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# Purification and Characterization of *Escherichia coli* Formamidopyrimidine-DNA Glycosylase That Excises Damaged 7-Methylguanine from Deoxyribonucleic Acid†

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ABSTRACT: A DNA glycosylase that excises 7-methylguanines with alkali-opened imidazole rings (formamidopyrimidines) from DNA has been purified more than 8000-fold from *Escherichia coli* cell extracts. The enzyme does not cleave 3-methyladenine, uracil, and intact 7-methylguanine from DNA. In assays containing pyrimidine analogues like oxauracil, 2,4,6-triaminopyrimidine, 2,5,6-triamino-2-hydroxypyrimidine sulfate, formamidopyrimidine, and 5-nitroso-2,4,6-triaminopyrimidine, only the last two compounds showed end product inhibition of the enzyme. The enzyme has been named formamidopyrimidine-DNA glycosylase. It has a molecular weight of 30000 and a Stokes radius of 26.4 Å. The enzyme prefers double-stranded to single-stranded DNA and is stimulated by the presence of 0.1 M KCl in the reaction mixture.

The formation of 7-methylguanine (7-meGua)[1] adducts is the predominant reaction observed when DNA is treated with $Me_2SO_4$ or MMS (Lawley & Shah, 1972b). The presence of these adducts does not interfere with DNA replication (Prakash & Strauss, 1970). There is also no evidence of miscoding when the copolymers uridylic acid and 7-methylguanylic acid are transcribed in vitro by *Escherichia coli* RNA polymerase (Ludlum, 1970). Although the slow rate of release of 7-MeGua ($t_{1/2} \simeq 150$ h) at 37 °C and pH 7.0 (Brooks & Lawley, 1963; Lawley & Orr, 1970; Singer, 1979) had earlier been taken to indicate that enzymatic mechanisms for 7-MeGua removal are not essential (Lawley & Warren, 1976), the discovery of bacterial and animal DNA glycosylases that excise these adducts from DNA has now been reported (Laval et al., 1981; Singer & Brent, 1981; Margison & Pegg, 1981% Cathcart & Goldthwait, 1981).

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[1] Abbreviations used: MMS, methyl methanesulfonate; $Me_2SO_4$, dimethyl sulfate; AP, apurinic–apyrimidinic; FAPY, formamidopyrimidine, shortened form of 2,6-diamino-4-hydroxy-5-(*N*-methylformamido)pyrimidine; EDTA, ethylenediaminetetraacetic acid; 1 × SSC, 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); 7-MeGua, 7-methylguanine; 7-MeGuo, 7-methylguanosine; 3-MeAde, 3-methyladenine; DNA, deoxyribonucleic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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Several enzymes which remove abnormal bases from DNA have now been identified. There are unique DNA glycosylases that remove from DNA such nonconventional bases as hypoxanthine (Karran & Lindahl, 1978), 3-methyladenine (Laval, 1977; Riazuddin & Lindahl, 1978; Brent, 1979), and uracil (Lindahl, 1974; Friedberg et al., 1975; Lindahl et al., 1977). These enzymes release free bases by cleaving the *N*-glycosyl bond.

Apurinic–apyrimidinic (AP) endonucleases hydrolyze the phosphodiester bonds at the resulting AP sites (Verly et al., 1973; Weiss, 1976), paving the way for a DNA polymerase catalyzed repair–synthesis of the missing oligomer. Apparently, the AP sites can also be repaired by a direct reinsertion of the proper base (Linsley et al., 1977; Livneh et al., 1979).

In addition to the glycosylases mentioned above, we have recently identified in *E. coli* B cell extracts another DNA glycosylase that removes the 7-MeGua residues with opened imidazole rings from DNA (Chetsanga & Lindahl, 1979). The particular form of ring-opened 7-MeGua excised by this enzyme is 2,6-diamino-4-hydroxy-5-(*N*-methylformamido)-pyrimidine (Haines et al., 1962), hereafter formamidopyrimidine (FAPY). The formamidopyrimidine-DNA (FAPY-DNA) glycosylase activity obtained from extracts of *E. coli* B was also found in an *E. coli* strain deficient in 3-methyladenine-DNA glycosylase activity (Karran & Lindahl, 1978) and in *E. coli* strain BD 10 deficient in uracil-DNA glycosylase activity (Duncan et al., 1978). A similar activity has now been identified in rat and hamster liver extracts (Margison & Pegg, 1981). In this paper we describe the further purification and characterization of FAPY-DNA glycosylase from *E. coli* W cells.

## Experimental Procedures

*Materials. Escherichia coli* W obtained from Grain Processing Corp. (Muscatine, IA) was grown in trypticase soy broth at 37 °C and harvested in mid log phase. Cell pellets were stored at −70 °C and thawed at the time of use. Bio-Gel P-60 and Affi-Gel Blue were obtained from Bio-Rad Laboratories. Whatman 3 MM chromatography paper and phosphocellulose P-11 were obtained from Whatman. [*methyl*-$^3$H]Dimethyl sulfate was obtained from New England Nuclear Corp. Aqueous counting scintillant (ACS) was obtained from Amersham Corp. The pyrimidine analogues 5-nitroso-2,4,6-–triaminopyrimidine, oxauracil, 2,4,6-triaminopyrimidine, 2,4,5-triamino-6-hydroxypyrimidine sulfate, and 2,4,5-triamino-2-hydroxypyrimidine sulfate, Sephadex G-10 and G-75, 7-MeGua, 7-MeGuo, uracil, calf thymus DNA, *Micrococcus luteus* DNA, Pipes, and Hepes buffer were obtained from Sigma Chemical Co.

