Purification and Properties of Mitochondrial Uracil-DNA Glycosylase from Rat Liver†

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Received April 5, 1988; Revised Manuscript Received May 20, 1988

ABSTRACT: Uracil-DNA glycosylase from rat liver mitochondria, an inner membrane protein, has been purified approximately 575 000-fold to apparent homogeneity. During purification two distinct activity peaks, designated form I and form II, were resolved by phosphocellulose chromatography. Form I constituted ~85% while form II was ~15% of the total activity; no interconversion between the forms was observed. The major form was purified as a basic protein with an isoelectric point of 10.3. This enzyme consists of a single polypeptide with an apparent M_r of 24 000 as determined by recovering glycosylase activity from a sodium dodecyl sulfate–polyacrylamide gel. A native M_r of 29 000 was determined by glycerol gradient sedimentation. The purified enzyme had no detectable exonuclease, apurinic/apyrimidinic endonuclease, DNA polymerase, or hydroxymethyluracil-DNA glycosylase activity. A 2-fold preference for single-stranded uracil-DNA over a duplex substrate was observed. The apparent K_m for uracil residues in DNA was 1.1 μM, and the turnover number is about 1000 uracil residues released per minute. Both free uracil and apyrimidinic sites inhibited glycosylase activity with K_i values of approximately 600 μM and 1.2 μM, respectively. Other uracil analogues including 5-(hydroxymethyl)uracil, 5-fluorouracil, 5-aminouracil, 6-azauracil, and 2-thiouracil or analogues of apyrimidinic sites such as deoxyribose and deoxyribose 5′-phosphate did not inhibit activity. Both form I and form II had virtually identical kinetic properties, and the catalytic fingerprints (specificity for uracil residues located in a defined nucleotide sequence) obtained on a 152-nucleotide restriction fragment of M13mp2 uracil-DNA were almost identical. These properties differentiated the mitochondrial enzyme from that of the uracil-DNA glycosylase purified from nuclei of the same source.

Uracil-DNA glycosylase catalyzes the specific removal of uracil from DNA by cleaving the N-glycosylic bond linking the base to the deoxyribose phosphate backbone. As products, free uracil and an apyrimidinic site in DNA are produced (Lindahl et al., 1977). Uracil residues found in DNA either as a result of dUMP incorporation by DNA polymerase or as a product of deamination of cytosine residues are recognized by this enzyme (Lindahl et al., 1977). The former situation results in a U·A base pair while the latter leads to a U·G mispair which, if not corrected, will generate a G·C to A·T transition mutation (Duncan & Miller, 1980). Genetic evidence in *Escherichia coli* (Duncan et al., 1978; Duncan & Weiss, 1982), *Bacillus subtilis* (Makino & Munakata, 1978), and *Saccharomyces cerevisiae* (Burgers & Klein, 1986) suggests that uracil-DNA glycosylase functions to prevent the mutagenic effects of uracil residues in DNA. On the basis of the properties of the purified *E. coli* enzyme, Lindahl (1977) first proposed that uracil-DNA glycosylase catalyzes the initial step in a base-excision repair pathway which functions to eliminate uracil from bacterial DNA. This pathway is a multistep process involving the concerted action of uracil-DNA glycosylase, apurinic/apyrimidinic (AP)[1] endonucleases, DNA polymerase, and DNA ligase.

