lesions in DNA. Both bacteria and mammalian cells have efficient and specific AP endonucleases that catalyze the hydrolysis of the phosphodiester bond 5′ to this lesion (Weiss & Grossman, 1987; Doetsch & Cunningham, 1990). Initial chain cleavage at an AP site in DNA may also occur by β-elimination 3′ to the lesion, catalyzed by an AP lyase activity associated with some DNA glycosylases (Bailly & Verly, 1987; Mazumder et al., 1991). However, this minor alternative repair pathway for AP sites is apparently only employed during enzymatic removal of base lesions generated by oxidative damage (Cunningham et al., 1986; Lindahl, 1990).

The noncoding deoxyribose phosphate (dRp) residue located at the 5′-terminus of a hydrolytically incised AP site in DNA must be removed in connection with enzymatic gap filling and ligation. AP endonucleases are unable to catalyze this reaction (Grafstrom et al., 1982; Franklin & Lindahl, 1988). A number of mechanisms, not necessarily mutually exclusive, could operate at this particular step in the base excision–repair process. The dRp residue might be enzymatically excised, either in free form or as part of a small oligonucleotide, by a separate enzyme. In this regard, no known 5′→3′ exonuclease has been observed to release free dRp. The 5′→3′ exonuclease function of *E. coli* DNA polymerase I does not catalyze this reaction (Franklin & Lindahl, 1988). However, this enzyme may liberate dRp as part of a small oligonucleotide (Gossard & Verly, 1978; Au et al., 1989). In vitro studies with *E. coli* DNA polymerase I have established that nick translation is inefficient at a strand break with a 5′-terminal dRp residue and the enzyme instead performs strand displacement DNA synthesis (Mosbaugh & Linn, 1982).

The major 5′→3′ exonuclease activity[2] of mammalian cell nuclei, DNase IV, has biochemical properties very similar to those of the 5′→3′ exonuclease function of *E. coli* DNA polymerase I, although the mammalian exonuclease is not covalently attached to a DNA polymerase domain (Lindahl et al., 1969b; Lindahl, 1971a,b; Grondal-Zocchi & Verly, 1985). The bacteriophage T5-encoded 5′→3′ exonuclease is also an enzyme of this type (Sayers & Eckstein, 1990). The main role of DNase IV appears to be the removal of one or two 5′-terminal ribonucleotide residues remaining at primer–DNA junctions after RNaseH I action to allow gap filling and ligation during discontinuous DNA replication (Ishimi et al., 1988; Kenny et al., 1988; Goulian et al., 1990). These functional data strengthen the analogy with the 5′→3′ exo-

[1] Abbreviations: AP, apurinic/apyrimidinic; dRp, deoxyribose 5-phosphate; HPLC, high-performance liquid chromatography; DEAE, (diethylamino)ethyl; dRpase, DNA deoxyribophosphodiesterase; FPLC, fast-performance liquid chromatography; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

[2] Cunningham and Laskowski (1953) introduced the names "DNase I" and "DNase II" for the two widely used reagent DNA endonucleases isolated from mammalian tissues. When the major 3′→5′ and 5′→3′ exonucleases of mammalian cell nuclei were characterized, they were called DNase III and DNase IV (Lindahl et al., 1969a) to avoid confusion with the extensively investigated *E. coli* exonucleases.

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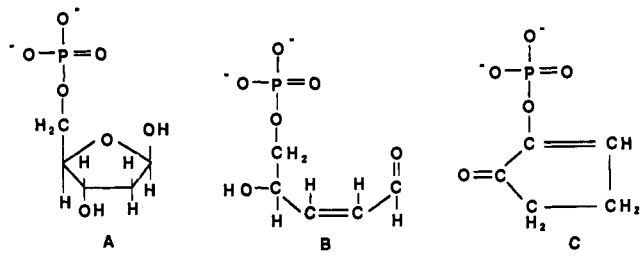

FIGURE 1: Sugar phosphate moieties that may be released from 5′-termini in DNA during the repair of abasic sites. (A) Deoxyribose 5-phosphate, excised by enzymatic hydrolysis. (B) 4-Hydroxy-2-pentenal 5-phosphate, released by β-elimination. This compound may be rearranged to the ring-closed form, (C) 3-oxocyclopent-1-enyl 2-phosphate, under alkaline conditions (Jones et al., 1968).

nuclease activity of *E. coli* DNA polymerase I. DNase IV is present at a higher level in proliferating than in nonproliferating mammalian cells (Mezzina et al., 1989).

An AP lyase such as the *E. coli* Nth protein (endonuclease III, redoxyendonuclease, pyrimidine hydrate–DNA glycosylase, thymine glycol–DNA glycosylase) is unable to cleave at the 3′-side of a dRp residue subsequent to a hydrolytic incision on the 5′-side by an AP endonuclease (Franklin & Lindahl, 1988; Levin & Demple, 1990). However, slow nonenzymatic release of 5′-terminal residues has been observed (Grafstrom et al., 1982; Bailly & Verly, 1989), leading these authors to suggest that enzymatic liberation of the residue may not be required in vivo. The release of a 5′-terminal sugar phosphate from an AP site by β-elimination would generate the ring-opened unsaturated aldehyde 4-hydroxy-2-pentenal 5-phosphate (Jones et al., 1968; Mazumder et al., 1991) instead of dRp (Figure 1).