Scintillation bags were obtained from Sybron/Nalge Corp. For purposes of clarity, the DNA samples containing uracil, 7-MeGua, and FAPY will be referred to as uracil-DNA, 7-MeGua-DNA, and FAPY-DNA. The protein determination kit and the electrophoresis reagent kit were obtained from Bio-Rad laboratories. Molecular weight marker proteins were obtained from Schwarz/Mann.

*Methods.* (*1*) *Preparation of Formamidopyrimidine Standards.* About 100 mg of 7-MeGuo was dissolved in 2 mL of 2 N $NH_4OH$ and incubated at 24 °C for 90 min. Although this treatment has been reported to open the imidazole ring of 7-MeGuo at the $C^8$–$N^9$ position to form 2-amino-6-hydroxy-5-(*N*-methylformamido)-4-(*N*-β-ribofuranosylamino)pyrimidine (Haines et al., 1962), we have found that high-performance liquid chromatography resolves the products into two peaks (C. J. Chetsanga, B. Bearie, and C. Makaroff, unpublished observations). After lyophilization the compound was dissolved in 2 mL of 85% formic acid and stored at 24 °C for 72 h; this reaction removes the ribose residue to provide the aglycon of the compound.

The material was lyophilized, dissolved in 2 mL of 3 mM NaCl, neutralized, and fractionated by chromatography on a Sephadex G-10 column (1 × 100 cm) (Sweetman & Nyhan, 1968) equilibrated with 3 mM NaCl. Elution with 3 mM NaCl resolved the material into a major peak followed by a minor one. The material in the major peak had a $\lambda_{max}$ 265 and $\lambda_{min}$ 242 and behaved chromatographically as formamidopyrimidine (Haines et al., 1962). The orcinol reaction showed the material in the minor peak to be largely ribose sugar.

(*2*) *Preparation of DNA Substrates.* Substrates were prepared from *M. luteus* or calf thymus DNA. In order to expedite the opening of the imidazole ring of guanine by alkali, we first methylated guanine residues with [methyl-$^3$H]dimethyl sulfate (New England Nuclear Corp., sp act. 3.4 Ci/mmol) which resulted in DNA with 7-[$^3$H]methylguanine adducts. A 5-mL methylation reaction mixture containing 0.2 M cacodylic acid–KOH (pH 7.2), 1 mM EDTA, 15 mg of DNA, and 5 mCi of [*methyl*-$^3$H]dimethyl sulfate was incubated for 1 h at 37 °C. After ethanol precipitation the DNA was dissolved in 1 mL of 0.1 × SSC. The DNA was dialyzed against three changes of 1000 mL of 0.2 M NaOH at 25 °C for 24 h to further remove unreacted dimethyl sulfate and to open the imidazole rings of 7-MeGua residues in DNA. The DNA was dialyzed against 2000 mL of 2 × SCC and subsequently allowed to reanneal at 65 °C for 48–72 h. About 85% of the 7-MeGua residues of the DNA was converted to formamidopyrimidine. The DNA was dialyzed against two changes of 2000 mL of 0.05 M Tris-HCl (pH 7.4) and 1 mM EDTA and frozen in small aliquots for subsequent use as substrate.

(*3*) *Enzyme Assay.* The release of formamidopyrimidine as a free base from FAPY-DNA was measured in 50 μL of a standard assay system consisting of 0.05 M Hepes–KOH (pH 7.4), 0.1 M KCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 2 μg of [$^3$H]FAPY-DNA (4000 cpm), and 6 microunits of enzyme. Incubation was for 20 min at 37 °C. The reaction was terminated by chilling the tubes in an ice bath and adding 10 μL of heat-denatured carrier DNA and 150 μL of chilled 95% ethanol. After being stored for 20 min at −20 °C, the samples were centrifuged at top speed in an Eppendorf 5412 centrifuge for 15 min, and 150 μL of supernatant was counted in 3 mL of ACS fluor in scintillation bags housed in counting vials. Alternatively, the 150 μL of supernatant was concentrated to 25 μL by flash evaporation and cochromatographed with marker FAPY on Whatman 3 MM paper. The chromatogram was developed in a methanol–ethanol–concentrated HCl–$H_2O$ (50:25:6:19) solvent system. The UV-absorbing spots were cut out and eluted with 2 mL of water; the radioactivity was measured in 15 mL of ACS fluor. One unit of FAPY-DNA glycosylase is defined as the amount of enzyme which catalyzes the release of 1 μmol of free FAPY under the standard reaction conditions described above. In control experiments 3-MeAde-DNA glycosylase (Riazuddin & Lindahl, 1978) and uracil-DNA glycosylase (Lindahl et al., 1977) were used in place of FAPY-DNA glycosylase.