E. coli provided the first source for purifying uracil-DNA glycosylase to apparent homogeneity (Lindahl et al., 1977). The enzyme was characterized as a monomeric protein M_r of 24 500 which did not require a cofactor, preferred single-stranded DNA as substrate, exhibited a turnover number of 800 uracil residues per minute, and was inhibited by free uracil. A similar activity has since been isolated from various other bacterial (Cone et al., 1977; Leblanc et al., 1982, Kaboev et al., 1985), viral (Caradonna & Cheng, 1981; Caradonna et al., 1987), plant (Talpaert-Borle & Liuzzi, 1982; Blaisdell & Warner, 1983; Bensen & Warner, 1987), and mammalian sources [Kuhnlein et al., 1978; Caradonna & Cheng, 1980; Krokan & Wittwer, 1981; Talpaert-Borle et al., 1982; Colson & Verly, 1983; Domena & Mosbaugh, 1985; Seal et al., 1987; for a review, see Mosbaugh (1988)]. Anderson and Friedberg (1980) first recognized that human KB cells contained both a nuclear and a mitochondrial form of uracil-DNA glycosylase. Distinct nuclear and mitochondrial enzyme activities have since been reported in other eukaryotic systems (Gupta & Sirover, 1981; Wittwer & Krokan, 1985; Domena & Mosbaugh, 1985; Burgers & Klein, 1986). In several cases, different biochemical properties have distinquished these two subcellular forms, although one could argue that such differences merely reflect variations in purity of the mitochondrial and nuclear enzymes since the former enzyme has, in most cases, not been extensively purified. To the contrary, evidence in *S. cerevisiae*

† This work was supported by Grant GM32823 from the National Institutes of Health.

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[1] Abbreviations: AP, apurinic/apyrimidinic; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetate adjusted to pH 8.0; DTT, dithiothreitol; form I DNA, covalently closed supercoiled duplex DNA; form II DNA, relaxed duplex DNA containing one or more phosphodiester bond discontinuites; poly(dA)·poly(dT+AP), annealed homopolymers of poly(dA) and poly(dT) in which the latter contains apyrimidinic sites; SDS, sodium dodecyl sulfate; HMU, 5-(hydroxymethyl)uracil; BSA, bovine serum albumin; BBOT, 2,5-bis(5-*tert*-butylbenzoxazol-2-yl)thiophene; PMSF, phenylmethanesulfonyl fluoride; TMEG buffer, 20 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 1 mM EDTA, and 10% (w/v) glycerol; TDEG buffer, 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 1 mM EDTA, and 10% (w/v) glycerol.

0006-2960/88/0427-6742$01.50/0

suggests that genetically separate subcellular forms exist since mutants defective in nuclear uracil-DNA glycosylase still possess a mitochondrial enzyme activity (Burgers & Klein, 1986). However, the biochemical relationship between these two enzymes remains to be determined.

In this paper, we describe the purification to apparent homogeneity of the mitochondrial uracil-DNA glycosylase from rat liver and characterization of its physical and catalytic properties. In addition, we provide evidence that two distinguishable forms of the mitochondrial enzyme exist. Finally, we compare the properties of each mitochondrial form with those of a homogeneous nuclear enzyme from the same tissue.

Experimental Procedures

Materials

E. coli CJ236[*dut*-1,*ung*-1,*thi*-1,*rel* A1/pCJ105 (Cm^r)] and M13mp2 bacteriophage were provided by T. A. Kunkel (National Institute of Environmental Health Sciences, Research Triangle Park, NC). DEAE-Sephadex A-50 and Pharmalyte ampholytes (pH range 7.5–10) were purchased from Pharmacia; phosphocellulose P-11 was from Whatman; hydroxylapatite (HTP) and Dowex 1-X8 (chloride form) resin were from Bio-Rad, and unlabeled deoxyribonucleoside triphosphates, calf thymus DNA, digitonin, uracil, uracil analogues, deoxyribose, and deoxyribose 5′-phosphate were from Sigma. [^{3}H]dUTP and [^{14}C]-5-hydroxytryptamine creatinine sulfate were obtained from Amersham, [^{3}H]dTTP was from New England Nuclear, and [γ-^{32}P]ATP was from ICN Radiochemicals.

E. coli uracil-DNA glycosylase (fraction V) was purified as previously described by Lindahl et al. (1977) except that the DNA–agarose column was eluted with a 400-mL linear gradient from 0 to 400 mM NaCl in 30 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, and 5% (w/v) glycerol. Homogeneous rat liver nuclear uracil-DNA glycosylase was purified as described by Domena and Mosbaugh (1985). One unit of glycosylase is defined as the amount required to release 1 nmol of uracil from DNA per hour at 37 °C under standard reaction conditions. *E. coli* DNA polymerase I (large fragment) and *Eco*RI restriction endonuclease were obtained from New England Biolabs, *Bgl*I and T4 polynucleotide kinase were from Bethesda Research Laboratories, and calf intestinal phosphatase was from Boehringer Mannheim.

