Hypoxanthine-DNA Glycosylase from *Escherichia coli*

PARTIAL PURIFICATION AND PROPERTIES*

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Hypoxanthine-DNA glycosylase from *Escherichia coli* was partially purified by ammonium sulfate fractionation and by chromatography on Sephacryl S-200, DEAE-cellulose, and phosphocellulose F-11 columns. Analysis of the enzymatic reaction products was carried out on a minicolumn of DEAE-cellulose and/or by paper chromatography, by following the release of the free 6x174 DNA from [*H]hypoxanthine from [*H]dIMP-containing 6x174 DNA. In native conditions, the enzyme has a molecular mass of 60 ± 4 kDa, as determined by gel filtration on Sephadex G-150 and Sephacryl S-200 columns. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis revealed a major polypeptide band of an apparent molecular mass of 56 kDa, and glycerol gradient centrifugation indicated a sedimentation coefficient of 4.0 S. Hypoxanthine-DNA glycosylase from *E. coli* has an obligatory requirement for Mg** and is totally inhibited in the presence of EDTA. C0** can only partially replace Mg**. The enzyme is inhibited by hypoxanthine which at 4 mM causes 85% inhibition. The optimal pH range of the enzymatic activity is 5.5–7.8, and the apparent K_m value is 2.5 × 10^{-7} M.

Hypoxanthine residues can occur in DNA as a result of the modification of adenines with nitrous acid in vitro (1) or by spontaneous deamination of adenine residues (2). Likewise, hypoxanthine can arise in DNA in vivo by deamination of dATP to dITP, followed by misincorporation of dITP during DNA replication (3–5). Hypoxanthine residues in DNA is potentially mutagenic since it can give rise to transition mutations during DNA replication (3). Hypoxanthine residues can occur in DNA as a result of spontaneous deamination of adenine residues (2). Likewise, hypoxanthine residues in DNA are potentially mutagenic since it can give rise to transition mutations during DNA replication (3).

The standard reaction mixture (50 pl) contained 10 mM sodium phosphate (pH 7.5), 10 mM MgSO_4, 50 ng/ml BSA, unlabeled dATP, dCTP, and dTTP (20 nM each), 2 µM [*H]dITP, 5 units of *E. coli* DNA-polymase I, and 1 µl of DNase I from a stock solution of 1 µg/ml. After incubation for 30 min at 14 °C, the reaction was stopped by the addition of 5 µl of 0.2 M EDTA, followed by incubation for 5 min at 65 °C to inactivate the DNase-I. The reaction mixture was loaded onto a Sephadex G-50 column (0.7 × 20 cm), equilibrated with 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. The nick-translation DNA was eluted in the void volume in 95% yield and had a specific activity of 230,000 cpm/µg. This substrate will hereafter be referred to as [*H]dIMP-6x174 DNA.

**Enzyme Assay**

The standard reaction mixture (50 µl) contained 10 mM sodium phosphate (pH 7.0), 8 mM MgSO_4, 0.12 µg of [*H]dIMP-6x174 DNA, and a limited amount of enzyme. After 15 min at 39 °C, the reaction was terminated by adding 3 µl of hypoxanthine from a stock solution of 10 µg/ml, and the mixture was chilled in an ice-water bath. One unit of hypoxanthine-DNA glycosylase activity is defined as the amount of enzyme that catalyzes the release of 1 nmol of hypoxanthine/min at 39 °C.

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Hepes/NaOH (pH 6.8), 20 mM NaCl, 1 mM 2-mercaptoethanol, and 10% glycerol (v/v) and dialyzed for 5 h against two changes of the same buffer. The dialysate is designated Fraction II.

**Sephacryl S-200 Chromatography**—Fraction II was applied to a column (3 × 100 cm) of Sephacryl S-200 superfine (Pharmacia LKB Biotechnology Inc.) equilibrated with enzyme buffer. The column was eluted with enzyme buffer, and fractions of 6 ml were collected. Column eluates were monitored by UV absorbance at 280 nm, and fractions were assayed for enzymatic activity. Fractions 44–52 (see Fig. 3), which contained most of the activity, were combined and designated Fraction III.

