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DNA deoxyribophosphodiesterase

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A previously unrecognized enzyme acting on damaged termini in DNA is present in *Escherichia coli*. The enzyme catalyses the hydrolytic release of 2-deoxyribose-5-phosphate from single-strand interruptions in DNA with a base-free residue on the 5′ side. The partly purified protein appears to be free from endonuclease activity for apurinic/apyrimidinic sites, exonuclease activity and DNA 5′-phosphatase activity. The enzyme has a mol. wt of ~50 000–55 000 and has been termed DNA deoxyribophosphodiesterase (dRpase). The protein presumably is active in DNA excision repair to remove a sugar-phosphate residue from an endonucleolytically incised apurinic/apyrimidinic site, prior to gap filling and ligation.

Key words: DNA repair/AP endonucleases/2-deoxyribose-5-phosphate/*Escherichia coli*

Introduction

The loss of an individual base residue from DNA causes the formation of an apurinic/apyrimidinic (AP) site. These frequently occurring DNA lesions are generated by non-enzymatic hydrolysis of base–sugar bonds, a reaction known to proceed at a significant rate at neutral pH, and by the enzymatic liberation of altered base moieties. In the latter process, at least six different DNA glycosylases convert a variety of damaged nucleotide residues to AP sites by removing deaminated, oxidized or alkylated bases from DNA. Uracil misincorporated instead of thymine is also corrected in the same fashion. The resulting AP sites, which are devoid of genetic information, have cytotoxic and mutagenic effects (Lindahl, 1982; Loeb and Preston, 1986).

AP sites in DNA are removed by an efficient excision-repair process, occurring both in prokaryotes and eukaryotes. This repair pathway is shown in Figure 1. A single-strand break is first introduced on the 5′ side of a base-free deoxyribose-phosphate residue through phosphodiester bond hydrolysis by an AP endonuclease. Several such enzymes from different sources have been characterized in detail. In *Escherichia coli*, the reaction is catalysed either by the endonucleolytic function of exonuclease III or by the inducible endonuclease IV (Cunningham *et al.*, 1986; Chan and Weiss, 1987; Levin *et al.*, 1988). The major mammalian AP endonucleases also act in a similar fashion (Mosbaugh and Linn, 1982; Weiss and Grossman, 1987). The sugar-phosphate residue is then excised to generate a short gap which appears usually to remain the size of a single nucleotide (Kataoka and Sekiguchi, 1982). The missing nucleotide residue is then replaced by a DNA polymerase, and the chain is joined by a DNA ligase.

The post-incision event of deoxyribose-phosphate removal at an AP site has not been well characterized, although this step would seem to be obligatory prior to gap filling and ligation. One possiblity might be that an exonuclease degrading DNA in the 5′→3′ direction could excise the sugar-phosphate residue. However, the main candidate for this role in *E.coli*, the 5′ exonuclease function of DNA polymerase I, is blocked by a 5′-terminal sugar-phosphate residue at a strand break in DNA. The polymerase is unable to perform nick-translation of a template with 5′ deoxyribose-phosphate termini, but can instead displace such parental strands during DNA synthesis at pH 7.5 (Mosbaugh and Linn, 1982). Release of the terminal deoxyribose-phosphate residue as part of a di- or trinucleotide can, however, be achieved *in vitro*, at pH 9.2, by employing high concentrations of purified DNA polymerase I (Gossard and Verly, 1978). Another possibility for the liberation of a deoxyribose-phosphate residue from DNA would result from cleavage by AP endonucleases on both sides of the base-free site. However, no AP endonuclease with the ability to hydrolyse the phosphodiester bond on the 3′ side of the lesion has so far been detected. A putative candidate, *E.coli* endonuclease III, has been shown to be a DNA glycosylase acting on pyrimidine hydrates, and this basic protein can also promote a β-elimination reaction at AP sites in DNA (Bailly and Verly, 1987; Kim and Linn, 1988). It is not known whether such β-elimination occurs to a significant extent *in vivo*. Cleavage by endonuclease III only accounts for a very minor proportion of DNA chain breakage at AP sites in crude cell-free extracts (Lindahl, 1982; Cunningham and Weiss, 1985).

In the present study, we have investigated the enzymatic release of radioactively labelled deoxyribose-phosphate residues from polydeoxyribonucleotide 5′ termini by *E.coli* extracts. The major activity is due to a previously unrecognized function which is neither a nuclease nor a DNA 5′-phosphatase. We have termed the enzyme DNA deoxyribophosphodiesterase, or dRpase.