Methods

Preparation of DNA Substrates. Calf thymus [*uracil*-^{3}H]DNA (210–450 cpm/pmol of uracil) was prepared as previously described by Domena and Mosbaugh (1985). Supercoiled PM2 [*thymine*-^{3}H]DNA (1650 cpm/pmol, >90% form I) was isolated from phage grown on *Alteromonas espejiana* Bal 31, as described by Espejo and Canelo (1968) and modified by Kuhnlein et al. (1976). Partial depurination of PM2 [^{3}H]DNA was carried out at 70 °C for 15 min in 10 mM sodium citrate (pH 5.0) and 100 mM NaCl so as to generate about two apurinic sites per DNA molecule. Bacteriophage SP01 [5-(*hydroxymethyl*)*uracil*-^{3}H]DNA was provided by S. M. Linn (University of California, Berkeley). Oligonucleotides GCCAGTGAATTCGTAATC (18-mer) and CATTCGCCATTCAGGCTGCGC (21-mer) were prepared with an Applied Biosystems 380A oligonucleotide synthesizer by W. R. Folk (University of Texas).

Poly(dA)·poly(dT) containing [^{3}H]uracil residues was synthesized in a reaction mixture (24.4 mL) containing 70 mM potassium phosphate buffer (pH 7.5), 1 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 165 μM dTTP, 81 μM [^{3}H]dUTP (660 cpm/pmol), 2.5 mM poly(dA), 1.1 mM oligo(dT)$_{12-18}$, and 183 units of *E. coli* DNA polymerase I. After a 30-min incubation at 37 °C, the reaction was adjusted to 500 mM NaCl and the polymerase inactivated by heating for 10 min at 70 °C. Unincorporated deoxyribonucleoside triphosphates were removed by chromatography on a NACS 52 (BRL) column, with [*uracil*-^{3}H]poly(dA)·poly(dT) being eluted in buffer containing 10 mM Tris-HCl (pH 7.2), 1 mM EDTA, and 2 M NaCl. The synthetic polymer was then dialyzed into TE buffer [10 mM Tris-HCl (pH 7.2), 1 mM EDTA].

Poly(dA)·poly(dT) containing apyrimidinic sites was produced in a reaction (9.1 mL) containing 70 mM Hepes–KOH (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, 8.1 μM [*uracil*-^{3}H]poly(dA)·poly(dT), and 573 units of *E. coli* uracil-DNA glycosylase. The reaction was carried out at 37 °C for 30 min and resulted in >95% of the [^{3}H]uracil being released. To remove *E. coli* uracil-DNA glycosylase, the reaction was adjusted to 1.25 M NaCl and concentrated with a Centriprep 30 (Amicon; M_r 30 000 cutoff) filter which was washed several times with 5 M NaCl. This treatment resulted in the loss of >99% of free [^{3}H]uracil and glycosylase activity from the DNA preparation. The poly(dA)·poly(dT+AP) product containing apyrimidinic sites was freed of NaCl and the buffer exchanged by washing with 10 mM Hepes–KOH (pH 7.5) containing 1 mM EDTA. As control, [*uracil*-^{3}H]poly(dA)·poly(dT) not exposed to uracil-DNA glycosylase was treated by the same procedure.