**Second Ammonium Sulfate Fractionation**—Fraction III was treated with ammonium sulfate (65% saturation) and centrifuged as described above. The precipitate was resuspended in enzyme buffer and dialyzed for 12 h against three changes of enzyme buffer. The dialysate is designated Fraction IV.

**DEAE-cellulose Fractionation**—Fraction IV was applied to a column (20 × 3 cm) containing DEAE-cellulose (Whatman DE-52) equilibrated in enzyme buffer. The column was washed with 90 ml of enzyme buffer and eluted by a gradient (600 ml) of 20–1000 mM NaCl in enzyme buffer. Fractions of 9 ml were collected and assayed for enzyme activity and protein concentration. Fractions 28–33 (see Fig. 4) contained the enzymatic activity and were combined to form Fraction V.

**Phosphocellulose Chromatography**—Fraction V was concentrated by treatment with polyethylene glycol-8000 (Sigma) or by ultrafiltration in an Amicon unit with a Diaflo PM-10 membrane. It was then dialyzed against enzyme buffer and applied to a column (5 × 1 cm) filled with phosphocellulose P-11 (Whatman) equilibrated with the same buffer. The column was eluted with 25 ml of a linear gradient of 10–1,000 mM NaCl in enzyme buffer. Hyp-DNA glycosylase activity eluted at 400–500 mM NaCl. At this stage the enzyme was separated from exonuclease activities, as indicated by the absence of release of [3H]dIMP by fractions that released hypoxanthine.

**Glycerol Density Gradient Centrifugation**

Samples were applied to a glycerol gradient (20–40%) containing 20 mM Tris-HCl (pH 7.2). The gradient was centrifuged in a Beckman SW 50.1 rotor at 48,000 rpm for 22 h at 4 °C. Beef liver catalase (240 kDa, 11 S), bovine serum albumin (68 kDa, 4.3 S), and lysozyme (14.4 kDa, 2.11 S), used as standards, were added to the samples containing Hyp-DNA glycosylase. Fractions of 200 μl were collected from the bottom of the tubes. The positions of the protein markers were determined by SDS-PAGE and by protein assay according to Bradford (31).

**SDS-Polyacrylamide Gel Electrophoresis**

For SDS-PAGE we employed the gel and buffer system of Laemmli (32). Gels were run at room temperature at 150 V and then stained for 30 min at room temperature with Coomassie Brilliant Blue R-250 (Sigma) in acetic acid/methanol/water (1:4:5:4.5). The gels were then destained for 1–2 h at 45 °C in acetic acid/methanol/water (5:5:87). The destaining solution was changed four to five times.

**RESULTS**

**Assay of Enzymatic Activity**

Hyp-DNA glycosylase activity was detected by following the release of [3H]hypoxanthine from [3H]dIMP-containing DNA. This substrate was prepared by nick translation (27) of φX174 DNA with DNA polymerase I in the presence of dATP, dCTP, TTP, and [3H]dTTP instead of dGTP. The dIMP content of typical DNA preparations was about 0.5% of the total dGMP residues. The release of hypoxanthine from this substrate was linear with time until about 20% of the incorporated [3H]hypoxanthine had been released. The incubation time used in our standard assays (15 min) is within this linear range. For negative controls, we used preparations that had been heat-treated at 96 °C for 5 min prior to their analysis by the standard assay. This treatment totally inactivated the Hyp-DNA glycosylase activity, as judged by the release of background levels (20–30 cpm) of [3H]hypoxanthine from the substrate.
the proteins in the extracts. The purification procedure that by alkali (Fig. 2) gave the most satisfactory results is summarized in Table I. The UV-absorbing markers dIMP, deoxyinosine, and hypoxanthine are indicated. Fraction II, ammonium sulfate precipitate of the crude protein extract; Fraction VI, second ammonium sulfate precipitation, followed by phosphocellulose chromatography.