Results

E.coli DNA polymerase I and endonuclease III do not remove 2-deoxyribose-5-phosphate at an incised AP site

A radioactive polynucleotide with selectively labelled sugar-phosphate residues at incised AP sites was prepared according to the first steps of the repair pathway shown in Figure 1. Alternating poly(dA-dT) was synthesized with *E.coli* DNA polymerase I in the presence of dATP, TTP and [α-^{32}P]dUTP or [^{3}H]dUTP. The polymer was subsequently treated with *E.coli* uracil–DNA glycosylase and endonuclease IV to generate incised AP sites with a base-free sugar-phosphate residue on the 5′ side (Clements *et al.*,

1978). The enzymatic release of ^{32}P- or ^{3}H-labelled deoxyribose-phosphate residues from the polymer was determined by direct analysis of the reaction mixture with anion-exchange HPLC, or by following the release of radioactively labelled acid-soluble material that did not adsorb to Norit charcoal.

When the polymer substrate was incubated with a high concentration (50 U/ml) of DNA polymerase I for 30 min at 37°C under standard assay conditions at pH 7.8, no deoxyribose-phosphate (<1%) was released in free form from the substrate. However, 25% of the radioactive material was converted to acid-soluble oligonucleotides. DNA polymerase I at a concentration similar to that present in a crude cell extract of *E.coli* (0.5 U/ml) did not release significant amounts (<5%) of oligonucleotide material from 5′ termini. These data indicate that the 5′→3′ exonuclease function of DNA polymerase I cannot release free deoxyribose-phosphate from 5′ termini in DNA, and that the enzyme is relatively ineffective in bypassing the lesion to excise an oligonucleotide. This is in agreement with previous results by Mosbaugh and Linn (1982).

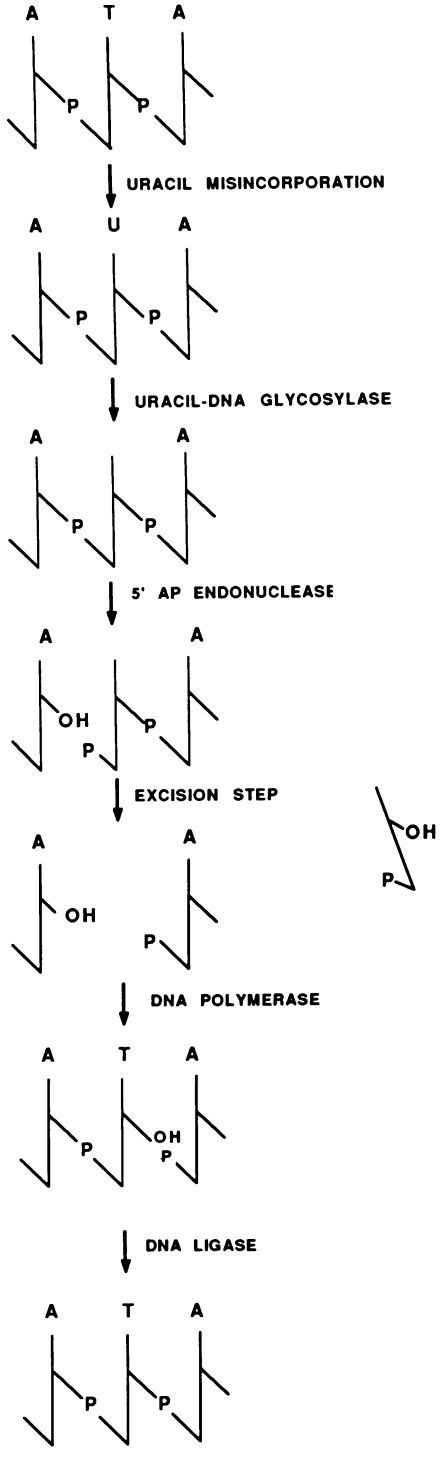

Fig. 1. The DNA base excision repair pathway for the removal of altered bases and AP sites. The excision of a misincorporated uracil residue from one strand in the DNA double helix is shown.

The effect of the *E.coli* pyrimidine hydrate−DNA glycosylase/endonuclease III on the 5′ labelled polymer substrate was also evaluated. Under standard reaction conditions, 5 U of the enzyme liberated <1% of the radioactive material as deoxyribose-phosphate (see below, Figure 4). Another DNA glycosylase with an associated ability to promote β-elimination at AP sites, *Micrococcus luteus* pyrimidine dimer−DNA glycosylase, also failed to release 5′-terminal sugar-phosphate residues from the polymer substrate (<1% release by 70 U). Thus, enzymes of this type seem unable to liberate deoxyribose-phosphate residues from previously incised AP sites.