M13mp2 uracil DNA was prepared essentially as described by Kunkel et al. (1987); some thymine residues in the phage DNA were replaced by uracil. A 152 nucleotide *Bgl*I–*Eco*RI single-stranded DNA fragment was isolated as a defined substrate for uracil-DNA glycosylase reactions. This target molecule was obtained by first annealing an oligonucleotide (18-mer; 6.3 ng/μg M13mp2 DNA), complementary to the single *Eco*RI restriction endonuclease site, to the M13mp2 DNA. The DNA was then linearized in a reaction (50 μL) containing 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 50 mM NaCl, and 48 units of *Eco*RI and 30 μg of M13mp2 DNA. Following incubation for 30 min at 37 °C, the DNA was phenol extracted, ethanol precipitated, and resuspended in 50 μL of buffer containing 52 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, and 10 mM $MgCl_2$. Calf intestinal alkaline phosphatase (0.01 unit/pmol of 5′-termini) was then added and incubated for 30 min at 37 °C. The phosphatase was then inactivated with 10 mM nitrilotriacetic acid and 0.5% SDS and heated at 68 °C for 30 min. Following sequential phenol and ether extractions, the linear DNA was precipitated with ethanol and resuspended in buffer (50 μL) containing 50 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 5 mM dithiothreitol, 10 mM $MgCl_2$, 0.1 mM spermidine, 0.9 μM [γ-^{32}P]ATP (7000 Ci/mmol), and 20 units of T4 polynucleotide kinase. The kinase reaction was terminated with 20 mM EDTA, diluted with 150 μL of buffer A [100 mM Tris-HCl (pH 7.5), 1 mM EDTA], and applied to a NENSORB 20 (Du Pont) column. After the column was washed with buffer A then with water, DNA was eluted with 50% methanol. The methanol was evaporated and [^{32}P]DNA resuspended in annealing buffer (25 μL) containing 100 mM Tris-HCl (pH 8.0) and 50 mM NaCl, and a second oligonucleotide (21-mer; 6.3 ng/μg M13mp2 DNA) complementary to the *Bgl*I restriction endonuclease site was added. After annealing, the mixture was adjusted to 10 mM $MgCl_2$, and 48 units of *Bgl*I was added. Incubation was for 30 min at 37 °C, and the product was phenol extracted and ethanol precipitated. The *Bgl*I digestion produced two "+" strand fragments, but only the 152-nucleotide fragment contained ^{32}P label. After the DNA pellet

was resuspended in 20 μL of 95% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% (w/v) xylene cyanol, and 0.1% (w/v) bromophenol blue, the sample was heated to 95 °C and loaded onto an 8% polyacrylamide–7 M urea gel. Electrophoresis was carried out in TBE buffer containing 90 mM Tris, 90 mM boric acid, and 2 mM EDTA (pH 7.5) at 1800 V. Autoradiography was performed with XAR-5 (Kodak) film; the 152-nucleotide [^{32}P]DNA fragment was located and then extracted from the gel by electroelution into TBE buffer. Finally, the fragment was precipitated with ethanol and resuspended in TE buffer.

Enzyme Assays. Standard uracil-DNA glycosylase reaction mixtures (100 μL) contained 70 mM Hepes–KOH (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, 25 mM NaCl, 25 μg of [*uracil*-^{3}H]DNA (3.5 μM uracil; 210–450 cpm/pmol of uracil), and 25 μL of enzyme (0.004–0.08 unit). When necessary, enzyme dilutions were made in 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 1 mM EDTA, 100 mM NaCl, 10% (w/v) glycerol, and 125 μg/mL bovine serum albumin. After incubation for 30 min at 37 °C, the reaction was terminated on ice with 250 μL of 10 mM ammonium formate (pH 4.2). The [*uracil*-^{3}H]DNA substrate was removed from free [^{3}H]uracil by applying 300 μL of the reaction mixture to a Bio-Rad 1-X8 (formate form) column (0.2 cm^2 × 2 cm) equilibrated with 10 mM ammonium formate (pH 4.2). [^{3}H]Uracil was washed through the column with 1.7 mL of equilibration buffer, four 500-μL fractions were collected, and the radioactivity was measured by a liquid scintillation spectrometer using Ready Protein$^+$ (Beckman) liquid scintillation fluor.

Hydroxymethyluracil-DNA glycosylase activity was measured under conditions as described above for uracil-DNA glycosylase except that SP01 [*5-(hydroxymethyl)uracil*-^{3}H]DNA (30 cpm/pmol of HMU) was substituted as the substrate.

