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Urea–DNA Glycosylase in Mammalian Cells†

Lars H. Breimer

ABSTRACT: Urea–DNA glycosylase, an enzyme presumed to be active in the repair of DNA damage caused by oxidizing agents, has been identified previously in *Escherichia coli*. This enzyme has now been shown to be present in cell extracts of calf thymus and human fibroblasts. It catalyzes the release of free urea from a double-stranded polydeoxyribonucleotide containing thymine residues fragmented by $KMnO_4$ and NaOH treatment. The calf thymus enzyme has been 400-fold purified and largely separated from previously identified mammalian DNA glycosylases. It has a molecular weight of about 25 000 and requires no cofactors. The identity of the enzymatically released product as unsubstituted urea has been verified by its susceptibility to urease.

The nitrogenous bases of DNA are susceptible to damage by oxidizing agents, and the lesions introduced by treatment with such agents resemble those observed after exposure to ionizing radiation (Howgate et al., 1968; Scholes, 1976; Teoule et al., 1977). This damage may be corrected through an excision-repair process initiated by a DNA glycosylase catalyzing the release of a degraded base residue. Extensive studies on DNA glycosylases have been performed in *Escherichia coli* [reviewed by Lindahl (1982)], and in several cases, similar activities have been demonstrated in mammalian cells. On the other hand, an enzyme such as the pyrimidine dimer–DNA glycosylase of phage T4 infected *E. coli* does not appear to have a counterpart either in uninfected *E. coli* or in mammalian cells (Demple & Linn, 1980). Two different DNA glycosylases which act on fragmented base residues have been detected. One activity liberates a substituted formamidopyrimidine, that is, a purine residue with an opened imidazole ring (Chetsanga & Lindahl, 1979). This formamidopyrimidine–DNA glycosylase has been found in mammalian cells (Margison & Pegg, 1981). Another enzyme catalyzes the release of free urea from an oxidized polydeoxyribonucleotide containing fragmented thymine residues (Breimer & Lindahl, 1980). Here it is shown that an analogous urea–DNA glycosylase is present in bovine and human cells.

Experimental Procedures

Materials. Phosphocellulose P11 was obtained from Whatman and Ultrogel AcA-54 from LKB Products. Single-stranded DNA–cellulose was made according to Alberts & Herrick (1971). Urease (crystalline, 79 units/mg) was purchased from Sigma.

Polydeoxyribonucleotide Substrates. The preparation of a polydeoxyribonucleotide substrate to measure urea–DNA glycosylase activity has been described (Breimer & Lindahl, 1980). Briefly, a poly(dA,[^{14}C]dT) copolymer containing 97% dAMP residues was synthesized with calf thymus terminal transferase and treated with $KMnO_4$ to convert the thymine moieties to a mixture of N-substituted urea and urea moieties.

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The copolymer was then incubated with 0.18 M NaOH at 37 °C for 15 min (see Results) prior to the addition of the complementary poly(dT) strand to generate a double-stranded polydeoxyribonucleotide.

A polydeoxyribonucleotide containing thymine glycol residues was obtained by incubation of the single-stranded poly(dA,[2-^{14}C]dT) copolymer with 7 mM OsO_4 in 0.4 M NH_4OH at 0 °C for 15 min (Burton & Riley, 1966). Excess OsO_4 was removed by ether extraction, and the polymer was extensively dialyzed against 10 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl)/0.2 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.5) prior to addition of a complementary poly(dT) strand. Under these conditions, 60% of the radioactive thymine residues were converted to thymine glycol, as determined by paper chromatography of a formic acid hydrolysate (Teoule et al., 1977).

Enzyme Assays. The standard reaction mixture (50 μL) for urea–DNA glycosylase was composed of 0.1 M KCl/0.07 M *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid–potassium hydroxide (Hepes–KOH) (pH 7.8)/1 mM EDTA/1 mM dithiothreitol/5 μg of double-stranded poly(dA)·poly(dT) containing scattered [^{14}C]urea residues (2500 cpm) in the poly(dA) strand and a limiting amount of enzyme. After 20 min at 37 °C, the reaction mixtures were chilled to 0 °C, and 10 μL of a 0.2% solution of heat-denatured calf thymus DNA, 5 μL of 5 M NaCl, and 150 μL of cold ethanol were added. The samples were kept at −20 °C for 1 h and centrifuged for 15 min at 4 °C in an Eppendorf centrifuge, and the radioactivity of each supernatant was determined.