In previous experiments, excision of a 5′-terminal dRp residue from DNA by *E. coli* cell-free extracts was observed to occur by hydrolysis rather than by β-elimination and was largely due to a previously unrecognized factor, DNA deoxyribophosphodiesterase (dRpase) (Franklin & Lindahl, 1988; Franklin et al., 1988). This enzyme cleaves the phosphodiester bond on the 3′-side of a 5′-terminal dRp residue in a Mg^{2+}-dependent reaction but has no detectable associated exonuclease, AP endonuclease, or DNA phosphatase activity. After either hydrolytic excision of the 5′-terminal dRp or β-elimination, a DNA molecule containing a one-nucleotide gap would be produced.

We have investigated whether this mechanism of dRp excision has been retained during evolution and also occurs in mammalian cells. A dRpase activity has been identified in human nuclear extracts, partially purified, and characterized. This enzyme accounts for the major part of the release of dRp residues from incised AP sites in DNA in vitro. We have also assessed the possibility that 5′-terminal dRp residues might be removed by alternative mechanisms involving β-elimination or exonuclease action. The ability of purified DNase IV to excise a 5′-terminal dRp residue at a previously incised AP site in DNA has been evaluated.

Materials and Methods

Preparation of Polynucleotide Substrates. Poly(dA-dT) containing ^{32}P-labeled AP sites was prepared essentially as previously described (Franklin & Lindahl, 1988) except for the use of Klenow fragment (Boehringer-Mannheim) in place of intact *E. coli* DNA polymerase I for synthesis of the polynucleotide. [α-^{32}P]dUTP was prepared from [α-^{32}P]dCTP (400 Ci/mmol, Amersham) by deamination with nitrous acid. Reaction mixtures (100 μL) for polynucleotide synthesis contained 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 0.5 μM [α-^{32}P]dUTP, 50 μM dTTP, 50 μM dATP, 1 μg of poly(dA-dT), and 1 unit of Klenow fragment. After incubation for 2 h at 37 °C, the reaction was stopped by incubation at 65 °C for 5 min with 20 mM EDTA. It was determined that 15–20% of the [α-^{32}P]dUTP residues were incorporated. The polynucleotide was precipitated with ethanol, resuspended in 100 μL of 50 mM Hepes–NaOH (pH 8.0)/1 mM DTT, and incubated for 2 h at 37 °C with 0.02 unit of *E. coli* uracil–DNA glycosylase (Lindahl et al., 1977). Approximately 60–75% of the dUMP residues were converted to AP sites in different preparations, as judged by experiments in which the polynucleotide was digested by snake venom phosphodiesterase and the products were analyzed by reverse-phase HPLC. The uracil–DNA glycosylase resistant dUMP residues may have been incorporated close to 3′-termini since such residues are refractory to enzymatic hydrolysis. An aliquot of the polymer, to be used as substrate for AP endonuclease assays, was precipitated with ethanol and stored at −20 °C in 10 mM citrate buffer (pH 6.2) prior to use. Substrates to be used in dRpase assays were incubated with 250 units of *E. coli* endonuclease IV (Ljungqvist, 1977) and 0.1 M NaCl for a further 1 h at 37 °C. Cleavage at AP sites was close to 100% as estimated from the release of ^{32}P-labeled material as free sugar phosphate by NaOH treatment. The polynucleotide was again precipitated with ethanol and stored at −20 °C in 10 mM citrate buffer (pH 6.2). To prepare a substrate containing AP sites incised on the 3′-side of the lesion, the uracil–DNA glycosylase treated polymer was incubated with *E. coli* Nth protein (endonuclease III) (Asahara et al., 1989) instead of endonuclease IV, without change of buffer, for 1 h at 37 °C followed by precipitation with ethanol and storage at −20 °C in 10 mM citrate buffer (pH 6.2).

Assays of 5′→3′ exonuclease activity employed [5′-^{32}P]oligo(dT)·poly(dA) as substrate. This double-stranded polymer was identical with a substrate for assaying DNA ligase activity, the preparation of which has been described (Tomkinson et al., 1990).

Enzyme Assays. The standard assay to estimate the excision of ^{32}P-labeled dRp from DNA termini measured the amount of radioactive material released in a form soluble in the presence of trichloroacetic acid and activated Norit charcoal. Reaction mixtures (100 μL) contained 50 mM Hepes–NaOH (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, poly(dA-dT) containing incised AP sites (1 ng, 3000 cpm), and a limiting amount of dRp-releasing activity. After incubation for 20 min at 37 °C, the reaction was stopped by chilling on ice and the addition of 50 μL of 0.5% calf thymus DNA and 17 μL of 50% trichloroacetic acid. After 5 min at 0 °C, 100 μL of 5% Norit charcoal in 1 mM HCl was added and the reaction mixtures were centrifuged at 10000*g* and 4 °C for 15 min. The radioactivity of the supernatant was determined by liquid scintillation counting. As previously described, (Franklin & Lindahl, 1988), 1 unit of dRpase activity was defined as the amount of enzyme catalyzing the excision of 1 pmol of [^{32}P]dRp from poly(dA-dT) containing incised AP sites in 30 min. Reactions with the *E. coli* dRpase (Franklin & Lindahl, 1988) contained 50 mM MES–NaOH (pH 6.8) in place of 50 mM Hepes–NaOH (pH 8.0).