DNA polymerase γ activity was measured in reaction mixtures (62.5 μL) containing 25 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 7.5 mM $MgCl_2$, 150 mM NaCl, 15 μM each of dATP, dGTP, dCTP, and [^{3}H]-dTTP (800–22 000 cpm/pmol), 15% (w/v) glycerol, 6.25 μg of activated DNA, and 12.5 μL of enzyme (0.0003–0.02 unit). After incubation at 37 °C for 30 min, 100 μL of 1 mg/mL BSA in 0.1 M sodium pyrophosphate was added on ice, and the DNA was precipitated with 500 μL of 10% trichloroacetic acid. Precipitates were collected on Schleicher & Schuell No. 30 glass fiber filters, washed with 15 mL of 1 N HCl in 0.1 M sodium pyrophosphate, and dried with 95% ethanol. Acid-insoluble radioactivity was measured with 0.4% 2,5-bis(5-*tert*-butylbenzoxazol-2-yl)thiophene (BBOT) in toluene being the scintillator. One unit of DNA polymerase activity catalyzes the incorporation of 1 nmol of total dNMP into activated DNA in 60 min at 37 °C.

Apurinic or nonspecific endonuclease activity was assayed in reactions (50 μL) containing 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.005% Triton X-100, and 5 nmol of either depurinated (ca. two AP sites per genome) or native PM2 [^{3}H]DNA, respectively. Reactions were incubated for 15 min at 37 °C and form II DNA molecules detected by the method of Kuhnlein et al. (1976). One unit of endonuclease activity will produce 1 pmol of nicks per minute at 37 °C. AP endonuclease activity generates nicks specifically in AP DNA.

Monoamine oxidase activity was measured in a reaction (20 μL) containing 50 mM potassium phosphate (pH 7.5) and 1 mM [^{14}C]-5-hydroxyltryptamine creatinine sulfate (6680 cpm/pmol). After 30 min at 37 °C, the reaction was adjusted to 6 N HCl. Unreacted and oxidized [^{14}C]-5-hydroxyltryptamine creatinine sulfate were separated by extraction with 120 μL of toluene/ethyl acetate (1:1), and 60 μL of the organic layer was removed for counting radioactivity in 5 mL of 0.4% BBOT in toluene.

Malate dehydrogenase was detected by monitoring the oxidation of NADH at 340 nm during the reduction of oxaloacetate to malate. Reactions (1 mL) contained 89 mM potassium phosphate (pH 7.4), 250 μM NADH, 20 μM oxaloacetate, and 10 μL of enzyme.

Molecular Weight Determination. Molecular weight was determined by centrifugation in 10–30% linear glycerol gradients (4.5 mL) containing 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA, 100 mM NaCl, and 0.015% Triton X-100. Samples (250 μL) were applied to the top of the gradient, and centrifugation was carried out at 45 000 rpm for 36 h at 4 °C in a Beckman SW 50.1 rotor. Fractions (175 μL) were collected from the bottom of the tube and assayed for uracil-DNA glycosylase activity. Each sample also contained bovine serum albumin (4.5 S, M_r 66 000) and lysozyme (2.1 S, M_r 14 000) as markers, and sedimentation coefficients were calculated by the method of Martin and Ames (1961).

Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis was performed according to Laemmli and Favre (1973) with a stacking gel containing 3% acrylamide with 0.24% *N,N'*-methylenebis(acrylamide). The resolving gel contained 12.5% acrylamide with 0.24% *N,N'*-methylenebis-(acrylamide). Samples (30–50 μL) were prepared by heating at 100 °C for 5 min with an equal volume of 50 mM Tris-HCl (pH 6.8), 140 mM 2-mercaptoethanol, 1% sodium dodecyl sulfate, 10% (w/v) glycerol, and 0.02% bromophenol blue. Electrophoresis was at 200 V until the tracking dye migrated about 90% the length of the gel. Following fixation in 10% acetic acid containing 40% methanol, protein bands were detected with Rapid-Ag-Stain (ICN). Recovery of mitochondrial uracil-DNA glycosylase activity from SDS–polyacrylamide gels was performed by a modification of the procedure described by Hager and Burgess (1980). Electrophoresis of uracil-DNA glycosylase (20–100 units) containing 10 μg of cytochrome *c* was as described above. After electrophoresis, the gel was immersed for 5 min each in water, then in 1 mM dithiothreitol, in 0.25 M KCl–1mM dithiothreitol, and finally in 1 mM dithiothreitol. Each lane of the gel was then sliced into either 2- or 4-mm sections, crushed, and extracted with 1 mL of extraction buffer containing 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 5 mM dithiothreitol, 150 mM NaCl, 0.1% SDS, and 100 μg of bovine serum albumin. Extraction was carried out for 5 h with vigorous agitation at 25 °C. Then the extraction buffer was recovered from the gel fragments, and 4 mL of cold (−20 °C) acetone was added to each sample to precipitate protein. After 20 min in a dry ice–acetone bath, the samples were centrifuged at 10000*g* for 20 min. The protein pellets were resuspended in 0.4 mL of buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 0.1 mM EDTA, 150 mM NaCl, 20% (w/v) glycerol, 100 μg/mL bovine serum albumin, and 6 M guanidine hydrochloride. After agitation for 50 min at 25 °C, the samples were dialyzed against 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA, 150 mM NaCl, 10% (w/v) glycerol, and 0.015% Triton X-100 to remove the guanidine hydrochloride and to renature the protein.