Since the enzymatic activity of Hyp-DNA glycosylase requires the presence of Mg2+ ions (see below), a concomitant activity of exonucleases was expected. To distinguish between these activities, we analyzed the monomeric reaction products by paper chromatography under conditions that enabled the separation between hypoxanthine and dIMP, the products of Hyp-DNA glycosylase and exonuclease activities, respectively. Fig. 1 illustrates a typical paper chromatographic analysis. It can be seen that the ammonium sulfate precipitate of the crude protein extract (Fraction II) contains an exonuclease activity that is responsible for the release of about 40% of the monomeric products as dIMP (solid circles). However, upon further purification (Fraction VI), the amount of exonuclease activity was reduced to less than 10%, while that of Hyp-DNA glycosylase activity increased, respectively (open circles).

Fig. 1 also shows that deoxyinosine is not liberated from the substrate DNA. Therefore, hypoxanthine is the only monomeric product released from the DNA that is expected not to bind to DEAE-cellulose. We thus use the flow-through fraction of the DEAE-cellulose minicolumn (see “Experimental Procedures”) for routine assays of Hyp-DNA glycosylase activity.

Fig. 2 indicates that the release of free base is accompanied by the formation of an apurinic site in the DNA. Hypoxanthine-containing DNA that had been treated with an active enzyme preparation (Fraction VI) was partially hydrolyzed by alkali (Fig. 2, lane 3), while reduction with NaBH4 subsequent to enzyme treatment rendered the DNA insensitive to alkaline hydrolysis (Fig. 2, lane 4). The same DNA preparation, on the other hand, was hydrolyzed by the AP endonuclease activity of exonuclease III (Fig. 2, lane 5), under reaction conditions that inhibit the exonuclease activity of the enzyme of an untreated DNA substrate (Fig. 2, lane 2) but maintain its AP endonuclease activity on reduced apurinic DNA (29, 30, and data not shown).

**Purification Procedure**

Several attempts to purify the Hyp-DNA glycosylase activity to homogeneity failed, mainly due to its scarcity and apparent instability. The main difficulty in purifying Hyp-DNA glycosylase was that in most fractionation steps, the enzymatic activity could not be separated from the bulk of the proteins in the extracts. The purification procedure that gave the most satisfactory results is summarized in Table I. It consisted of an essential streptomycin sulfate treatment to remove the nucleic acids, followed by ammonium sulfate precipitation. Separation of most of the proteins was achieved by exclusion chromatography through Sephacryl S-200 (Fig. 3). About 60% of the proteins were eluted in the excluded volume, while the Hyp-DNA glycosylase activity was eluted in the included volume, slightly after a marker of BSA. A second ammonium sulfate fractionation was then employed in which about half of the remaining proteins that accompanied Hyp-DNA glycosylase were removed with almost no loss of the enzymatic activity. Further enrichment was achieved by anion exchange chromatography (Fig. 4), which separated 70% of the bulk proteins while retaining about 80% of the remaining activity. Following the final step of cation exchange chromatography, the enzymatic activity was almost free of accompanying proteins (Fig. 5). However, at this stage, the enzyme became labile as manifested by the low recovery yield of the enzymatic activity. For storage purposes, the eluate from the phosphocellulose column was concentrated 10-fold by ultrafiltration, and glycerol was added to 50%. No apparent loss of enzymatic activity could be detected after 6 months of storage at -20 °C.
while aliquots from preceding fractions totally nicked the DNA under identical incubation conditions (data not shown).

The pooled fractions containing the enzymatic activity eluted from the DEAE-cellulose column were refractionated by phosphocellulose chromatography. The elution profile of a sample from the DEAE-cellulose purification step (Fraction VI) was demonstrated by paper chromatographic analysis (Fig. 1). The second ammonium sulfate precipitate (Fraction VI) was also devoid of AP endonucleases, as shown by alkaline agarose-gel electrophoresis of NaBH₂O₃-reduced apurinic DNA that had been treated with enzyme preparations as described by Weinberger and Sperling (28). We observed that reduced apurinic DNA treated by Fraction VI was not degraded by the alkaline treatment, while treatment with the crude extract completely degraded the DNA (data not shown). The depletion of exonucleases from Fraction VI was demonstrated by paper chromatographic analysis (Fig. 1) which shows that the amount of dIMP released by Fraction VI is diminished, relative to Fraction II, with a concomitant increase of the amount of hypoxanthine.