AP endonuclease activity was assayed essentially according to Levin and Demple (1990). Reaction mixtures (100 μL) contained 50 mM Hepes–NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, ^{32}P-labeled poly(dA-dT) containing nonincised AP sites (5000 cpm), and limiting amounts of enzyme. Incubations were for 15 min at 37 °C. NaOH (5 M) was then added to a final concentration of 0.25 M, and the incubation

continued for a further 15 min at 37 °C to obtain complete alkali-catalyzed β-elimination on the 3'-side of the AP sites. The amount of free sugar phosphate was measured as in the dRpase assays.

Reaction mixtures for exonuclease assays (100 μL) contained 50 mM Hepes–NaOH (pH 8.2), 3 mM $MgCl_2$, 1 mM DTT, [5'-^{32}P]oligo(dT)·poly(dA) (1 ng, 5000 cpm), and limiting amounts of enzyme. Incubations were for 10 min at 37 °C. Reactions were stopped by addition of 10 μL of 1% bovine serum albumin and 13 μL of 50% trichloroacetic acid followed by centrifugation at 10000*g* and 4 °C for 15 min and determination of the radioactivity in the supernatant.

Product Analysis. The β-elimination product, 4-hydroxy-2-pentenal 5-phosphate (Figure 1B) is susceptible to addition reactions across its 2,3 C=C double bond with nucleophiles such as thiols. Bricteux-Gregoire and Verly (1989) have used this reactivity to facilitate the separation of the β-elimination product from dRp. When an anionic thiol such as sodium thioglycolate is present at the time of the β-elimination reaction, an addition product is produced that is retarded relative to dRp on anion-exchange chromatography. This procedure has been used here to improve the HPLC separation of reaction products by addition of 0.1 M sodium thioglycolate to the standard reaction mixture in dRpase assays. The entire reaction mixtures (100 μL) were injected onto a Brownlee MPLC AX (4.6 mm × 3 cm) cartridge column and eluted with 5 mL of 25 mM KH_2PO_4 (pH 3.5), followed by 10 mL of 250 mM KH_2PO_4 (pH 3.5), at a flow rate of 1 mL/min. Fractions (0.5 mL) were collected, and the radioactivity was determined by liquid scintillation counting. Unretarded material appeared after 0.5–1 min, dRp after 1.5–2 min as indicated by colorimetric estimation using 2,4-dinitrophenylhydrazine, P_i after 3–4 min, and the β-elimination sugar phosphate product after 9–12 min.

Reaction products from exonuclease assays were analyzed by reverse-phase HPLC using a Varian Micropak MCH-10 column (4 mm × 30 cm). A gradient of 2% methanol/0.1 M ammonium formate (pH 5.0) to 100% methanol was applied over 20 min at 1 mL/min. Fractions were collected at 0.5-min intervals. P_i, dRp, and the β-elimination products eluted after 3–4 min and dTMP eluted after 9.5–11 min.

Source of Enzymes. E. coli dRpase was purified as described previously (Franklin & Lindahl, 1988). DNase IV was purified from HeLa cells as described for "factor pL" (Guggenheimer et al., 1984) by gel filtration, single-stranded DNA–cellulose chromatography, and (carboxymethyl)cellulose chromatography. One unit of enzyme was defined as the amount that released 1 pmol of [^{32}P]dTMP from [5'-^{32}P]oligo(dT)·poly(dA) in 15 min.

Cell Lines. Human lymphoblastoid cell lines GM 0892B, GM 1310B, and GM 6315A from normal individuals, GM 1311A from an individual with myeloma, and GM 2782A from an individual with ataxia-telangiectasia, were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Cells were grown in suspension culture at 37 °C in RPMI 1640 medium supplemented with 10% fetal calf serum.

Calf thymus cell extracts were made as previously described (Tomkinson et al., 1990) but with buffers containing 0.35 M NaCl in the place of 0.1 M NaCl.

Size Fractionation of Whole Cell Extracts. Human lymphoblastoid cells (5 × 10^8) were collected by centrifugation and washed twice in phosphate-buffered saline at 4 °C. The cells were resuspended in 500 μL of 0.35 M NaCl/50 mM Tris-HCl (pH 8.0)/1 mM DTT/1 mM EDTA/10% glycerol (buffer A), supplemented with the protease inhibitors 1 mM phenylmethanesulfonyl fluoride, 10 μM leupeptin, 1 μM pepstatin, and 10 μM chymostatin and aprotinin (1.9 μg/ml), and disrupted by sonication for 3 periods of 5 s. After 1 h on ice, the lysate was centrifuged at 10000*g* for 30 min at 4 °C and batch adsorbed to 250 mg of DEAE-cellulose (Whatman DE52) (preequilibrated with buffer A) to remove nucleic acids. The resin was removed by centrifugation at 10000*g* for 30 min at 4 °C, and 200 μL of the supernatant (approximately 2.5 mg of protein) was immediately applied to a Superose 12 FPLC column (Pharmacia) equilibrated with ice-cold 0.5 M NaCl/50 mM Tris-HCl (pH 8.0)/1 mM DTT/1 mM EDTA/10% glycerol (buffer B). Proteins were eluted with the same buffer at a flow rate of 0.4 mL/min, and 0.2 mL fractions were collected. Fractions were assayed for dRpase activity and protein concentrations were estimated by the Coomassie Blue method (Bradford, 1976).