Results

Association of Uracil-DNA Glycosylase Activity with Mitochondria

Using mitochondria isolated by differential centrifugation,

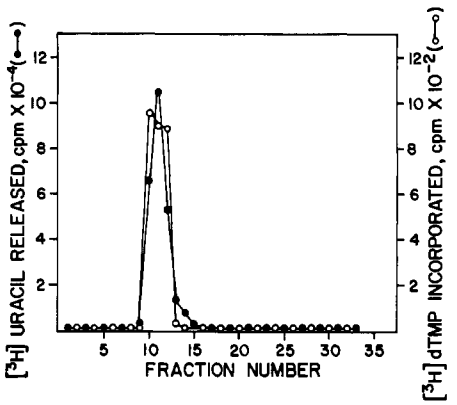


FIGURE 1: Cosedimentation of uracil-DNA glycosylase and DNA polymerase γ with mitochondria. Isolated mitochondria (1 g wet weight) were layered onto a linear sucrose gradient (1–1.9 M) containing 10 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, and 1 mM EDTA. The sample was centrifuged in a Beckman SW28 rotor at 20000 rpm for 2 h at 4 °C. Fractions (1.2 mL) were collected from the bottom of the gradient, and 2 mL of buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA, 100 mM NaCl, 10% (w/v) glycerol, 1% Triton X-100, and 1 mM phenylmethanesulfonyl fluoride was added to each tube. Each sample was subjected to two 15-s pulses with a Vibra Cell (Sonic Materials) sonicator at maximum power to disrupt mitochondria. Samples were then assayed for uracil-DNA glycosylase (●) and DNA polymerase (○) activity as described under Experimental Procedures. Polymerase activity was also found to be stimulated by 250 mM NaCl and inhibited by 10 mM *N*-ethylmaleimide. Sedimentation was from right to left.

we previously identified a mitochondrial-associated uracil-DNA glycosylase (Domena & Mosbaugh, 1985). To determine whether this activity was truly a mitochondrial enzyme and not merely a cytoplasmic contaminant, we sedimented intact mitochondria through a linear sucrose gradient. After centrifugation, uracil-DNA glycosylase was found to cosediment with a mitochondrial enzyme marker, DNA polymerase γ (Figure 1). No uracil-DNA glycosylase activity was detected near the top of the gradient as would be expected for a soluble protein. Further evidence that this represented a mitochondrial enzyme came from submitochondrial fractionation experiments. Isolated mitochondria were treated with digitonin and fractionated into matrix, inner membrane, and outer membrane plus intermembrane space. Monoamine oxidase, an outer membrane enzyme (Greenawalt & Schnaitman, 1970), malate dehydrogenase, a matrix enzyme (Schnaitman & Greenawalt, 1968), DNA polymerase γ, an inner membrane enzyme (Adams & Kalf, 1980), and uracil-DNA glycosylase were assayed to establish the location of each (Table I). The majority (83%) of the uracil-DNA glycosylase activity was located with DNA polymerase γ in the inner membrane compartment. Taken together, these results suggest that uracil-DNA glycosylase resides not only as a mitochondrial component but as an inner mitochondrial membrane-associated protein. Whether any association exists between the glycosylase and DNA polymerase γ in the membrane remains unclear.