We observed that deoxyribose-phosphate residues at non-incised AP sites could be removed by first exposing the polymer to endonuclease III, in order to allow β-elimination on the 3′ side of the base-less residue, followed by hydrolytic release of the 3′ sugar-phosphate by exonuclease III. These results are in agreement with data by others (Kim and Linn, 1988; Bailly and Verly, 1987).

Active release of deoxyribose-phosphate residues

Crude cell extracts of several *E.coli* strains were found to contain an activity that could release free 2-deoxyribose-5-phosphate from the 5′ termini of incised AP sites in the polynucleotide substrate (Figure 2). Under the standard assay conditions, 10−20% release of 2-deoxyribose-5-phosphate was achieved with 10 μg extract protein from different strains. A smaller, variable amount of free phosphate was also liberated, presumably due to a separate phosphatase activity. No radioactive free dUMP could be detected in the solubilized material by reverse-phase HPLC analysis.

Similar levels of active release of 2-deoxyribose-5-phosphate residues were observed with cell extracts from several different wild-type, *polA*, and *nth* strains, including a strain that overproduced the nth^{+} gene product. These results confirm that DNA polymerase I and endonuclease III do not seem responsible for the release of deoxyribose-phosphate residues. Furthermore, the activity was present at an unchanged level in *xon*, *xth*, *xse* and *alkB* mutants and, consequently, it could not be ascribed to the exonucleases I, III or VII or the AlkB protein. In summary, the data indicate that *E.coli* contains a deoxyribophosphodiesterase (dRpase) activity for the release of deoxyribose-phosphate from 5′ incised AP sites in DNA which is not identical with a previously known nuclease.

Purification of dRpase

The dRpase activity has been purified 430-fold from an *E.coli polA xth* strain. The procedure is summarized in Table I. On gel-filtration chromatography, the dRpase activity eluted in the 50−60 kd range (Figure 3). This purification

step removed most of the contaminating DNA phosphatase activity, which was of higher mol. wt, as well as several nucleases. The dRpase activity did not adsorb to single-stranded DNA cellulose, and this property was employed to remove contaminating enzymes acting on DNA. Following SDS–PAGE, the most purified enzyme fraction (fraction V) showed four protein bands, all within the 45 000–60 000 mol. wt range. The sedimentation coefficient of the dRpase activity was ~4.6S, as determined by sucrose gradient centrifugation with bacterial alkaline phosphatase, carbonic anhydrase and lysozyme as references (Martin and Ames, 1961). This value indicates a mol. wt of ~55 000 for a globular protein. Analytical gel filtration (Siegel and Monty, 1966) of the dRpase activity (fraction V) together with bacterial alkaline phosphatase, bovine serum albumin, carbonic anhydrase and cytochrome *c* also indicated a mol. wt of dRpase of 50 000–55 000 (data not shown)

Enzymatic properties of dRpase

A number of experiments were performed with fraction V of the purified enzyme. 2-Deoxyribose-5-phosphate was released from the standard polymer substrate by dRpase as a function of time, whereas purified endonuclease III was unable to catalyse such release (Figure 4). The rate of release

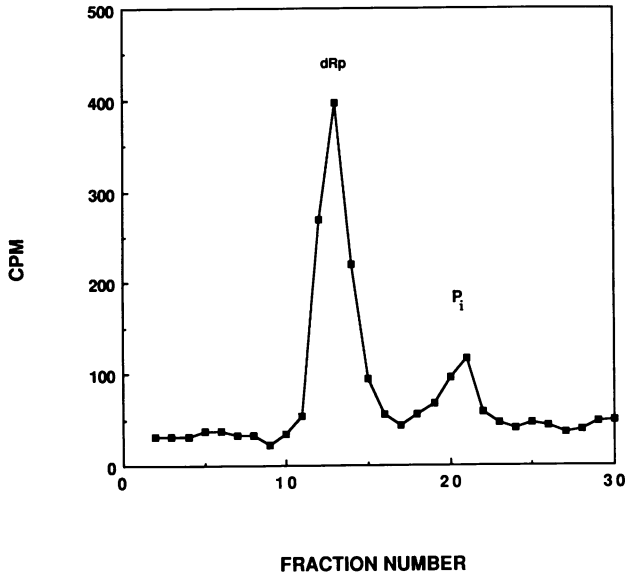

Fig. 2. Separation by SAX HPLC of released radioactive material following incubation of the ^{32}P-labelled polynucleotide substrate containing incised AP sites with a crude cell lysate of *E.coli* strain AB1157. The elution position of 2-deoxyribose-5-phosphate, as determined by the diphenylamine reaction, is shown as dRp; the elution position of inorganic phosphate, as determined with authentic $^{32}PO_4$, is denoted as P_i.