Uracil–DNA glycosylase was assayed according to Lindahl et al. (1977), 3-methyladenine–DNA glycosylase and 7-methylguanine–DNA glycosylase according to Karran et al. (1982), hypoxanthine–DNA glycosylase according to Karran & Lindahl (1980), and formamidopyrimidine–DNA glycosylase according to Chetsanga & Lindahl (1979). Thymine glycol–DNA glycosylase activity (Demple & Linn, 1980) was measured as in the standard assay for urea–DNA glycosylase, except that the poly(dA)·poly(dT) substrate (5 μg, 2500 cpm) contained 2% [^{14}C]thymine glycol residues in the dA strand instead of urea residues.

DNA endonuclease activity was assayed by incubation of 0.5 μg of double-stranded, covalently closed circular phage M13 replicative form DNA with purified enzyme fractions under the standard reaction conditions for urea–DNA glycosylase, followed by separation of closed circular DNA from nicked circular and linear DNA by agarose gel electrophoresis in the presence of ethidium bromide (Sharp et al., 1973). Duplicate samples were incubated in reaction mixtures supplemented with 5 mM $MgCl_2$ and analyzed in the same fashion. For assays of endonuclease activity at apurinic sites, the DNA was heated at 70 °C and pH 5.0 for 10 min prior to use to introduce two to three apurinic sites per double-stranded circular molecule (Lindahl & Andersson, 1972). Protein concentrations were determined with Coomassie Brilliant Blue (Bradford, 1976).

Cells. Fresh calf thymus was collected at a local slaughterhouse and stored at −70 °C. Human fibroblasts from a normal control individual (CRL 1141) were purchased from the American Type Culture Collection (Rockville, MD) and grown in minimal Eagle's medium supplemented with 15% newborn calf serum. Fibroblasts from patients with Fanconi's anemia (CRL 1196), ataxia telangiectasia (CRL 1343), basal cell carcinoma (CRL 1169), and inherited retinoblastoma were kindly provided by Dr. Stefan Söderhäll, Department of Clinical Genetics, Karolinska Hospital, Stockholm, Sweden.

Human Fibroblast Extracts. Approximately 30 mg of cells was suspended in 0.1 mL of 0.1 M NaCl/50 mM Tris-HCl (pH 7.5)/1 mM dithiothreitol/1 mM EDTA. The cells were disrupted in a small ground-glass hand homogenizer. Cell homogenates were transferred to standard 1.5-mL Eppendorf microtubes and centrifuged at 12000 rpm for 20 min in a Sorval SS-34 rotor with microtube adaptors. Supernatant solutions were recovered, dialyzed for 14 h against the extraction buffer supplemented with 5% glycerol, and either assayed directly or stored at −70 °C.

Partial Purification of the Calf Thymus Enzyme. All extraction and purification steps were carried out at 0–4 °C.

(*A*) *Crude Extract.* Thawed pieces of calf thymus (250 g) were homogenized for two 30-s periods in a Waring blender in 750 mL of 0.1 M NaCl/50 mM Tris-HCl (pH 7.4)/10 mM dithiothreitol/1 mM EDTA/0.1 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 15000*g* for 30 min and the supernatant solution collected (fraction I, 730 mL).

(*B*) *Ammonium Sulfate Fractionation.* Fraction I was slowly supplemented with 0.33 volume of 10% streptomycin sulfate in the extraction buffer. After 30 min of gentle agitation, the suspension was centrifuged at 10000*g* for 30 min. The supernatant (870 mL) was recovered, and 326 g of ammonium sulfate (60% saturation) was slowly added with gentle stirring. After 30 min, the suspension was centrifuged at 10000*g* for 30 min and the supernatant discarded. The precipitate was dissolved in 80 mL of 10 mM potassium phosphate (pH 7.4)/7 mM 2-mercaptoethanol/1 mM EDTA/10% glycerol (buffer A) and dialyzed for 5 h against buffer A. A small precipitate was removed by centrifugation, and the solution was diluted to 360 mL with buffer A (fraction II).