(*4*) *Fate of 7-Methylguanine in DNA.* The first group of experiments was designed to monitor the spontaneous release of 7-MeGua from DNA under physiological conditions. For this study 7-[$^{14}$C]methylguanine-DNA in 0.1 M KCl, 0.05 M Hepes–KOH (pH 7.4), 0.01 M $MgCl_2$, and 1 mM EDTA was incubated at 37 °C and 25-μL aliquots (500 cpm) were col-

lected at selected time intervals ranging from 0 h to 14 days.

Each collected aliquot was then frozen for the duration of the remaining incubation period. The aliquots were subsequently resolved by descending chromatography on Whatman 3 MM paper together with marker 7-MeGua for 18 h in a methanol–ethanol–concentrated HCl–$H_2O$ (50:25:6:19) solvent system. The position of the markers was established by scanning the chromatograms with ultraviolet light. The UV-absorbing spots were cut out and extracted with 2 mL of water, and the radioactivity was measured in 15 mL of the ACS fluor.

In the second set of experiments we were looking for the lowest pH at which 7-MeGua in DNA is converted to FAPY. Nine buffer conditions were used (sodium acetate, pH 5.1; Pipes, pH 6.5 and 6.8; $KH_2PO_4$, pH 7.2 and 7.5; Tris-HCl, pH 8.0 and 8.5; glycine, pH 9.0; sodium borate, pH 9.4) in a solution of 0.1 M KCl, 0.01 M $MgCl_2$, and 1 mM EDTA. The 7-[$^{14}$C]methylguanine-DNA was incubated at 37 °C for 8 days under the different pH conditions. Subsequently, the nine samples were made 0.1 M in HCl and incubated 16 h at 37 °C to hydrolyze the bases. The material was then fractionated by descending paper chromatography together with marker FAPY and 7-MeGua in the solvent system used above. The spots corresponding to the markers were cut out and measured for radioactivity content.

(*5*) *Other Methods.* The protein concentration in column fractions was estimated from $A_{280}$ measurements. More accurate estimates of protein concentration were obtained by the method of Bradford (1976) as modified by Bio-Rad Laboratories. DNA concentration was determined by the diphenylamine reaction (Burton, 1956). Molecular weight and Stokes' radius estimates were obtained from analytical gel filtration (Siegel & Monty, 1966).

## Results

*Enzyme Purification.* (*a*) *Cell Extracts.* Crude cell extracts were obtained by suspending 20 g of *E. coli* W in 50 mL of buffer A (0.05 M Tris-HCl, pH 7.4, 1 mM 2-mercaptoethanol, 1 mM EDTA, and 5% glycerol) and blending with glass beads. This extraction as well as all subsequent steps was performed at 0–4 °C. The enzyme is very sensitive to the rise in temperature that frequently develops during blending with glass beads. Cell disruption at −20 °C in a modified Hughes press as reported elsewhere (Chetsanga & Lindahl, 1979) was found to be the most satisfactory procedure for cell lysis. The homogenate was gently stirred with 1% streptomycin sulfate for 15 min, followed by centrifugation at 36000*g* for 30 min. The supernatant was collected as fraction I.

(*b*) *Ammonium Sulfate Fractionation.* Chilled solid ammonium sulfate (0.27 g/mL) was added slowly to fraction I to a final concentration of 45%. The extract was stirred for 30 min and the precipitated material removed by centrifugation (32000*g* for 30 min). Additional ammonium sulfate (0.17 g/mL) was slowly added to the supernatant to a final concentration of 70% and the precipitated material collected by centrifugation (32000*g* for 30 min). FAPY-DNA glycosylase was recovered in this precipitate. The pellet was suspended in 6 mL of buffer B (0.5 M KCl, 0.05 M Tris-HCl, pH 8.0, 1 mM 2-mercaptoethanol, 1 mM EDTA, and 5% glycerol), dialyzed against the same buffer, and designated fraction II.

(*c*) *Gel Filtration.* Fraction II was loaded on a 4 × 100 cm column of Sephadex G-75 preequilibrated with buffer B. The column was developed with two column volumes of buffer B; the activity was recovered in fractions collected immediately following the $A_{280}$ peak (Figure 1). The fractions containing the enzyme activity were pooled, and the protein was precipitated with 70% ammonium sulfate. After centrifugation (36000*g* for 30 min) the pellet was dissolved in 5 mL of buffer C (0.1 M KCl, 0.05 M Tris-HCl, pH 7.4, 1 mM 2-mercaptoethanol, and 5% glycerol) and dialyzed against three changes of 1000 mL of the same buffer (fraction III).