Partial Purification of Human dRpase. Nuclear extracts of human lymphoblastoid cells were prepared as described (Jehn et al., 1972). GM 6315A cells (7 × 10^8) were washed in phosphate-buffered saline at 4 °C, suspended in 70 mL of 0.25 M sucrose/10 mM Tris-HCl (pH 7.2)/1 mM $MgCl_2$/0.5 mM $CaCl_2$/0.5% Nonidet P-40 and incubated on ice for 10 min. After centrifugation for 7 min at 1000*g* and 4 °C, the cytoplasmic fraction was removed, and the nuclear pellet was resuspended in 500 μL of buffer A supplemented with protease inhibitors as above and incubated on ice for 1 h with occasional agitation. After centrifugation (fraction I) and batch adsorption to DEAE-cellulose, two aliquots (200 μL) were applied in two consecutive runs to a Superose 12 FPLC column, and fractions were collected as above and assayed for dRpase activity and protein concentration. Peak fractions from the two eluates were pooled (fraction II) and applied to an 0.5 × 4 cm phenyl-Sepharose (Pharmacia) column equilibrated with buffer B. Stepwise elution was performed with use of 20 mL of buffer B, 20 mL of buffer B containing 0.1 instead of 0.5 M NaCl and 20% ethylene glycol, and finally 20 mL of buffer B containing 0.02 M NaCl and 60% ethylene glycol. Fractions (2.0 mL) were collected and assayed for dRpase activity and protein concentration. The dRpase activity was recovered in the 0.1 M NaCl fraction (fraction III).

Results

Nonenzymatic Release of 5'-Terminal dRp. In order to assess the stability of 5'-terminal dRp residues at incised AP sites, the rate of spontaneous release of sugar phosphate residues from such sites was measured. In agreement with previous investigations (Grafstrom et al., 1982; Bailly & Verly, 1989), nonenzymatic liberation of terminal residues was observed from poly(dA-dT) containing incised ^{32}P-labeled AP sites. However, this reaction was slow, with approximately 3% of the 5'-terminal dRp residues being released from the incised AP sites per hour at pH 8.0 (Figure 2), indicating a half-life of 5'-terminal dRp residues of approximately 30 h at 37 °C and pH 8.0. Grafstrom et al. (1982) obtained similar results and observed release of approximately 20% of the 5'-terminal dRp residues after incubation of a polynucleotide containing incised AP sites at 37 °C and pH 7.6 for 6 h. Bailly and Verly (1989) reported a shorter half-life of 2 h at 37 °C and pH 8.2. Internal AP sites in DNA are cleaved by β-elimination at 37 °C and pH 7.4 with a half-life of approximately 200 h, or 50-100 h in the presence of Mg^{2+} and basic amino acids (Lindahl & Andersson, 1972). These data show that the phosphodiester bond on the 3'-side of a dRp residue is more labile at a terminal than at an internal site but that the bond would still persist for many hours under "physiological" solvent conditions.

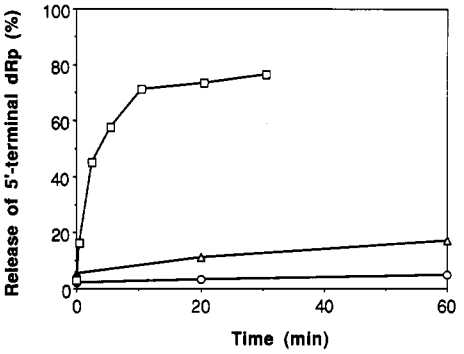

FIGURE 2: Kinetics of excision of 5′-terminal dRp from poly(dA-dT) containing incised 5′-^{32}P-labeled AP sites. The polynucleotide (1 ng, 3000 cpm) was incubated at 37 °C for the times shown in reaction mixtures (100 μL) containing 50 mM Hepes–NaOH (pH 8.0), 10 mM $MgCl_2$, and 1 mM dithiothreitol supplemented with (○) no addition, (△) 0.5 mM spermidine, or (□) 10 μg of protein from fraction II of GM 1311A whole cell extract.