The protein composition of the fractions obtained during the different stages of purification was analyzed by SDS-PAGE (Fig. 6). Lanes b-f show the protein profile of Fractions I-V (see Table I). Lanes g and i contain duplicate samples of the phosphocellulose purification step (Fraction VI). Following this step, most of the bulk proteins were removed, leaving a band of a major protein of an apparent molecular mass of 56 kDa.

**Native Molecular Weight Determination**

Native molecular mass was determined by gel filtration and glycerol gradient centrifugation. Fig. 7 shows the elution profile of a sample from the DEAE-cellulose purification step (Fraction V) on a calibrated column of Sephadex G-150. Hyp-DNA glycosylase eluted with an apparent molecular value of 60 ± 4 kDa as compared to elution volumes of the indicated molecular mass markers.

To estimate the sedimentation coefficient of Hyp-DNA glycosylase, a sample from Step VI (Table I) was centrifuged through a 5 ml 20–40% linear glycerol gradient with the following proteins as markers: beef liver catalase (11.3 S), BSA (4.3 S), and lysozyme (2.11 S). Marker proteins were monitored by SDS-PAGE and by Bradford protein assay (31), and all fractions were assayed for Hyp-DNA glycosylase activity. The last purification step also separated the Hyp-DNA glycosylase activity from the majority of the accompanying activities of endo- and exonucleases. Endonuclease activity was assayed by the method of Helling et al. (33), using ϕX174 RFI DNA as substrate. We found that no nicking of the DNA occurred when it was incubated with aliquots of Fraction VI, while aliquots from preceding fractions totally nicked the DNA under identical incubation conditions (data not shown).
FIG. 7. Molecular mass determination of Hyp-DNA glycosylase by gel filtration. A sample of the pooled enzymatic activity from the DEAE-cellulose step (Fraction V) was loaded onto a column of Sephardex G-150 and eluted with 10 mM Hepes/NaOH (pH 6.8), 20 mM NaCl, 1 mM 2-mercaptoethanol, and 10% glycerol (v/v). Fractions of 2.5 ml were collected and assayed for Hyp-DNA glycosylase activity.

Lower panel, profile of enzymatic activity; upper panel, molecular mass markers: BSA (68 kDa), carbonic anhydrase (CA) (30 kDa), and lysozyme (14 kDa).

TABLE II

| Additives | Concerto | Enzymatic activity |
|-----------|----------|---------------------|
| Mg$^{2+}$ | 2        | 65                  |
| Mg$^{2+}$ | 4        | 100                 |
| Mg$^{2+}$ | 4*       | 3                   |
| Co$^{2+}$ | 2        | 38                  |
| Co$^{2+}$ | 4        | 42                  |
| Ca$^{2+}$ | 2        | 12                  |
| Ca$^{2+}$ | 4        | 6                   |
| Mn$^{2+}$ | 2        | 12                  |
| Mn$^{2+}$ | 4        | 12                  |
| Zn$^{2+}$ | 2        | 14                  |
| Zn$^{2+}$ | 4        | 10                  |

* Control containing heat-inactivated enzyme.

FIG. 8. Glycerol gradient centrifugation of Hyp-DNA glycosylase. Purified enzyme sample (150 μl from purification step VI of Table I) was sedimented along with three protein markers through a 5-ml linear glycerol gradient (20-40%) in a Beckman SW 50.1 rotor for 22 h at 48,000 rpm and 2 °C. Fractions were collected from the bottom of the tube and assayed for Hyp-DNA glycosylase activity. Lower panel, Hyp-DNA glycosylase activity across the gradient; upper panel, calibration curve using the following protein markers as indicated by the arrows: 1, beef liver catalase (11 S); 2, BSA (4.3 S); 3, lysozyme (2.11 S).

FIG. 9. Effect of magnesium cations on Hyp-DNA glycosylase activity. The standard enzymatic assay, using the indicated Mg$^{2+}$ concentrations, was carried out with proteins from purification step V (Table I).