(*C*) *Phosphocellulose Chromatography.* Fraction II was applied to a phosphocellulose column (6.7 × 16 cm) equilibrated with buffer A. The column was then washed with 2 L of buffer A, followed by elution of bound proteins with buffer A supplemented with 0.75 M NaCl. The peak of protein-containing fractions in the eluate was pooled (214 mL) and dialyzed for 5 h against buffer A containing 50 mM NaCl. A small precipitate was removed by centrifugation (fraction III, 212 mL).

(*D*) *DNA–Cellulose Chromatography.* Fraction III was applied to a column of single-stranded DNA–cellulose (4 × 28 cm) equilibrated with buffer A containing 50 mM NaCl. After the column was washed with the same buffer, bound proteins were eluted in three steps by increasing NaCl concentrations of buffer A to 0.15, 0.3, and 0.75 M NaCl. The urea–DNA glycosylase activity was eluted with 0.75 M NaCl. The most active fractions were pooled and concentrated by dialysis against an 80% saturated, neutralized ammonium sulfate solution containing 50 mM Tris-HCl (pH 7.4)/10 mM dithiothreitol/3 mM EDTA for 16 h. The resulting turbid solution was centrifuged at 10000*g* for 30 min, the supernatant discarded, and the precipitate suspended in 0.4 mL of 0.5 M NaCl/15 mM potassium phosphate (pH 7.4)/7 mM 2-mercaptoethanol/1 mM EDTA/5% glycerol (buffer B) supplemented with 10 mM dithiothreitol. The suspension was dialyzed against buffer B (without dithiothreitol) for 5 h to yield a clear protein solution (fraction IV, 0.7 mL).

(*E*) *Ultrogel Chromatography.* Fraction IV was applied to a column of Ultrogel AcA-54 (1 × 110 cm) equilibrated with buffer B and chromatographed at a flow rate of 3 mL h^{-1}. The most active fractions (Figure 1) were pooled, dialyzed against buffer B containing 10 mM dithiothreitol and 50% glycerol, and stored at −20 °C (fraction V, 0.8 mL). Fraction

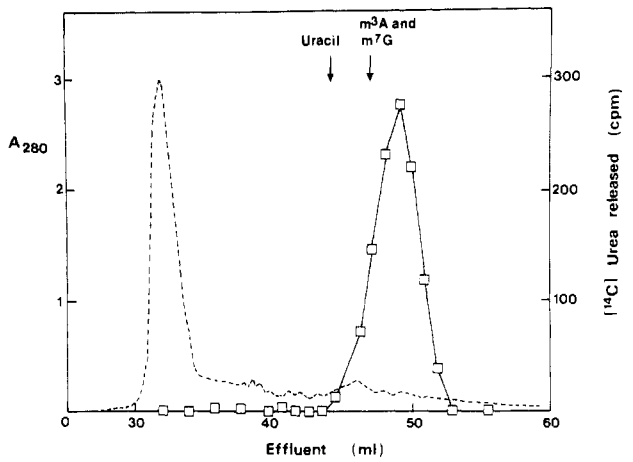

FIGURE 1: Gel filtration of partially purified urea–DNA glycosylase (fraction IV) on an Ultrogel AcA-54 column. (□) Urea–DNA glycosylase activity; (---) A_{280}. Arrows indicate the elution positions of the peaks of uracil–, 3-methyladenine–, and 7-methylguanine–DNA glycosylase activities.

Table I: Purification of Urea-DNA Glycosylase from Calf Thymus

fraction	protein (mg)	total act. (units)[a]	sp act. (units/mg)	yield (%)
(I) crude extract	5740	17900	3.1	100
(II) ammonium sulfate	2230	11400	5.1	64
(III) phosphocellulose	660	8100	12.3	45
(IV) DNA-cellulose	8.5	1920	225	11
(V) Ultrogel AcA-54	0.4	520	1290	3

[a] One unit of enzyme activity releases 1 pmol of urea in 1 min under the standard assay conditions.