by dRpase was three times faster at pH 6.5, which appeared optimal, than at pH 7.8 (Figure 4). All the radioactive material excised by dRpase had the chromatographic properties of deoxyribose-phosphate, indicating that the purified enzyme was free from DNA 5′-phosphatase activity (Figure 5, see also Figure 2). The reaction product was identified as authentic 2-deoxyribose-5-phosphate by ion-exchange HPLC, reverse-phase HPLC and paper chromatography. The enzymatic release of the sugar-phosphate by dRpase was absolutely dependent on the presence of Mg^{2+} in the reaction mixture, with an optimal concentration of 10 mM $MgCl_2$, but no other cofactors were required.

In order to define the mechanism of release of deoxyribose-phosphate, the standard polymer substrate was treated with sodium borohydride to reduce the aldehyde group at position 1 of the base-free sugar. The corresponding alcohol is no longer susceptible to release by a β-elimination mechanism. The reduced polynucleotide remained a substrate for dRpase, and the enzymatically solubilized material was identified by anion-exchange HPLC as the reduced form of 2-deoxyribose-5-phosphate (data not shown). We conclude that dRpase acts by hydrolytic cleavage of the phosphodiester bond (see Figure 1), and not by a β-elimination reaction.

The dRpase (fraction V) was free from endonuclease activity. When covalently closed circular plasmid DNA containing about four AP sites per molecule was challenged with increasing amounts of dRpase, no detectable conversion to nicked circles was detected. In contrast, these covalently closed DNA molecules were incised during incubation with *E.coli* endonuclease IV (Figure 6). Thus, the dRpase does not possess an intrinsic AP endonuclease activity. Therefore, the enzyme can only act to remove sugar-phosphate residues from AP sites subsequent to endonucleolytic incision.

The ability of dRpase to act as a DNA 5′→3′ exonuclease at single-strand breaks was assessed with a poly(dA)·oligo-[5′-^{32}P](dT) substrate. Incubation of this polymer with dRpase (10 U, 1 h at 37°C) resulted in no detectable release of [5′-^{32}P]dTMP. Furthermore, incubation of plasmid pBR322 DNA, linearized by *Eco*RI treatment, with dRpase (10 U) caused no detectable inhibition of the subsequent ability of T4 DNA ligase to re-circularize the plasmid DNA (data not shown). These experiments strongly indicate that dRpase does not act as an exonuclease.

Fraction V contained an activity that would release 3′ sugar-phosphate residues after cleavage at AP sites by β-elimination with endonuclease III. The amount of this enzyme activity seemed equivalent to the 5′ dRpase activity. We believe both activities are due to the same protein. However, this point will only be completely settled by purification of dRpase to physical homogeneity. At present, it would appear that dRpase acts to remove any exposed base-free sugar-phosphate residue at strand interruptions in DNA.

Table I. Purification of dRpase from *E.coli*

Fraction	Protein (mg)	Sp. act. (U/mg)	Total activity (U)	Purification
I. Crude extract	1230	3.5	4300	–
II. Polymin-P/ammonium sulphate	185	19.8	3670	6
III. AcA-44	31	107	3320	31
IV. DNA–cellulose/DEAE–Sepharose	3.3	488	1610	140
V. MonoQ	1.0	1500	1440	430

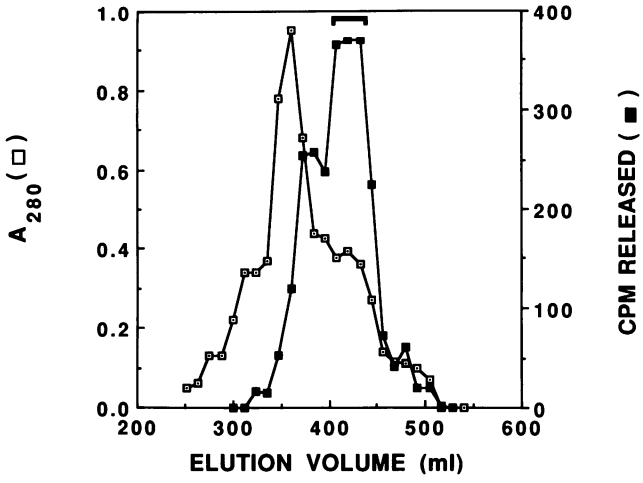

Fig. 3. Gel filtration chromatography of the dRpase activity on Ultrogel AcA-44. The pooled dRpase activity (fraction III) is indicated by the bracket. The release of acid-soluble material following incubation of the polynucleotide containing incised AP sites with fractions eluting from 375–400 ml is due to phosphatase activity.