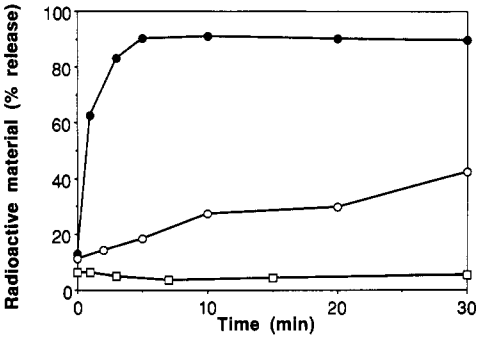

FIGURE 3: Kinetics of release of 5′-terminal dRP residues from polydeoxynucleotides by DNase IV. Reaction mixtures contained 50 mM Hepes–NaOH (pH 8.2), 3 mM $MgCl_2$, 1 mM dithiothreitol, polynucleotide substrate (1 ng, 5000 cpm), and 0.05 unit of DNase IV and were incubated at 37 °C. Data shown represent (□) release of 5′-terminal dRp in free form from poly(dA-dT) containing ^{32}P-labeled incised AP sites, (○) release of TCA-soluble radioactive material (i.e., dRp and small oligonucleotides containing dRp) from the same polynucleotide, or (●) release of TCA-soluble radioactive material from [5′-^{32}P]oligo(dT)·poly(dA).

Spermidine, a polyamine abundantly present in the cell nucleus, promotes the cleavage of internal AP sites by β-elimination (Lindahl & Andersson, 1972). In this regard, it was the most effective small molecule tested. To ascertain whether similar data would be obtained for incised AP sites, poly(dA-dT) containing incised 5′-^{32}P-labeled AP sites was incubated with 0.5 mM spermidine in the standard dRpase reaction mixture. Approximately 17% of the 5′-terminal dRp was released in 1 h (Figure 2). The data indicate a moderate (5-fold) increase in the rate of release of 5′-terminal dRp from AP sites in the presence of spermidine. The activity was inefficient compared to that of human cell extracts (data not shown) or partially purified protein fractions (Figure 2).

Excision of 5′-Terminal dRp Residues at AP Sites by DNase IV. The 5′→3′ exonuclease DNase IV catalyzes the release of a mixture of mononucleotides and dinucleotides from the 5′-ends of intact double-stranded DNA and larger oligonucleotides from DNA containing nonconventional residues such as pyrimidine dimers (Lindahl, 1971a) or ribonucleotides at 5′-termini (Goulian et al., 1990). We investigated the activity of DNase IV purified from HeLa cells on poly(dA-dT) containing 5′-terminal dRp residues at incised AP sites. Incubation of 0.05 unit of DNase IV with this substrate for 30 min under standard enzyme assay conditions did not result in a detectable increase in the amount of TCA- and Norit-

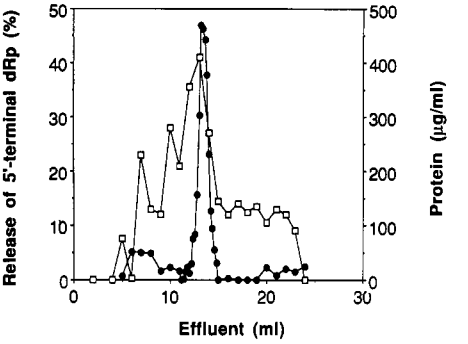

FIGURE 4: Release of dRp by size-fractionated human cell extract. A whole cell extract of GM 1311A lymphoblastoid cells was prepared and fractionated on a Superose 12 FPLC column (as described under Materials and Methods). One microliter of each fraction was assayed for release of dRp from poly(dA-dT) containing incised AP sites. (□) Protein concentration; (●) 5′-terminal dRp residues excised from the polynucleotide substrate. The column was precalibrated with thyroglobulin (670 kDa), γ-globulin (158 kDa), [*methyl*-^{14}C]bovine serum albumin (69 kDa) (NEN), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin D (1.4 kDa) (Bio-Rad).

soluble radioactive material generated (Figure 3). Thus, DNase IV does not excise 5′-terminal dRp residues as free sugar phosphates from incised AP sites. However, 0.05 unit of DNase IV catalyzed the release of approximately 40% of the 5′-terminal dRp moieties in a TCA-soluble form from poly(dA-dT) containing incised ^{32}P-labeled AP sites in 30 min (Figure 3). Under the same conditions, 0.05 unit of DNase IV excised the 5′-terminal nucleotide residues from [5′-^{32}P]-oligo(dT)·poly(dA) to an extent of more than 50% in 1 min and 90% in 5 min (Figure 3). In several experiments, DNase IV was 10–40 times more active on [5′-^{32}P]oligo(dT)·poly(dA) than on poly(dA-dT) containing incised AP sites with 5′-terminal [^{32}P]dRp residues. HPLC analysis of the radioactive products obtained from the latter substrate indicated that the 5′-terminal dRp residues were released as part of both dinucleotides and trinucleotides by DNase IV.

Grondal-Zocchi and Verly (1985) reported that DNase IV purified from rat liver chromatin by phosphocellulose and hydroxyapatite chromatography acted as a weak catalyst of β-elimination and suggested that this reaction might be of relevance with regard to excision of dRp residues. We have made similar observations with partly purified preparations of DNase IV. However, the β-elimination activities can be completely removed from active DNase IV by single-stranded DNA–cellulose and (carboxymethyl)cellulose chromatography (data not shown). These weak β-elimination catalysts are possibly basic nuclear proteins but have not been further characterized. Thus, DNase IV is unable to liberate free sugar phosphate residues from incised AP sites either by hydrolysis or by β-elimination, but the enzyme catalyzes the slow release of such residues as part of small oligonucleotides.