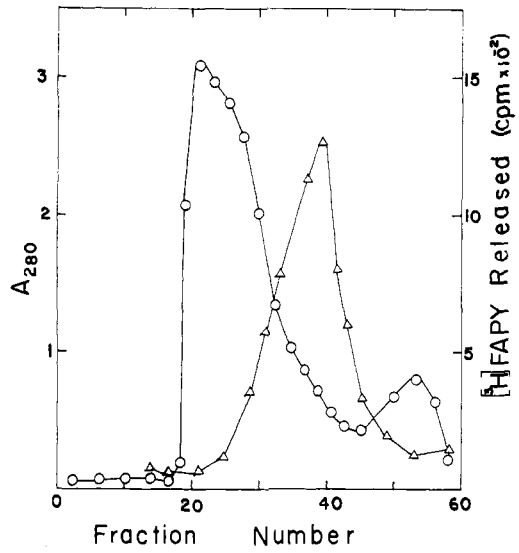


FIGURE 1: Sephadex G-75 chromatography of fraction II. About 312 mg of protein in buffer B was loaded on Sephadex G-75 column. The column was washed with loading buffer, and 20-mL fractions were collected. In the assays, 10 μL of each fraction was used. $A_{280}$ (○); cpm in [$^3$H]FAPY released (△).

The FAPY-DNA glycosylase in fraction III was ~12-fold purified. The enzyme activity is stable and can last for several months at −20 °C. Enzyme stability is enhanced by using 0.5–1.0 M KCl in the buffer solution. If the enzyme is stored in a solution with >0.5 M KCl, it must be dialyzed to reduce its salt content before being assayed under the enzyme assay conditions given under Experimental Procedures above.

(*d*) *Affi-Gel Blue Affinity Chromatography.* Fraction III was applied to an Affi-Gel Blue column (0.8 × 12 cm) equilibrated with buffer C. After the column was washed with 100 mL of loading buffer, the enzyme was eluted with a linear gradient of 0.1–0.8 M KCl in 0.05 M Tris-HCl, 1 mM 2-mercaptoethanol, and 5% glycerol. Fractions (2 mL) were collected and analyzed for absorbance at 280 nm, enzyme activity, and electrical conductivity measured with an ElectroMark Analyzer (Markson Science, Inc.). The fractions with the highest activity eluted at 0.26 M KCl (Figure 2). Such fractions were pooled (fraction IV) and dialyzed against buffer C.

Enzymes with binding sites for nucleotides have an affinity for Affi-Gel Blue (Heyns & deMoor, 1974; Wilson, 1976). The blue chromophore of this adsorbent bears some structural similarity to nucleotides (Thompson et al., 1975). The affinity of FAPY-DNA glycosylase for Affi-Gel Blue is thus based on the resemblance of FAPY residues to components of this affinity adsorbent. This was the most effective step in the purification of FAPY-DNA glycosylase. The enzyme recovered from the Affi-Gel Blue column is 7000-fold purified.

Fraction IV is unstable and was found to be very sensitive to temperature fluctuations caused by freezing and thawing routines. The enzyme retains activity for up to 2 weeks at 0 °C in a buffer solution containing 0.1 M KCl and 5% glycerol. Increasing the glycerol concentration was not effective in stabilizing enzyme activity.

(*e*) *Bio-Gel P-60 Chromatography.* Fraction IV (1 mL) was applied to a 0.9 × 25 cm column of Bio-Gel P-60. The protein was eluted with buffer C. The fractions containing

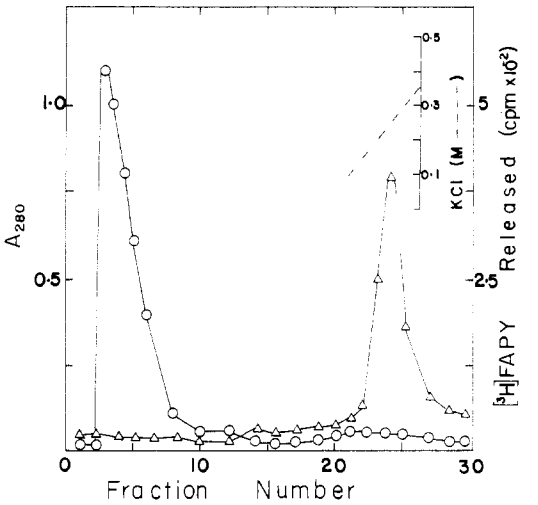


FIGURE 2: Chromatography of fraction III on Affi-Gel Blue. About 42 mg of protein in buffer C was applied to the column (0.8 × 12 cm). After the column was washed with loading buffer, the enzyme was eluted with a linear gradient of 0.1–0.8 M KCl in 0.05 M Tris-HCl, pH 7.4, 1 mM 2-mercaptoethanol, and 5% glycerol. After the $A_{280}$ was measured 10 μL of the indicated fractions was used in enzyme assays. $A_{280}$ (O); [$^3$H]FAPY cpm released (Δ); KCl linear gradient (M) (---).