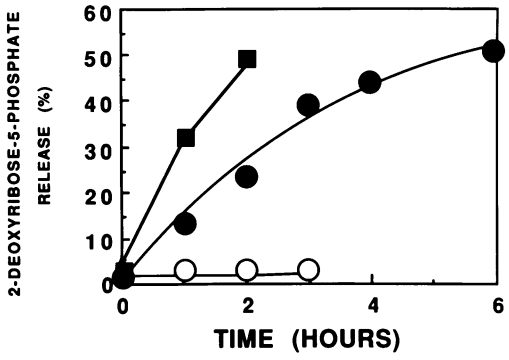

Fig. 4. Kinetics of enzymatic release of 2-deoxyribose-5-phosphate at 30°C. The polynucleotide containing incised AP sites (5000 c.p.m.) was incubated with either 5 U of dRpase (fraction V) in 50 mM Hepes–KOH, pH 7.8, 10 mM $MgCl_2$, 5 mM DTT (●), 5 U of dRpase (fraction V) in 50 mM Hepes–KOH, pH 6.5, 10 mM $MgCl_2$, 5 mM DTT (■), or 5 U of endonuclease III in 50 mM Hepes–KOH, pH 7.8, 1 mM NaEDTA, 5 mM DTT (○). The release of 2-deoxyribose-5-phosphate was determined by precipitation with TCA in the presence of Norit charcoal.

Discussion

The existence of a separate enzyme that catalyses the release of deoxyribose-phosphate residues from 5′ incised AP sites in DNA provides a satisfactory explanation for this excision step in the DNA repair pathway shown in Figure 1. The removal of the sugar-phosphate residue would otherwise constitute a missing link in the postulated pathway, because functions such as the 5′→3′ exonuclease activity of DNA polymerase I or endonuclease III appear unable to undertake this reaction. The situation seems similar with regard to DNA repair in mammalian cells, since we have recently detected and purified a bovine dRpase (unpublished data), and DNA glycosylases and AP endonucleases are already known to occur in cells from higher organisms.

No enzyme of the approximate molecular size of dRpase has been found during previous extensive studies on the nucleases of *E.coli*. The apparent absence of exonuclease

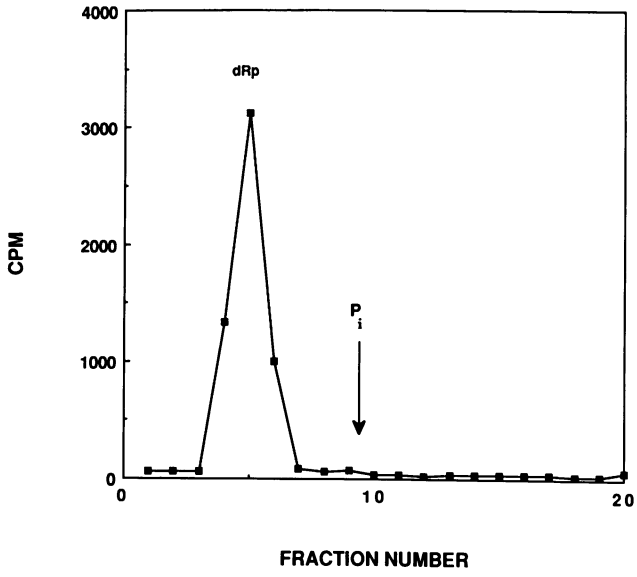

Fig. 5. Release of 2-deoxyribose-5-phosphate by purified dRpase, as determined by MPLC AX HPLC. The polynucleotide containing incised AP sites (5000 c.p.m.) was treated with 5 U of dRpase (fraction V) under standard assay conditions. The elutions of 2-deoxyribose-5-phosphate (dRp) and inorganic phosphate (P_i) were determined as described in the legend to Figure 2. The void volume for this column was 0.5 ml (fraction 1).