Identification and Partial Purification of a Human dRpase Activity. When poly(dA-dT) containing preincised AP sites with radioactively labeled 5′-dRp residues was incubated with soluble extracts from human lymphoblastoid cells, the 5′-terminal dRp residues were rapidly excised (data not shown). Extracts of HeLa cells and calf thymus seemed slightly less active but also yielded similar results. In order to further characterize the activities involved, cell extracts were size fractionated by gel chromatography. A single major peak of dRp-excising activity was observed. This activity eluted immediately after the major protein peak (Figure 4). An activity of the same size was present in similar amounts in extracts from several different human lymphoblastoid cell lines (GM 0892B, GM 1310B, GM 1311A, GM 2782A, and GM

Table I: Partial Purification of dRpase from Human Lymphoblastoid Cells (7×10^8 GM 6315A Cells)

fraction	protein (μg)	sp act.[a] (units/mg)	total act. (units)
I nuclear extract	1100	2.5	2.8
II Superose 12	60	15	0.9
III phenyl-Sepharose	6	33	0.2

[a] One unit of dRpase activity was defined as the amount of enzyme catalyzing the excision of 1 pmol of [^{32}P]dRp from poly(dA-dT) containing incised AP sites in 30 min.

6315A). By comparison with reference proteins, the activity had an apparent molecular mass of approximately 47 kDa, assuming that it was a typical globular protein.

In order to investigate the subcellular localization of the dRpase, lymphoblastoid cell nuclei were prepared in isotonic sucrose and nuclear and cytoplasmic extracts were assayed separately. About 80% of the dRpase activity but only 20% of the total protein was present in the nuclear extract. On size fractionation of a nuclear extract, a distinct peak of dRp-excising activity was again observed at a molecular mass of 45–50 kDa. The elution profile of the nuclear extract was similar to that of the whole cell extract. In some experiments, a smaller peak of β-eliminating activity associated with a larger protein (200–300 kDa) was also observed (data not shown).

The dRpase activity was purified from the nuclear extract (Table I). Peak fractions from size fractionation experiments were pooled (fraction II) and purified by hydrophobic chromatography on phenyl-Sepharose. The dRpase activity was retained by this column whereas 33% of the total protein and a number of contaminating activities did not adsorb. The latter included all detectable DNase IV activity and >99% of the AP endonuclease activity of the extract. The dRpase was subsequently eluted by buffer B containing 20% ethylene glycol and 0.1 M NaCl (fraction III), while most of the adsorbed protein remained bound to the column. The peak fraction (fraction III) was recovered and stored at −20 °C.

A major difficulty in these purification experiments was the lability of the dRpase activity. Fraction III from nuclei or whole cells lost 50% of its activity in approximately 30 h at −20 °C. Attempts to stabilize the activity by omitting EDTA or including additional protease inhibitors, neutral detergents, or high concns. of glycerol in buffers were unsuccessful. Thus, the rapid partial purification procedure described here was employed to obtain moderate yields of total activity.

Fraction III containing dRpase activity had no detectable 5′→3′ exonuclease activity (<3 units/mg) or 5′-phosphatase activity (<1 pmol of P_i released by 1 mg of protein from poly(dA-dT) containing incised 5′-^{32}P-labeled AP sites). Moreover, fraction III contained only a trace amount of AP endonuclease activity, corresponding to 0.5–1% of the AP endonuclease activity of fraction II.

Mechanism of Action of Human dRpase. Fraction III catalyzed the efficient release of 5′-dRp residues from incised AP sites. To determine whether the enzyme would also liberate sugar phosphate residues from 3′-termini, we investigated the activity of fraction III on poly(dA-dT) containing ^{32}P-labeled AP sites incised by the *E. coli* Nth protein. Fraction III showed no detectable activity on this substrate (<1 unit/mg). It would appear from these experiments that the mammalian enzyme is specific for 5′-terminal dRp residues at preincised AP sites in DNA.

The human dRpase might liberate 5′-terminal dRp residues from DNA either by promoting β-elimination or by catalyzing the hydrolysis of the phosphodiester bond 3′ to the 5′-terminal dRp. In order to define the mechanism of action of the activity, assays were performed in the presence of 0.1 M sodium thioglycolate to facilitate product analysis [as described by Bricteux-Gregoire and Verly (1989)] and the reaction products analyzed by anion-exchange HPLC. The materials released from the standard substrate by the mammalian activity, by spermidine, which promotes β-elimination, and by the *E. coli* dRpase, which is known to act by hydrolysis (Franklin et al., 1988), were compared. Figure 5 shows data for the release of 5′-terminal sugar phosphates after incubation of poly(dA-dT) containing incised ^{32}P-labeled AP sites with either 2 mM spermidine, 0.002 unit of *E. coli* dRpase, or 0.004 unit of human dRpase (from GM 1311A cells). The HPLC profile of the products obtained by incubation with spermidine showed a double peak eluting at 9–10 min and 11.5–13 min. This might be due to the formation of addition products of sodium thioglycolate with both compounds B and C (Figure 1) (Jones et al., 1968). Alternatively, the double peak may represent the addition product of compound B and sodium thioglycolate and another addition compound formed with spermidine (Mazumder et al., 1991). In contrast, the product of incubation of the polynucleotide substrate with *E. coli* dRpase coeluted with unlabeled authentic dRp on anion-exchange chromatography and clearly represented the hydrolysis product. Similarly, the material released by the human activity coeluted with unlabeled dRp and with the product of the *E. coli* dRpase on anion-exchange chromatography. It also cochromatographed with unlabeled dRp on reverse-phase HPLC. These data confirm that the product excised by the *E. coli*