V retained more than 50% of its activity after 8 months. The entire purification procedure is summarized in Table I. On a sodium dodecyl sulfate–polyacrylamide gel, the approximately 400-fold purified fraction V displayed three strong and six weak bands and clearly was not a homogeneous preparation.

Results

General Properties of Mammalian Urea–DNA Glycosylase. In the final gel filtration purification step, the calf thymus urea–DNA glycosylase activity showed the chromatographic properties of a protein with a molecular weight of 25 000 (Figure 1). The activity had a broad pH optimum at pH 7.0–8.0. It showed no cofactor requirements and was neither stimulated nor inhibited by the addition of 5 mM $MgCl_2$ to the standard reaction mixture. No product inhibition (<10%) was observed with 2 mM urea. Further, in contrast to several other mammalian DNA glycosylase activities such as those liberating hypoxanthine, 7-methylguanine, and 3-methyladenine, the urea–DNA glycosylase was not detectably inhibited (<5% inhibition) by addition of single-stranded DNA (10 μg mL^{-1}) to reaction mixtures.

Product Identification. The ethanol-soluble product enzymatically excised from the $KMnO_4$- and NaOH-treated polydeoxyribnonucleotide substrate exhibited the chromatographic properties of free urea in three different paper chromatography systems [systems I, IV, and V of Breimer & Lindahl (1980)]. No enzymatic release of substituted derivatives of urea from the substrate was detected (Figure 2). I have observed (by paper chromatography of formic acid hydrolysates) that incubation of the $KMnO_4$-treated polymer with 0.18 M NaOH at 37 °C for 15 min, prior to the addition

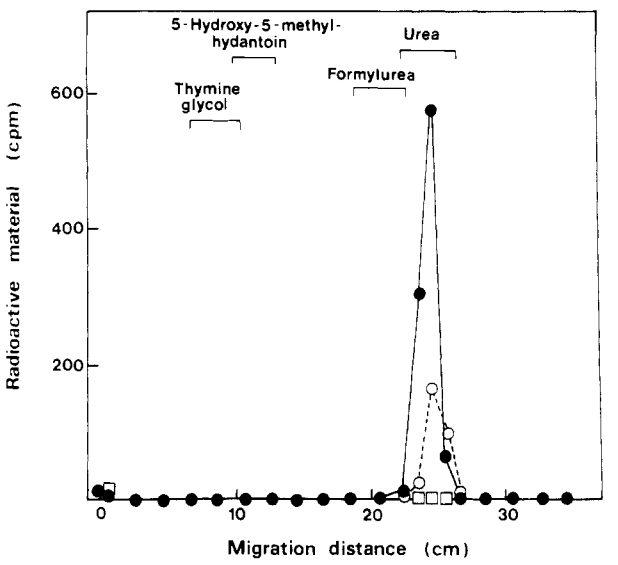

FIGURE 2: Urease sensitivity of the enzymatically released product. The ethanol-soluble fraction from a standard reaction mixture (0.5 mL, 12 000 cpm) containing 40 units of urea–DNA glycosylase was evaporated to dryness under reduced pressure. The residue was dissolved in 0.5 mL of 0.1 M potassium phosphate, pH 6.1. Aliquots of 100 μL were incubated with varying amounts of urease at 30 °C for 30 min. These samples were then applied to Whatman 3MM paper and chromatographed in isobutyric acid/H_2O/0.1 M EDTA/concentrated NH_3/toluene (160:22:3:2:20). Analysis of reference compounds and radioactive material was performed as described (Breimer & Lindahl, 1980). (●) No urease; (○) 0.1 unit of urease; (□) 0.5 unit of urease.

of a complementary poly(dT) strand, caused the conversion of a large proportion of the N-substituted urea derivatives to unsubstituted urea, without release of the latter residues from the polymer. Thus, while only 10–15% of the radioactive material could be released by enzyme extracts from a $KMnO_4$-treated double-stranded substrate in our previous work (Breimer & Lindahl, 1980), 50% of the total radioactivity could be enzymatically liberated from NaOH-treated substrates. For additional verification of the structure of the released product, its sensitivity to degradation with urease was investigated. Figure 2 shows that the radioactive material was completely susceptible to degradation into volatile products by urease, as expected for unsubstituted urea (Reithel, 1971).