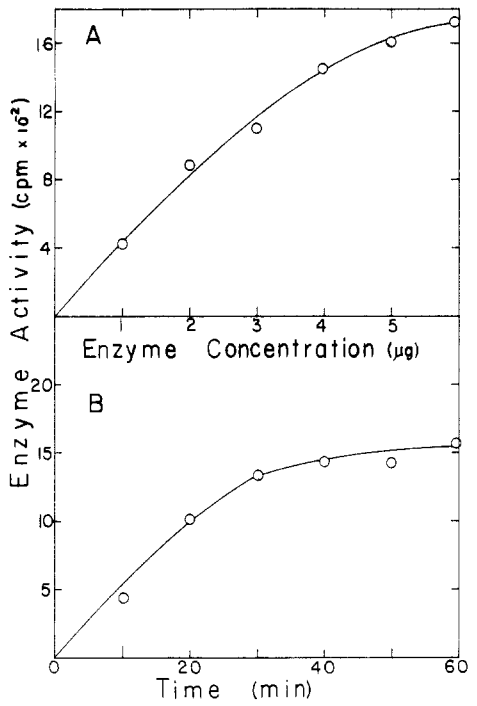


FIGURE 3: Enzyme activity as a function of protein concentration and reaction time. Reaction mixtures contained 2 μg of [$^3$H]FAPY-DNA (4000 cpm). (A) The reaction measured the release of FAPY by increasing amounts of enzyme during a 20-min incubation. (B) Kinetics of removal of FAPY by 4 microunits enzyme over a 60-min time period.

enzyme activity were pooled (fraction V) and used in the studies reported in this paper. At this stage 0.04 mg of the original 1104 mg of protein was recovered.

Fraction V was more unstable than fraction IV. To minimize loss of activity, we carried out Bio-Gel P-60 chromatography immediately after affinity column fractionation. The sensitivity of the enzyme to further handling made it impossible to purify it beyond this step.

Figure 3A depicts the relation between the rate of FAPY removal and increasing enzyme concentration (Fraction V). The reaction rate began to level off as the amount of enzyme in the reaction mixture exceeded 4 μg, presumably due to substrate depletion.

Table I: Purification of Formamidopyrimidine-DNA Glycosylase from 20 g of *E. coli*

| | volume (mL) | protein (mg) | sp. act. (microunits/mg) | total act. (microunits) |
|---|---|---|---|---|
| (I) crude extract | 57 | 1 104 | 2.1 | 2 700 |
| (II) ammonium sulfate | 8.6 | 312 | 5.4 | 2 100 |
| (III) Sephadex G-75 | 60 | 42 | 26.1 | 1 402 |
| (IV) Affi-Gel Blue | 3 | 0.14 | 14 402 | 362 |
| (V) Bio-Gel P-60 | 4.5 | 0.041 | 16 600 | 178 |

The time course of the reaction showed a linear rate of release of FAPY during the first 20 min and reached steady-state kinetics after 30 min (Figure 3B). As a result of these observations, we routinely carry out FAPY-DNA glycosylase assays over a 20-min time period. The steady-state phase seems to be due to a combination of substrate depletion and loss in enzyme activity because the addition of more enzyme resulted in further release of small amounts of FAPY (data not shown).

Analysis of fraction V by $NaDoDSO_4$–polyacrylamide slab gel electrophoresis (Weber & Osborn, 1969) showed three peaks of protein, suggesting that the preparation was not homogeneous. The extreme sensitivity of the enzyme to this manner of handling made it impossible for us to identify the peak with enzyme activity.

*General Properties of FAPY-DNA Glycosylase.* The results of a typical FAPY-DNA glycosylase preparation are summarized in Table I. The five-step purification of the enzyme resulted in a >8000-fold purification and an overall recovery of 6%. After sephadex G-75 filtration the affinity chromatography step achieved 550-fold enzyme purification while the final gel filtration raised this to 638-fold purification. This final step did not contribute much to raising the specific activity of the enzyme even though it eliminated as much as 70% of the material that had shown affinity for the Affi-Gel Blue adsorbent. The apparent discrepancy probably results from the increasing loss of enzyme activity that is observed during this final step of purification. Analytical gel filtration on a calibrated Bio-Gel P-60 column gave a molecular weight estimate of 30 000 and a Stokes radius of 26.4 Å for the protein.

The lability of FAPY-DNA glycosylase presents problems that are aggravated by the prolonged handling that is necessary for achieving enzyme purity. We would like to point out some of our observations in this respect. The enzyme is unstable in low ionic strength solutions but will retain activity for 1 week when stored in buffered 0.5 M KCl at 0 °C. Catalytic activity is lost when purified enzyme (fraction IV or V) is exposed to extreme temperature fluctuations; no activity was recovered when the enzyme stored at −20 or −80 °C was subsequently thawed for assaying. The enzyme also rapidly loses activity when a conventional pressure cell (Amicon PM-10 membrane) is used to concentrate it.

The stability of most enzymes is usually enhanced when they are stored in 50% glycerol or sucrose. In the case of FAPY-DNA glycosylase such a concentration of glycerol destabilizes it during storage. The presence of >10% glycerol in the reaction mixture causes a 60% reduction in enzyme activity. Addition of proteins like bovine serum albumin to fractions IV and V did not result in a significant stabilization.