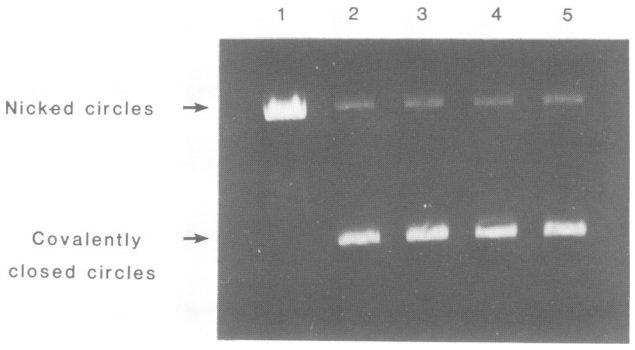

Fig. 6. Reaction of pAT153 DNA containing an average of four AP sites per plasmid with endonuclease IV and purified dRpase (fraction V). Plasmid DNA (0.5 μg) was reacted with either dRpase under standard assay conditions or with endonuclease IV in 50 mM Hepes–KOH, pH 7.8, 1 mM NaEDTA, 5 mM DTT for 30 min at 37°C. The reaction products were resolved by electrophoresis on a 1% agarose gel. **Lane 1**, 100 U endonuclease IV; **lane 2**, no enzyme added; **lane 3**, 5 U dRpase; **lane 4**, 10 U dRpase; **lane 5**, 25 U dRpase.

activity in dRpase readily accounts for the observed 'very short patch' repair of AP sites in DNA, because dRpase would be unable to continue excision at a 5′ terminus by removing nucleotide residues subsequent to the release of a deoxyribose-phosphate moiety. For this reason, it seems likely that the patch size at a repaired AP site is constrained to a single nucleotide residue. This situation is different from the excision-repair of cyclobutane pyrimidine dimers and other DNA lesions causing major helical distortion, because such damage is recognized by the UvrABC nuclease, which introduces strand breaks on either side of the lesion to generate DNA gaps ~13 nucleotide residues in size (Sancar and Rupp, 1983).

The *E.coli* enzymes exonuclease III and endonuclease IV

not only act as efficient AP endonucleases, they can also excise a variety of damaged 3′-terminal moieties, e.g. 3′ sugar-phosphate residues and the 3′-phosphoglycolaldehyde esters, occurring at strand breaks with fragmented sugar residues generated by oxygen radicals (Levin *et al.*, 1988). The latter enzyme activities are required for the removal of lesions that block DNA repair synthesis at 3′ termini. In addition to exonuclease III and endonuclease IV, a third, recently detected enzyme is present in *E.coli* which can excise 3′-phosphoglycolaldehyde esters from DNA. The latter enzyme requires Mg^{2+} for activity and has a mol. wt of ~55 000 (B.Demple, personal communication). Although its action on damaged 5′ termini has not yet been investigated, this protein may well be identical with the dRpase described here, which has a mol. wt of ~50 000–55 000 and apparently has the ability to excise sugar-phosphate residues both from 5′ and 3′ ends at AP sites in DNA. *E.coli* seems well equipped to repair damaged 3′ termini, having access to at least three different enzymes for the purpose. However, two of these, exonuclease III and endonuclease IV, are unable to excise the 5′ 2-deoxyribose-5-phosphate residues they generate when acting as AP endonucleases. Consequently, these enzymes would have to act in concert with an activity such as the 5′ dRpase described here to repair AP sites in DNA.

The assignment of a role in DNA repair to dRpase so far rests on the biochemical evidence for a very restricted substrate specificity. The enzyme effectively catalyses the hydrolytic release of deoxyribose-phosphate from incised AP sites in DNA, but it seems unable to act as an AP endonuclease, exonuclease or phosphatase. These data should now be complemented by genetic experiments, to define further the physiological role of this novel type of enzyme acting on damaged DNA.

Materials and methods

Enzymes and reagents

E.coli uracil–DNA glycosylase (Lindahl *et al.*, 1977), endonuclease III (Breimer and Lindahl, 1984) and endonuclease IV (Ljungquist, 1977) were prepared as described. DNA polymerase I (endonuclease-free, 6600 U/mg) was obtained from Boehringer Mannheim, and *E.coli* exonuclease III, T4 DNA ligase and *Eco*RI restriction nuclease were obtained from New England Biolabs. *M.luteus* pyrimidine dimer–DNA glycosylase was obtained from Applied Genetics Inc., Freeport, NY. Non-radioactive poly(dA-dT) for use as primer-template in polymer synthesis was purchased from Boehringer Mannheim. Poly(dA)·oligo[5′-^{32}P](dT) was prepared as described (Arrand *et al.*, 1986). Plasmid pAT153 DNA was heated at pH 5 and 70°C to introduce an average of four AP sites per molecule as described (Lindahl and Andersson, 1972). 2-Deoxyribose-5-phosphate and single-stranded DNA–cellulose were obtained from Sigma, and Ultrogel AcA-44 was from LKB, Inc.