Substrate Specificity. The calf thymus urea–DNA glycosylase efficiently catalyzed the excision of urea from the double-stranded polymer substrate. However, it showed very little, if any, activity on the single-stranded oxidized polydeoxyribonucleotide in the absence of a complementary poly(dT) strand (<1% of the activity with the double-stranded substrate, Figure 3). It would consequently appear that the enzyme requires a double-stranded polydeoxyribonucleotide as a substrate.

Fraction V of the enzyme, as well as fractions I–IV and side fractions from the gel column (Figure 1), was assayed for several different known DNA glycosylase activities. Since the individual members of this group of enzymes have many properties in common, such as approximate size and affinity for binding to DNA, they are difficult to separate completely from each other (Karran, 1981). For example, calf thymus uracil–DNA glycosylase was largely removed from the urea–DNA glycosylase in the DNA–cellulose chromatography step, and remaining uracil–DNA glycosylase activity chromatographed as a single symmetrical peak of molecular weight 30 000 (Caradonna & Cheng, 1980), eluting before the urea–DNA glycosylase on gel filtration (Figure 1). Due to a small degree of overlap of these two separate peaks of ac-

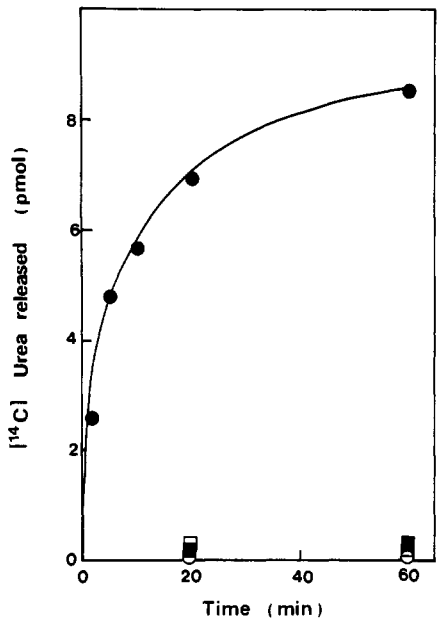

FIGURE 3: Kinetics of enzymatic release of [^{14}C]urea from a poly(dA-dT) polymer containing fragmented base residues. The substrate was employed both with and without a complementary poly(dT) strand. The reaction was allowed to proceed for different times and terminated by ethanol precipitation. The reaction mixture contained 1600 cpm (18 pmol) of ^{14}C-labeled modified base. Circles indicate double-stranded polymer, and squares indicate single-stranded polymer; filled symbols indicate 1.3 units of glycosylase added, and open symbols indicate no enzyme added.

tivity, however, trace amounts of uracil–DNA glycosylase (less than 0.05% of the amount in the crude cell extract) were still present in fraction V. Similarly, hypoxanthine–DNA glycosylase, M_r 30000 (Karran & Lindahl, 1980), was eluted before the urea–DNA glycosylase and so were the DNA glycosylase activities excising 3-methyladenine and 7-methylguanine from alkylated DNA (Figure 1). These latter two activities cochromatographed, and it cannot be decided from these studies if they are associated with one or two proteins. No separate minor peak of activity occurred with any of these substrates at the elution position of urea–DNA glycosylase, and it is concluded that the latter enzyme does not catalyze the release of the deaminated and alkylated bases investigated. A similar conclusion, supported by mutant studies, has previously been reached for the *E. coli* urea–DNA glycosylase (Breimer & Lindahl, 1980).