*Substrate Specificity.* Figure 4 presents data on the activity of FAPY-DNA glycosylase toward three different DNA

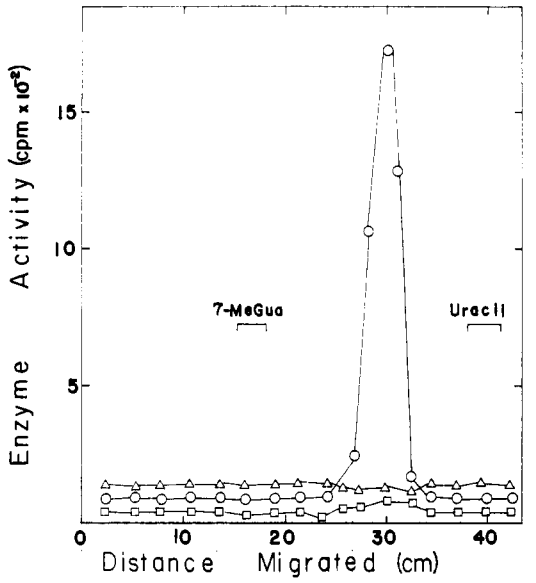


FIGURE 4: Substrate specificity of FAPY-DNA glycosylase. The activity of 6 microunits of enzyme on three different substrates was measured in the standard assay system. The material in the ethanol-soluble fraction was analyzed by paper chromatography. FAPY-DNA (○); 7-MeGua-DNA (□); uracil-DNA (△). Positions of marker 7-MeGua and uracil are indicated.

Table II: Substrate Specificities of Three DNA Glycosylases[a]

| enzyme | cpm in free base released from substrate: Me-DNA[b] | FAPY-DNA | uracil-DNA |
|---|---|---|---|
| FAPY-DNA glycosylase | 31 | 510 | 91 |
| 3-MeAde-DNA glycosylase | 397 | 28 | 82 |
| uracil-DNA glycosylase | 51 | 56 | 620 |

[a] Each reaction mixture contained 4 microunits of enzyme and 2 μg of DNA substrate (2200 cpm). [b] Me-DNA is [$^3$H]$Me_2SO_4$-alkylated DNA with ~15% 3-MeAde and 85% 7-MeGua or further processed by the method of Riazuddin & Lindahl (1978). The free base released was measured by paper chromatography.

substrates. The substrates tested were uracil-DNA, 7-methylguanine-DNA, and FAPY-DNA. The plot shows that the enzyme did not release either uracil or 7-methylguanine from DNA. When the enzyme was presented with a FAPY-DNA substrate, the plot shows that the enzyme released substantial amounts of FAPY residues from the DNA. The results in Table II further confirm the substrate specificity of FAPY-DNA glycosylase as well as those of uracil-DNA and 3-MeAde-DNA glycosylases. It is clear that FAPY-DNA glycosylase does not remove either 3-MeAde or uracil from DNA.

It should be pointed out that the enzyme has a preference for double-stranded DNA substrates, while denatured DNA is a poor substrate. If ring fission in guanine is produced by NaOH treatment, the DNA must be subsequently neutralized and then allowed to reassociate under the appropriate conditions before use as substrate. Under our reannealing conditions, most *M. luteus* DNA regains duplex structure while ~60% of calf thymus DNA reanneals (Britten & Kohne, 1968). The alternative procedure is to induce ring opening by solutions at pH 11.4; such solutions will cause ring fission without denaturing the DNA. Since the enzyme has a pH optimum between 7.2 and 8, the DNA substrates should be neutralized before use in assays. Because of the difficulties associated with ensuring the titrimetric accuracy of solutions buffered at pH 11.4, we have found it more satisfactory to generate FAPY-DNA substrates by NaOH treatment.

Table III: Effect of Pyrimidine Analogues on FAPY-DNA Glycosylase Activity[a]

| pyrimidine | concn (μg/mL) | enzyme act. (% of control) |
|---|---|---|
| none | | 100 |
| oxauracil | 7 | 100 |
| 2,4,6-triaminopyrimidine | 5 | 87 |
| 2,4,5-triamino-6-hydroxypyrimidine sulfate | 5 | 97 |
| 5-nitroso-2,4,6-triaminopyridine | 5 | 24 |
| 2,5,6-triamino-2-hydroxypyrimidine sulfate | 5 | 90 |
| formamidopyrimidine (FAPY) | 5 | 40 |

[a] The reaction protocol was that described under Methods. Enzyme activity values are averaged results from four experiments.