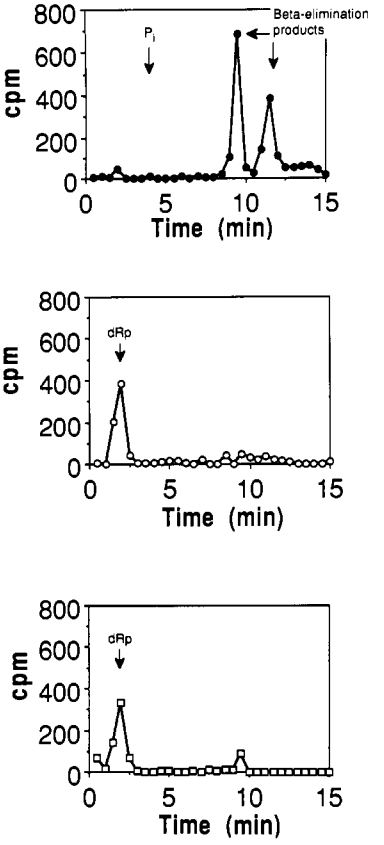

FIGURE 5: Product analysis by ion-exchange HPLC of residues released from 5′-termini at AP sites. Samples in standard reaction mixtures supplemented with 0.1 M sodium thioglycolate were loaded on to a Brownlee MPLC AX cartridge column, and radioactive material was eluted with 5 mL of 25 mM KH_2PO_4, (pH 3.5) followed by 10 ml of 250 mM KH_2PO_4, (pH 3.5) at a flow rate of 1 mL/min. Elution profiles are shown for reactions with (●) spermidine (2 mM), (○) *E. coli* dRpase (0.002 unit); or (□) fraction II (0.004 unit of dRpase) from the human lymphoblastoid cell line GM 1311A.

dRpase from incised AP sites is identical with dRp and further show that the human enzyme also catalyzes the release of dRp by hydrolysis of the phosphodiester bond 3′ to the incised 5′-AP site.

No single major activity that acted by β-elimination was identified in human or calf thymus cell extracts. Instead, there seemed to be numerous minor activities probably associated with different basic proteins and polyamines, which slowly promoted the release of 5′-terminal sugar phosphates (Figure 4).

No dRpase activity was detected in fraction III (<0.4 unit/mg) when reactions were performed in the absence of $MgCl_2$. The optimal Mg^{2+} concentration was 10 mM. The dRpase activity was similar in reaction mixtures containing 0 or 50 mM NaCl but was 3-fold reduced in the presence of 100 mM NaCl. The pH optimum of the human dRpase activity was 8.0, with 50% of the activity retained at pH 8.8 and 35% at pH 6.8. In the latter regard, the human enzyme is different from *E. coli* dRpase, which is optimally active at pH 6.8 (Franklin & Lindahl, 1988).

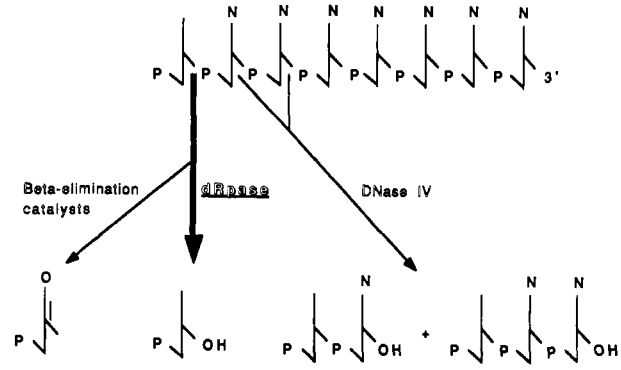

FIGURE 6: Modes of excision of 5′-terminal dRp residues from AP sites in human cells. The major activity in nuclear extracts, DNA deoxyribophosphodiesterase (dRpase), hydrolyzes the phosphodiester bond 3′ to the AP site, catalyzing the release of free dRp. Polyamines and basic proteins promote the slow release of 4-hydroxy-2-pentenal 5-phosphate by β-elimination. DNase IV, the major 5′→3′ exonuclease, excises the 5′-terminal dRp as part of a small oligonucleotide, but this reaction appears to be less efficient than the excision of free dRp by dRpase.