A formamidopyrimidine–DNA glycosylase, which catalyzes the excision of certain fragmented purine residues from DNA, has been found in *E. coli* (Chetsanga & Lindahl, 1979) and in rat liver cells (Margison & Pegg, 1981). In *E. coli* extracts, this enzyme can be chromatographically separated from the urea–DNA glycosylase (Breimer & Lindahl, 1980). Similar results were obtained here for the corresponding calf thymus activities. Thus, fraction IV of the urea–DNA glycosylase liberated 0.5 pmol of formamidopyrimidine min^{-1} (mg of protein)$^{-1}$ in the standard assay for that activity, but the formamidopyrimidine–DNA glycosylase activity was eluted before the urea–DNA glycosylase on gel filtration and was not detectable in fraction V of the latter enzyme. While these observations confirm the report of Margison & Pegg (1981) on the existence of a mammalian formamidopyrimidine–DNA glycosylase, the activities of all fractions investigated here were low, and the enzyme could not be clearly detected in crude cell extracts. This is in apparent agreement with the recent discovery that formamidopyrimidine, derived from ring-opened 7-methylguanine, is a relatively persistent alkylation lesion in vivo in rat liver cells (Beranek et al., 1983).

In the survey of different DNA glycosylase activities, the enzymatic release of thymine glycol was also investigated. A small but significant amount of thymine glycol–DNA glycosylase activity was present in fraction V, with thymine glycol being released at 1–2% of the rate of urea from a similar polydeoxyribonucleotide substrate treated with OsO_4 instead of $KMnO_4$. The identity of the released material with authentic thymine glycol was confirmed by paper chromatography in several systems, including an isobutyric acid based system which affords excellent separation of thymine glycol and urea (Breimer & Lindahl, 1980). A thymine glycol–DNA glycosylase activity has been found previously in *E. coli*, where it appears to be an associated activity of DNA endonuclease III (Demple & Linn, 1980). However, this is the first report of such a DNA glycosylase activity in mammalian cells. Only very small amounts of thymine glycol–DNA glycosylase activity were observed in fractions III–V of the calf thymus enzyme preparation (1% of the urea–DNA glycosylase activity), and it could not be detected with certainty in crude cell extracts. For these reasons, it is not known if the thymine glycol- and urea–DNA glycosylase activities exhibited similar or identical chromatographic properties, or if the thymine glycol–DNA glycosylase was associated with an endonuclease activity. It is noteworthy that Nes (1980) has partly purified a DNA repair endonuclease from mouse cells, which cleaves DNA containing thymine glycol residues by an uncharacterized mode of incision.

Fraction V of calf thymus urea–DNA glycosylase was free from nonspecific DNA endonuclease activity, in both the presence and absence of $MgCl_2$, as estimated by a sensitive assay employing covalently closed circular DNA as substrate (<10% incision by 1 unit of urea–DNA glycosylase). However, a weak endonuclease activity for apurinic sites in DNA [or alternatively, a protein factor that promoted chain cleavage at such sites; see Pierre & Laval (1981)] was associated with fraction V. The activity was approximately 4-fold stimulated by the presence of $MgCl_2$ in the reaction mixture but was still observed in the presence of EDTA without added divalent metal ions. It is not known if this apparent nuclease activity was a contaminant or an intrinsic property of the enzyme, and attempts at further purification of the urea–DNA glycosylase were unsuccessful due to losses of activity. From the amount of cleavage observed at apurinic sites by 0.2 unit of urea–DNA glycosylase in fraction V, it may be estimated that the nuclease activity was present at a 20-fold lower level than the glycosylase activity under the standard reaction conditions. Thus, the initial reaction products would be expected to be free urea and a polydeoxyribonucleotide containing apyrimidinic sites, with chain cleavage at the latter sites occurring as a subsequent event.

Urea–DNA Glycosylase in Human Fibroblasts. Crude extracts of human fibroblasts, grown in tissue culture, contained low but detectable amounts of a urea–DNA glycosylase with the same general properties as the calf thymus enzyme. For the demonstration of the activity in human cell extracts, it was useful to perform assays at different KCl concentrations to verify that enzymatically catalyzed release of urea (stimulated at 0.1 M KCl) had occurred (Figure 4). Further, the released product was characterized as unsubstituted urea by paper chromatography in several systems.