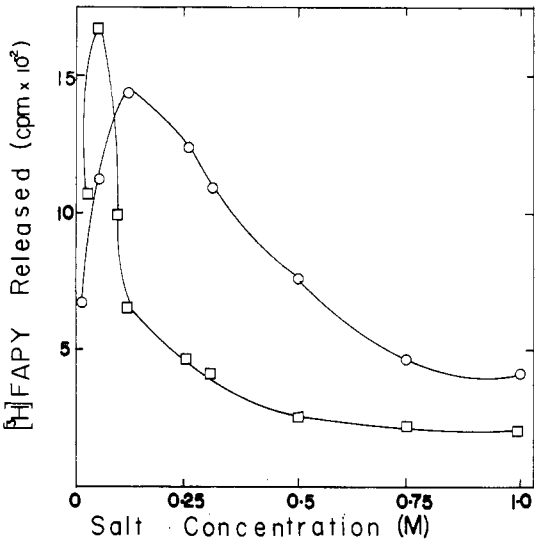


FIGURE 5: Effect of salt concentration on FAPY-DNA glycoylase activity. To the standard assay system were added increasing amounts of KCl or $KH_2PO_4$ buffer (pH 7.4). Incubation was for 20 min at 37°C. The [$^3$H]FAPY released into the ethanol-soluble supernatant was measured: KCl (○); $KH_2PO_4$ (□).

In order to further demonstrate the substrate specificity as well as the end product inhibition of FAPY-DNA glycosylase, we measured its activity in the presence of a wide variety of pyrimidine analogues. The enzyme was most inhibited by 5-nitroso-2,4,6-triaminopyrimidine followed by formamidopyrimidine (Table III). The inhibition by 2,4,6-triaminopyrimidine and 2,5,6-triamino-2-hydroxypyrimidine sulfate was at an insignificant level.

*Effect of Salt Concentration.* The activity of FAPY-DNA glycosylase toward FAPY-DNA was measured in the presence of varying salt concentrations. Figure 5 shows that a 0.1 M KCl or 0.05 M $KH_2PO_4$ (pH 7.4) concentration in the reaction mixture is optimal for enzyme activity. Lower concentrations of either reagent did not enhance enzyme activity while higher concentrations were inhibitory to the reactions.

*Effect of Different pH Conditions on 7-Methylguanine in DNA.* Exposure of DNA samples to physiological conditions (pH 7.4, 37 °C) over a 14-day time period showed a progressive, spontaneous release of 7-MeGua residues. About half of the 7-MeGua residues were released after 144 h. This rate of 7-MeGua depurination is in agreement with those obtained by Lawley (1975) and Singer (1979), who reported the release of this adduct with a half-life of 150 and 155 h, respectively, under comparable conditions.

The results summarized in Table IV show that the lowest pH at which ring opening was first observed is 9.0. This

Table IV: Fate of 7-Methylguanine in DNA Incubated under Different pH Conditions[a]

| buffer (0.05 M) | pH | 7-methylguanine (cpm) | FAPY (cpm) |
|---|---|---|---|
| sodium acetate | 5.1 | 293 | 0 |
| Pipes | 6.5 | 297 | 0 |
| Pipes | 6.8 | 320 | 0 |
| $KH_2PO_4$ | 7.2 | 303 | 0 |
| $KH_2PO_4$ | 7.5 | 322 | 0 |
| Tris-HCl | 8.0 | 311 | 0 |
| Tris-HCl | 8.5 | 318 | 0 |
| Glycine | 9.0 | 243 | 130 |
| sodium borate | 9.5 | 102 | 215 |

[a] Each buffer was in 0.1 M KCl, 0.01 M $MgCl_2$, and 1 mM EDTA. Processing details are given under Methods.

high-pH requirement for ring fission suggests that the intracellular mechanisms for the formation of FAPY are unlikely to be alkalinity.

## Discussion

The enzymatic release of 7-MeGua described in this paper is contingent upon the fission of the imidazole ring of 7-MeGua residues. There are two conditions under which the opening of the imidazole rings of adenine and guanine nucleosides or nucleotides has been observed. The radiogenic ring fission of such purines at $C^8$–$N^9$ to form FAPY derivatives (Hems, 1958, 1960; van Hemmen, 1971) appears to result from the action of the OH· and H· radicals (van Hemmen & Bleichrodt, 1971) generated by the radiolysis of solvent water molecules (Figure 6A). This mode of ring fission does not require alkylation of the purines.

The second agent causing ring fission is alkali. Alkaline ring fission requires the presence of N-7 alkylated guanines in DNA (Figure 6B) and can be induced by alkaline reagents starting at pH 9.0. While Haines et al. (1962) reported the formation of one pyrimide derivative, FAPY, from alkaline-treated 7-MeGuo, other investigators have reported the generation of four different products by this reaction (Lawley & Shah, 1972a). Our analysis of alkaline-treated 7-MeGuo by high-performance liquid chromatography resolves the ring-opened products into two distinct peaks; 90% of the enzyme-released FAPY cochromatographs with the product corresponding to the FAPY reported by Haines et al. (1962), and the other 10% is found in the second peak containing deformylated FAPY (C. J. Chetsanga, B. Bearie, C. Makaroff, unpublished observations). Garrett & Mehta (1972) showed that the imidazole ring of adenosine can be opened by 1.0 N NaOH at 80 °C; this fission does not require alkylated adenosine residues. The ring fission in 7-MeGua appears to be followed by an enolization of the carbonyl oxygen at C-4 and a deprotonation of N-3 (Hems, 1958) leading to the formation of 2,6-diamino-4-hydroxy-5-(*N*-methylformamido)pyrimidine (Haines et al., 1962). The resulting anomalous base pairing between the two pyrimidines, cytosine and formamidopyrimidine, is likely to have a mutagenic effect (Lawley & Brookes, 1961).