Discussion

The loss of a base residue from DNA weakens the phosphodiester bond 3′ to the abasic site by making the bond susceptible to β-elimination. This β-elimination process is promoted by polyamines and other basic molecules. Nevertheless, it may be estimated from the present experiments and previous data (Lindahl & Andersson, 1972; Grafstrom et al., 1982) that nonenzymatic cleavage of this bond would require 5–50 h under physiological conditions, either before or after enzymatic hydrolysis of the more stable phosphodiester bond 5′ to the lesion. Such estimates contrast with the rapid repair of AP sites in vivo. Moran and Ebisuzaki (1987) showed that after the introduction of large numbers of AP sites in human cells following exposure to ionizing radiation apparently complete DNA repair with conversion of the AP sites to alkali-stable nucleotide residues occurred in 2–4 min. No transient accumulation of chain breaks was detected during the removal of the AP sites, indicating that the postincision steps were efficient and not rate-limiting in the repair process. This indicates that enzyme-catalyzed chain cleavage occurs at AP sites in DNA, first by an AP endonuclease acting on the 5′-side of the lesion and then by an excision activity that removes the abasic residue.

AP endonucleases have been extensively characterized by biochemical and genetic methods (Doetsch & Cunningham, 1990), whereas the excision function(s) has received scant attention. The most active excision activity for the relevant lesion in soluble extracts of human cell nuclei, as well as in *E. coli* (Franklin & Lindahl, 1988), is a dRpase that catalyzes the hydrolytic release of 5′-terminal dRp residues in free form from incised AP sites in DNA (Figure 6). A likely fate of the excised dRp residue in vivo might be subsequent degradation to D-glyceraldehyde 3-phosphate and acetaldehyde by dRp aldolase (Racker, 1952). The human dRpase resembles the *E. coli* activity in being a Mg^{2+}-requiring enzyme of about 50 kDa that is free from detectable exonuclease activity.

A number of alternative routes of enzymatic excision of dRp residues also occur (Figure 6). It is shown here that the mammalian 5′→3′ exonuclease, DNase IV, can release 5′-terminal dRp residues as part of small oligonucleotides, but an incised AP site seems to be a poor substrate for this enzyme. Similarly, the 5′→3′ exonuclease function of *E. coli* DNA polymerase I apparently has some ability to remove 5′-terminal dRp residues in oligonucleotide form (Gossard & Verly, 1978; Au et al., 1989) although the excision product has not been isolated or characterized. Furthermore, the *E. coli* UvrABC nuclease can cleave DNA containing either AP sites or such sites preincised by an AP endonuclease to generate an oligonucleotide containing the lesion, but this is an inefficient process compared to the action of the enzyme at pyrimidine dimers and other bulky DNA lesions (Lin & Sancar, 1989; Snowden et al., 1990). The corresponding human activity may act in a similar fashion. A number of mammalian exonucleases other than DNase IV also have been suggested to have a possible role in the excision of dRp residues from DNA (Bose et al., 1978; Mosbaugh & Linn, 1983; Ivanov et al., 1988).

In all cases investigated, 5′→3′ exonucleases liberate 5′-terminal dRp residues as part of small oligonucleotides, since they are unable to release such residues as free dRp. Consequently, a minimum repair patch size of two nucleotides would occur if an exonuclease was involved in the removal of a dRp residue from an AP site. In contrast, it may be predicted that the concerted action of an AP endonuclease and a dRpase at an AP site would only generate a gap of a single nucleotide. More detailed studies of the patch size during cellular repair of AP sites might help to clarify the relative importance of dRpase and exonucleases in the excision process. Repair of AP sites occurs by very short patch excision–repair (Regan & Setlow, 1974), but it is at present unclear whether only one or 2–4 residues are replaced. In this regard, Wiebauer and Jiricny (1990) found that repair of an AP site, apparently generated as an intermediate during mismatch correction of a G-T base pair by a nuclear extract from HeLa cells, was associated with the replacement of only a single nucleotide residue. In apparent contrast, Matsumoto and Bogenhagen (1989) observed that repair of a reduced analogue of an AP site by a *Xenopus laevis* oocyte extract occurred in a small patch of not more than 3–4 nucleotides, but significant repair synthesis also occurred at the nucleotides immediately adjacent to the AP residue. A possible problem with the latter experiment is that the tetrahydrofuran analogue of an abasic site employed might not be a substrate for *X. laevis* dRpase, since excision of this residue seemed inefficient and rate-limiting under the experimental conditions used. In addition, the unusually high concentration of DNA replication enzymes present in a *X. laevis* oocyte extract may have resulted in short strand displacement DNA synthesis at the 3′-OH terminus (Mosbaugh & Linn, 1982) affecting the normal repair process.

Repair of AP sites in DNA can occur by initial cleavage on the 3′-side of the lesion by an AP lyase activity, followed by excision of the 3′-terminal baseless residue by an AP endonuclease or 3′→5′ exonuclease. A problem with this pathway is that the unsaturated ring-opened terminal residue (Figure 1B) is highly reactive and might form cytotoxic lesions such as DNA–protein cross-links (Lefrancois et al., 1990; Bricteux-Gregoire & Verly, 1991; Mazumder et al., 1991). This complication is avoided in the major repair pathway, which employs initial hydrolytic cleavage on the 5′-side of the lesion by an AP endonuclease followed by enzymatic excision. The dRpase described here is a likely candidate for this excision function in human cells.

Registry No. dRpase, 119940-18-0.

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