Several fibroblast lines derived from individuals with inherited disorders associated with increased sensitivity to ionizing radiation were also employed as sources of cell extracts. This was in an attempt to find an enzyme-deficient cell line,

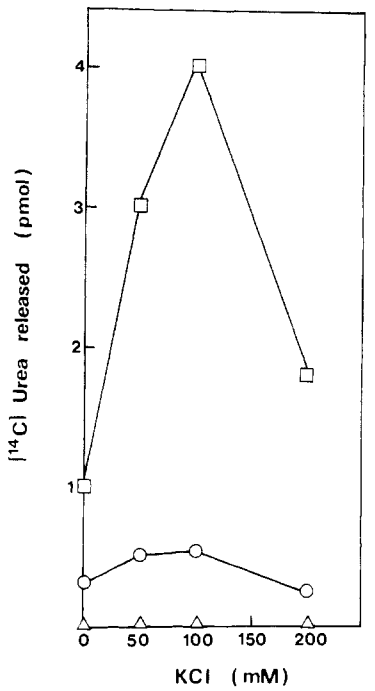

FIGURE 4: Urea–DNA glycosylase activity at different KCl concentrations. A cell extract of normal human fibroblasts (0.03 unit) (○) and 0.2 unit of calf thymus enzyme (fraction V) (□) were compared. (△) Reaction mixture without enzyme.

since conversion of thymine to urea residues in DNA occurs as one of several radiation lesions (Teoule et al., 1977). However, the cells investigated from individual cases of Fanconi's anemia, ataxia telangiectasia, basal cell carcinoma, and inherited retinoblastoma all contained 0.3 ± 0.07 unit of urea–DNA glycosylase activity per mg of protein. This value was also observed for normal human fibroblasts. Thus, the fibroblast extracts had a 10-fold lower level of the enzyme than calf thymus cell extracts.

Discussion

The main purpose of this work has been to document the existence of a urea–DNA glycosylase activity in mammalian cells. The enzyme described here from bovine and human cells has properties similar to those of the analogous *E. coli* enzyme (Breimer & Lindahl, 1980). On the other hand, the substrate specificity and fractionation properties of the partly purified calf thymus urea–DNA glycosylase show that it is clearly a different enzyme from previously described mammalian DNA glycosylases, which catalyze the release of uracil (Caradonna & Cheng, 1980; Krokan & Wittwer, 1981), hypoxanthine (Karran & Lindahl, 1980), formamidopyrimidine (Margison & Pegg, 1981), 3-methyladenine, and 7-methylguanine (Singer & Brent, 1981; Cathcart & Goldthwait, 1981) from DNA. In the course of these studies, a thymine glycol–DNA glycosylase activity, not previously reported in mammalian cells, was also detected in small amounts in the partially purified calf thymus enzyme preparation. Because of its low level of activity, at least under the in vitro conditions described here, it is presently unclear if this latter function is physically separable from the urea–DNA glycosylase. Moreover, the relationship (if any) of the urea–DNA glycosylase to DNA repair endonucleases such as those described by Bacchetti & Benne (1975) and Nes (1980) remains unclear. A more detailed investigation of the substrate specificity of urea–DNA glycosylase would require a highly purified, preferably homogeneous enzyme. Such studies are in progress with the *E. coli* urea–DNA glycosylase.

X irradiation of DNA causes a variety of lesions, including the degradation of thymine residues to urea (Teoule et al., 1977). Of the bases in DNA, thymine seems to be particularly vulnerable to oxidizing agents (Hayatsu & Ukita, 1967) and ionizing radiation (Scholes, 1976). In agreement with this, recent work by Levin et al. (1982) has shown that a *Salmonella* strain designed to monitor alterations at a critical A·T base pair is much more efficient at identifying mutagens such as H_2O_2 and ionizing radiation than previously employed strains which follow changes at G·C pairs. Consequently, cells may require specific mechanisms of correction of oxidized and fragmented thymine residues in DNA, and a possible physiological role for the urea–DNA glycosylase might be in the repair of such damage.

Acknowledgments

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Registry No. Urea–DNA glycosylase, 85976-58-5; thymine glycol–DNA glycosylase, 85976-57-4.