Radioactive [α-^{32}P]dUTP was prepared by deamination of dCTP. A typical reaction mixture (1 ml) contained 100 μCi of [α-^{32}P]dCTP (Amersham) (400 Ci/mmol) or 100 μCi of [1′,2′,5-^{3}H]dCTP (Amersham) (66 Ci/mmol) in 6 M $NaNO_2$, 1 M sodium acetate pH 4.0. After incubation for 12 h at 37°C, a small amount of Norit charcoal was added and the mixture was centrifuged in an Eppendorf centrifuge for 2 min at room temperature. The Norit pellet was washed four times with water, and then 1 ml of 3% NH_3 in 50% ethanol was added to the Norit pellet and left at room temperature for 20 min. The sample was then centrifuged for 2 min, and the supernatant was passed through an Acro LC13 microfiltration unit (Gelman Sciences) and lyophilized. The conversion of dCTP to dUTP was >99%, as estimated by HPLC.

Polynucleotide-containing incised AP sites

Poly(dA-dT) containing [^{32}P]dUMP residues was synthesized with *E.coli* DNA polymerase I (Schachman *et al.*, 1960; Clements *et al.*, 1978). The polymer contained ~0.02% of its nucleotide residues as [^{32}P]dUMP. The polynucleotide (1 μg) was incubated with 0.03 U of uracil–DNA glycosylase in 50 μl 50 mM Hepes–KOH, pH 8.0, 1 mM NaEDTA, 5 mM DTT. After incubation for 1 h at 37°C, 0.2 M NaCl and 500 U of endonuclease IV were added, and the reaction was allowed to continue for 1 h, followed by heating for 5 min at 65°C. The polymer was precipitated with ethanol, lyophilized, and dissolved in 50 mM Hepes–KOH, pH 7.8, 1 mM NaEDTA.

Bacterial strains and lysates

The following *E.coli* strains were used: AB1157 (wild type), AB3027 (*xth polA*) (Ljungquist *et al.*, 1976), KLC381 (*xse*) (Chase and Richardson, 1974), HK117 (*alkB*) (Kataoka *et al.*, 1983), JG112 (*polA1*) (Gross and Gross, 1969), and NH5049 (*xon*) (Mackay and Linn, 1974). Strains BW372 (*nth*), BW415 (Δ*nth*) and BW531, an overproducer of the *nth*$^{+}$ gene product, were supplied by Dr B.Weiss. Bacteria were grown as 50-ml cultures in Luria broth to late log phase, collected by centrifugation and washed once in extraction buffer (50 mM Tris–HCl, pH 7.8, 300 mM NaCl, 5 mM DTT, 2 mM NaEDTA, 5% glycerol). After centrifugation, the cells were pelleted by centrifugation and suspended in 2 ml of the extraction buffer. The cells were disrupted by sonication, and debris was removed by centrifugation at 10 000 *g* for 30 min. Protein concentrations were determined by the Coomassie-blue method (Bradford, 1976).

Enzyme assays

Reaction mixtures (100 μl) contained the polynucleotide with incised AP sites (5000 c.p.m., 0.3 nmol nt residues), 50 mM Hepes–KOH, pH 7.8, 10 mM $MgCl_2$, 5 mM DTT, 50 μg/ml tRNA, 1 mM Na phosphate and a limiting amount of dRpase. The additions of tRNA and inorganic phosphate served to inhibit DNA endonuclease I (Lehman *et al.*, 1962) and alkaline phosphatase activities in crude extracts, and these compounds were usually excluded in experiments with purified dRpase. Following incubation at 37°C for 30 min, the samples were either directly injected onto an HPLC column fitted with a guard column to adsorb polymeric material (see below), or were precipitated by the addition of 20 μg carrier DNA, 110 μl 10% TCA and 30 μl 5% Norit suspension, and centrifuged in an Eppendorf centrifuge for 15 min at 4°C. Radioactive material contained in the supernatant was measured by liquid scintillation counting, and radioactive material contained in the Norit pellet was determined by Cerenkov counting. One unit of dRpase activity is defined as the release of 1 pmol of 2-deoxyribose-5-phosphate at 37°C in 30 min in the presence of substrate excess. Enzyme assays were usually performed at substrate concentrations below the K_m to limit consumption of the radioactive polymer.