Substrate Specificity—To test the activity of Hyp-DNA glycosylase on single-stranded DNA, we used heat- or alkali-denatured [3H]dIMP-φX174 DNA as substrate. S1 nuclease analysis, described by Bryant and Lehman (34), confirmed the persistence of more than 95% of this substrate in a single-stranded form under the reaction conditions. We have found that Hyp-DNA glycosylase acts about twice as fast on single-stranded DNA as on double-stranded DNA. On the other hand, the enzyme does not act on monomeric derivatives of hypoxanthine as indicated by the inability of Hyp-DNA glycosylase to release hypoxanthine from dITP.

pH Optimum—The enzymatic activity was determined in the pH range of 4-10 in phosphate buffer at a constant ionic strength of 25 mM. The optimal activity of Hyp-DNA glycosylase is in the pH range of 5.5-7.8.

Effect of Divalent Cations—The enzymatic activity of Hyp-DNA glycosylase has an obligatory requirement for magnesium cations and is totally inhibited in the presence of 1 mM EDTA. Fig. 9 shows the enzymatic activity as a function of Mg$^{2+}$ concentration. The minimal Mg$^{2+}$ concentration required for optimal enzymatic activity is 4 mM. Table II summarizes the effect of other divalent cations. Mn$^{2+}$, Zn$^{2+}$, and Ca$^{2+}$ did not replace Mg$^{2+}$, while Co$^{2+}$ could only partially replace the requirement for Mg$^{2+}$.

Inhibition of Hyp-DNA Glycosylase by Hypoxanthine and Related Purine Derivatives—Hyp-DNA glycosylase is inhibited by the end product of the reaction, as has been found for uracil-DNA glycosylase (9). Hypoxanthine at 2 mM caused...
indicated concentrations of hypoxanthine. The enzyme was from described under “Experimental Procedures,” in the presence of the matic activity from the activity of Hyp-DNA glycosylase is about 39 dIMP was estimated to be 2.5 determined by employing the standard assay at the tempera-
ture range between 4 and 55 °C. The optimal temperature for optimal and is totally inhibited in the presence of Mg" or other divalent cations as cofactors. Third, end product inhibition is observed for the presently described enzymatic reaction, at a concentration of 5 mM.

The hypoxanthine-DNA glycosylase described here differs from the previously described enzyme (24, 25) in three features. First, its molecular mass appears to be 56 kDa, whereas the molecular mass of the previously described activity is 30 kDa. Second, the enzymatic activity described here is Mg"-dependent, while the previously described one has no requirement for Mg" or other divalent cations as cofactors. Third, end product inhibition is observed for the presently described activity, whereas it did not occur previously. As far as the first two features are concerned, our hypoxanthine-DNA glycosylase may occur in DNA in vivo, either by spontaneous deamination of adenine residues (25) or by misincorporation of dIMP residues from traces of dITP that might exist in the deoxynucleoside triphosphates pool. Although the latter possibility is less probable (25), the incorporation of dITP into DNA in vitro by E. coli DNA polymerase I (3, 35) and by DNA polymerase α from HeLa cells (4) indicates that such incorporation might also occur in vivo. For our studies, we have employed DNA that was nick-translated in the presence of [3H]dITP instead of dGTP. This substrate corresponds to DNA damaged by misincorporation and contains a stable Watson-Crick F.C base pair, rather than an I.T pair that results from deamination and may exist in the less stable “Wobble” configuration.

Hypoxanthine-DNA glycosylase was partially purified by ammonium sulfate fractionation, gel filtration, and ion-exchange chromatography. The partially purified enzyme preparation has been shown to be free of contaminating deoxyribonucleases that accompanied the initial stages of purification. In non-denaturing conditions, the enzyme exhibited a molecular mass of 60 kDa as determined by gel filtration methods. This observation was substantiated further by estimating the sedimentation coefficient of the enzyme using glycerol gradient centrifugation. The results show that the enzymatic activity (4.0 S) migrated close to BSA (4.2 S), which has a molecular mass of 68 kDa. Analysis of the purified preparation under denaturing conditions by polyacrylamide gel electrophoresis in the presence of SDS revealed a major band of 56 kDa (Fig. 6, lanes g and j). The sedimentation and gel filtration data are consistent with the assignment of this band to Hyp-DNA glycosylase. However, the presence of minor quantities of multiple polypeptide species in the enzyme preparation does not exclude other possibilities, for example, the persistence of multiple subunits in the 60-kDa native enzyme. We have also shown that the enzyme has an obligatory requirement for Mg" and is totally inhibited in the presence of EDTA. Inhibition of the enzymatic activity could also be achieved by hypoxanthine, the end product of the enzymatic reaction, at a concentration of 5 mM.