While the presence of FAPY in cells treated with either ionizing radiation or alkylating agents has not been demonstrated, Kriek & Emmelot (1964) observed a spontaneous ring fission in 35% of 7-MeGuo residues under physiological conditions in vitro. In this investigation we were unable to confirm such ring fission of 7-MeGua in DNA samples treated under comparable conditions. If FAPY is the true substrate of the glycosylase that we have purified, these results still leave unanswered the question about the cellular mechanisms for the conversion of 7-MeGua to FAPY. Although the alkaline

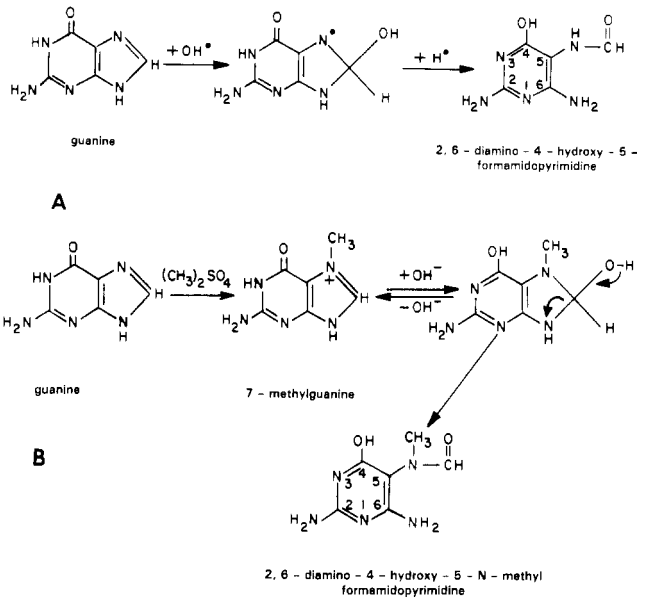


FIGURE 6: Proposed schemes for opening of imidazole ring of guanine by (A) ionizing radiation and (B) alkali to form formamidopyrimidine.

treatment of alkylated DNA has conveniently provided us with conditions that selectively generate ring-opened forms of methylated guanine (Kohn & Spears, 1967), the question as to whether methyl or other alkylating groups are required for the recognition of FAPY by the enzyme still needs to be resolved. It is hoped that radiogenic ring fission of nonalkylated guanines in DNA will provide FAPY-DNA substrates which will be suitable for resolving this question.

Finally, the affinity of the enzyme for the agarose-linked sulfonated polyaromatic dye, Affi-Gel Blue, provides some insight into the likely mechanism by which the enzyme binds substrate. This adsorbent is known to bind proteins that contain a dinucleotide fold which may consist of a nucleotide-specific allosteric site as well as a substrate binding site (Thompson et al., 1975). The strong affinity of FAPY-DNA glycosylase for Affi-Gel Blue suggests that the enzyme possesses a dinucleotide fold. We are seeking confirmation for the presence of such a fold in the enzyme by using appropriate probes. A knowledge of the nature of conformation around the catalytic site may enable us to understand the basis for the attack of ring-opened 7-methylguanine by FAPY-DNA glycosylase. Conceivably, other DNA glycosylases may interact with their substrates by a similar mechanism.

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# Effects of Detergent Micelles on the Recombination Reaction of Opsin and 11-*cis*-Retinal†

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ABSTRACT: When detergent-solubilized proteins interact with hydrophobic or amphiphilic molecules in the presence of detergent micelles, the solubility of the latter species in the micelles must be included in both thermodynamic and kinetic treatments. In this paper, we derive equations which describe the distribution of species present at equilibrium for a system in which a detergent-solubilized protein binds a hydrophobic (or amphiphilic) ligand. We have applied the formalism developed in this paper to the reaction describing the formation of rhodopsin from its apoprotein and 11-*cis*-retinal. Qualitatively, the results demonstrate that a significant portion of the observed decrease in the extent of recombination for rhodopsin solubilized in either sodium cholate or Tween 80 may be attributed to the partition of retinal into detergent micelles and that a detergent-induced protein denaturation need not be invoked to explain the data. We also discuss results for rhodopsin solubilized in a nonionic detergent (octaethylene glycol *n*-dodecyl ether) in which the detergent is clearly causing irreversible loss of the capability to recombine with 11-*cis*-retinal.

Whenever a detergent-solubilized membrane protein reacts with a hydrophobic or amphiphilic molecule, the solubility of the reactants in the detergent influences the thermodynamic and kinetic properties of the system. This paper addresses itself to this problem in terms of the recombination of 11-*cis*-retinal with opsin (bleached rhodopsin) to form the photosensitive protein rhodopsin.

The recombination of bleached rhodopsin and 11-*cis*-retinal occurs readily in intact disk membranes. When recombination is attempted with detergent-solubilized rhodopsin, the results depend on the detergent being used. With some detergents, no recombination is observed at all, and it is generally assumed that opsin has undergone irreversible denaturation in these

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