Enzyme purification

The purification procedure is summarized in Table I. *E.coli* AB3027 (*xth polA*) cells were grown in Luria broth in a fermenter, and harvested in late log phase. All enzyme purification steps were performed at 0–4°C. The cells (78 g) were suspended in 140 ml of buffer A (50 mM Tris–HCl, pH 7.8, 5 mM DTT, 2 mM NaEDTA, 5% glycerol) containing 300 mM NaCl and were disrupted by sonication. After centrifugation at 10 000 *g* for 1 h, the supernatant was recovered (fraction I, 150 ml), and a neutralized solution of 5% Polymin-P was slowly added under stirring to a final concentration of 0.4%. After 1 h, the mixture was centrifuged at 10 000 *g* for 30 min. To the recovered supernatant (150 ml), 47 g of ammonium sulphate was added slowly while stirring (50% saturation). After 30 min, the mixture was centrifuged at 10 000 *g* for 30 min. The pellet was suspended in 7 ml of buffer A containing 500 mM NaCl and dialysed against this buffer for 4 h. The dialysed protein solution was centrifuged to remove a small precipitate and this material (fraction II, 11 ml) was applied to an Ultrogel AcA-44 column (2.6 × 150 cm) equilibrated with buffer A containing 500 mM NaCl. The most active fractions (Figure 3) were pooled and dialysed against buffer A overnight (fraction III, 33 ml). The dialysed material was loaded onto a single-stranded DNA–cellulose column (0.8 × 4 cm) which had been equilibrated with buffer A, and the column was washed with 20 ml of buffer A. The entire flow-through material from the DNA–cellulose column was then applied to a DEAE–Sepharose column (1.5 × 8 cm; fast-flow, Pharmacia) which had been equilibrated with buffer A. After washing with 30 ml of buffer A, the column was eluted with a 100-ml gradient from buffer A to buffer A containing 500 mM NaCl. The dRpase activity eluted later than the majority of the proteins on this column, and the most active fractions were pooled and dialysed against buffer B (50 mM Hepes–KOH, pH 7.8, 5 mM DTT, 2 mM NaEDTA, 10% glycerol) overnight (fraction IV, 3.1 ml). This material was injected onto a MonoQ HR 5/5 FPLC column (Pharmacia) eluted with buffer B at a flow rate of 0.25 ml/min. Protein was eluted from the column with a 30-ml gradient from buffer B to buffer B containing 500 mM NaCl at a flow rate of 0.5 ml/min. The most active fractions were pooled. This material was dialysed overnight against 50 mM

Hepes–KOH, pH 7.8, 250 mM NaCl, 5 mM DTT, 2 mM NaEDTA, 50% glycerol and stored in aliquots at −70°C (fraction V).

Product analysis

HPLC separations were performed using either a Whatman 10 μm SAX column (4.6 mm × 25 cm) eluted with 50 mM KH_2PO_4, pH 3.15, at 1 ml/min, a Brownlee MPLC AX (4.6 mm × 3 cm) column eluted with 25 mM KH_2PO_4, pH 3.5, at 1 ml/min, or a Varian MCH-10 (4.6 mm × 25 cm) column eluted with 50 mM KH_2PO_4, pH 4.5, at 1 ml/min. Fractions were collected at 30-s intervals and the radioactivity contained in each fraction was determined. The elution position of 2-deoxyribose-5-phosphate was determined by the diphenylamine reaction (Burton, 1956).

To reduce the majority of base-free sugar sites, the polynucleotide containing incised AP sites (50 000 c.p.m.; 0.3 nmol) was incubated with 300 mM $NaBH_4$ for 30 min at 25°C (500 μl reaction volume) and excess $NaBH_4$ was removed by extensive dialysis against 50 mM Hepes–KOH, pH 7.8, 1 mM NaEDTA. The reduced polynucleotide (5000 c.p.m.) was incubated with 10 U of dRpase (fraction V) under standard assay conditions for 37°C for 30 min, and the sample was injected directly onto a Spherisorb 5-μm amino column (fitted with a guard column) and eluted with a linear KH_2PO_4 (pH 4.5) gradient (10–150 mM). Reaction products were also resolved by paper chromatography (Grafstrom *et al.*, 1982). The reference compounds were authentic 2-deoxyribose-5-phosphate and reduced 2-deoxyribose-5-phosphate, obtained by reduction of ^{32}P-labelled 2-deoxyribose-5-phosphate with $NaBH_4$.

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