The hypoxanthine-DNA glycosylase described here differs from the previously described enzyme (24, 25) in three features. First, its molecular mass appears to be 56 kDa, whereas the molecular mass of the previously described activity is 30 kDa. Second, the enzymatic activity described here is Mg"-dependent, while the previously described one has no requirement for Mg" or other divalent cations as cofactors. Third, end product inhibition is observed for the presently described activity, whereas it did not occur previously. As far as the first two features are concerned, our hypoxanthine-DNA glycosylase also differs from other DNA glycosylases that have been collectively described as polypeptides of 20-30 kDa in molecular mass that do not require Mg" for their activity. However, Micrococcus luteus uracil-DNA glycosylase (10) and E. coli 3-methyladenine DNA glycosylase (13) are inhibited by the respective free base end product, while 3-methyladenine from calf thymus has been reported not to be inhibited by 3-methyladenine (36).

The apparent discrepancy with respect to the requirement

50% inhibition of the enzymatic activity, whereas 4 mM caused 85% inhibition (Fig. 10). Other purine derivatives did not inhibit the enzymatic activity.

**Optimal Temperature of Hyp-DNA Glycosylase Activity**—The effect of temperature on the enzymatic activity was determined by employing the standard assay at the temperature range between 4 and 55 °C. The optimal temperature for the activity of Hyp-DNA glycosylase is about 39 °C. The observed release of hypoxanthine at temperatures higher than 40 °C is due to residual enzymatic activity, since incubation of the substrate at these temperatures in the absence of an enzyme did not cause the release of hypoxanthine.

**Heat Stability**—The effect of heat treatment on the enzymatic activity was tested on Fraction VI (phosphocellulose column). The half-life of the enzymatic activity at 55 °C is 2 min. The enzyme remained fully active for several days at 4 °C in enzyme buffer, and for several months at −20 °C in enzyme buffer containing 50% (v/v) glycerol.

**Kₐ Value**—The initial rate of hypoxanthine release was determined by the standard assay using DNA concentrations corresponding to a range of 7–20 nM [3H]dIMP. The Kₐ of dIMP was estimated to be 2.5 × 10⁻⁷ M (see Fig. 11).

**DISCUSSION**

This study presented the partial purification of an enzymatic activity from E. coli extracts that releases hypoxanthine from dIMP-containing DNA. Such activity was initially discovered by Karran and Lindahl in E. coli (24) and in calf thymus (25) and has been termed hypoxanthine-DNA glyco-
sylase. However, attempts to obtain the enzyme in a substan-
tially purified form have been hampered by its scarcity and apparent instability. Functionally and mechanistically, the enzyme belongs to a group of enzymes that initiate the repair of DNA-containing altered bases by hydrolyzing the glycosyl bond between the altered base and the deoxyribose, leading to the formation of apurinic or apyrimidinic sites.

The apparent discrepancy with respect to the requirement...
of Mg$$^{2+}$$ may be attributed to the nature of the substrates used for the enzymatic assay. We have used nick-translated native DNA that contains IC base pairs that presumably assumes the B DNA conformation, while the substrates used by Karran and Lindahl were poly(dL)-poly(dC), poly(dA,dL)-poly(dT), and deaminated DNA. The secondary structure of the synthetic polynucleotides may differ significantly from the B DNA structure (37, 38), while the thymine-containing polymer and the deaminated DNA contain I:T mismatches that may affect the glycosylase activity by introducing local structural changes in the substrate DNAs. On the other hand, the large difference in the reported mass and the contradictory effect of hypoxanthine strongly suggest that the two hypoxan-thine-DNA glycosylase activities reside in two different poly- 

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