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Biochemistry of Terminal Deoxynucleotidyltransferase: Characterization and Properties of Photoaffinity Labeling with 8-Azidoadenosine 5′-Triphosphate†

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ABSTRACT: We have found that 8-azidoadenosine 5′-triphosphate (8-azido-ATP) and its photolyzed product are competitive inhibitors of terminal deoxynucleotidyltransferase with respect to substrate deoxynucleoside triphosphates. A detailed characterization of the inhibitory effect of 8-azido-ATP revealed that its mechanism of inhibition is identical with that reported for ATP [Modak, M. J. (1978) *Biochemistry 17*, 3116–3120]. Photoactivation of the azido-ATP–enzyme complex results in the covalent binding of azido-ATP to terminal deoxynucleotidyltransferase. No significant incorporation of prephotolyzed azido-ATP or unsubstituted ATP into enzyme protein is noted when complexes of these nucleotides with enzyme were exposed to identical photoactivation conditions. The majority of incorporated analogue was associated with the 26 000-dalton subunit of terminal deoxynucleotidyltransferase. Incorporation of azido-ATP was further found to be absolutely dependent on the presence of a divalent cation. All four deoxyribonucleoside triphosphates as well as ATP and guanosine 5′-triphosphate were able to compete with azido-ATP during the incorporation experiment as judged by the competitive reduction in the cross-linking of the photoaffinity analogue to terminal deoxynucleotidyltransferase (TDT). In addition, substrate binding site directed inhibitors, pyrophosphate and pyridoxal 5′-phosphate, effectively blocked the incorporation of azido-ATP into enzyme protein, while several other inhibitors of TDT catalysis, namely, ethylenediaminetetraacetic acid, α,α'-dipyridyl, 1,10-phenanthroline, *p*-(chloromercuri)benzoate, Rose Bengal, and the presence of 0.5 M KCl, influenced the cross-linking reaction to varying degrees. A tryptic peptide analysis of the azido-ATP-labeled 26K subunit of TDT revealed that the majority of the incorporated photoaffinity analogue was present in two peptides.

All known DNA polymerases including terminal deoxynucleotidyltransferase (TDT)[1] bind substrate deoxynucleoside triphosphate (dNTP) in their metal chelate forms and subsequently incorporate the bound substrate into a DNA chain with concomitant release of pyrophosphate (Bollum, 1974). In addition, most of these enzymes also catalyze reversal of the synthetic reaction, namely, PP_i exchange and pyrophosphorolysis reactions (Kornberg, 1980; Srivastava & Modak, 1980a,b). TDT, however, is unique among DNA polymerases in that it does not obey template direction, and, consequently, all four dNTPs with the exception of Mn-dATP readily compete for binding to TDT (Bollum, 1974; Modak, 1979). The ability of ribonucleoside triphosphates (rNTPs) to compete with dNTPs for binding to TDT with subsequent enzyme inhibition is another unique feature of this enzyme (Kato et al., 1967; Bhalla et al., 1977; Modak, 1978, 1979). Thus, the substrate binding site in this enzyme appeared to be amenable to binding both dNTPs and rNTPs. With the development and availability of photoaffinity analogues of rNTPs in general and ATP and GTP in particular (Czarnecki et al., 1979; Geahlen & Haley, 1979; Khatoon et al., 1983) in recent years, it appeared plausible that specific photoaffinity labeling of the triphosphate binding site in TDT could be accomplished provided that the photoaffinity analogue has inhibitory properties identical with those of its parent compound. This report describes the results of the characterization of TDT inhibition by the photoaffinity analogue of ATP, 8-azido-ATP, and shows that it is identical with that inhibition caused by ATP as judged by kinetic as well as physical binding studies. Furthermore, we find that covalent binding of 8-azido-ATP to TDT may be achieved via photoactivation. Thus, the requirements and restrictions for covalent binding

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[1] Abbreviations: TDT, terminal deoxynucleotidyltransferase; dNTP, deoxynucleoside triphosphate; rNTP, ribonucleoside triphosphate; PP_i, sodium pyrophosphate; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; UV, ultraviolet; Tris, tris(hydroxymethyl)aminomethane; *p*CMB, *p*-(chloromercuri)benzoate.

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