Purification of Uracil-DNA Glycosylase from Mitochondria

In a previous paper (Domena & Mosbaugh, 1985) we described a partial purification of rat liver mitochondrial uracil-DNA glycosylase. It was necessary to radically change that purification scheme to obtain a homogeneous preparation with higher yield and stability.

Preparation of Mitochondrial Extract. Mitochondria were isolated by differential centrifugation as previously described by Domena and Mosbaugh (1985). All procedures were performed at 0–4 °C unless otherwise indicated. Generally, 80 g of frozen mitochondria (10 g of total protein) was disrupted by grinding with 50 g of levigated alumina. The paste was extracted with 200 mL of buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 1 mM EDTA, 1 M NaCl, and 1 mM phenylmethanesulfonyl fluoride. The alumina was removed by centrifugation at 500*g* for 10 min, and unbroken mitochondria in the supernatant fraction were pelleted at 10000*g* for 10 min. The resulting supernatant was then centrifuged for 1 h at 100000*g* in a Beckman 50.2 Ti rotor, and the soluble proteins were dialyzed against TMEG buffer containing 150 mM NaCl. The dialyzed extract was designated fraction I.

Table I: Uracil-DNA Glycosylase Activity in Various Submitochondrial Fractions[a]

fraction	enzyme activity (units)[b]			
	monoamine oxidase	malate dehydrogenase	DNA polymerase γ	uracil-DNA glycosylase
outer membrane-intermembrane space	29	398	1.8	274
inner membrane	0.3	555	26.2	1980
matrix	0.1	1110	14.1	126

[a] Mitochondria were isolated from 13 g of rat liver by the "fast, low yield" procedure and then fractionated by treatment with digitonin as described by Pedersen et al. (1978). [b] Enzyme activities were measured as described under Experimental Procedures.

Ammonium Sulfate Precipitation. Ammonium sulfate (solid) was slowly added to fraction I to give 35% saturation. Precipitated protein was removed by centrifugation at 15000*g* for 20 min. The supernatant fraction was then adjusted to 65% ammonium sulfate and the precipitate collected as described above. The pellet was suspended in TDEG buffer and dialyzed against the same buffer. This material constituted fraction II.

DEAE-Sephadex Chromatography. Fraction II was loaded onto a DEAE-Sephadex A-50 column (4.9 cm^2 × 70 cm) equilibrated with TDEG buffer. The column was washed with 175 mL of equilibration buffer, and a 300-mL linear gradient of 0–500 mM NaCl in TDEG buffer was applied. Uracil-DNA glycosylase eluted during the wash (Figure 2A). Since a physical association between human placental uracil-DNA glycosylase and a subunit of DNA polymerase α has been reported (Seal & Sirover, 1986), we monitored DNA polymerase γ activity to determine whether a complex might exist between the two corresponding mitochondrial enzymes. However, no polymerase activity was detected in the uracil-DNA glycosylase peak; nor was uracil-DNA glycosylase found associated with the polymerase γ peak. Fractions containing uracil-DNA glycosylase activity were pooled as fraction III.

Isoelectric Focusing. Fraction III was concentrated (~ 25-fold) by ultrafiltration and subjected to isoelectric focusing on an flat-bed apparatus (LKB). The sample (4.4 mL) was applied to a partially prefocused gel bed consisting of Sephadex G-75 and Pharmalyte (pH 7.5–10) ampholytes. After focusing was complete, the bed was partitioned into 30 equal sections, and each gel aliquot was placed into 5-mL syringes plugged with glass wool. The enzyme was eluted with 3 mL of TDEG buffer containing 0.015% Triton X-100 which was passed through the gel by centrifugation for 5 min in a clinical centrifuge. Following centrifugation the eluate was assayed for activity. Uracil-DNA glycosylase focused as a sharp peak with a p*I* value of 10.3 (Figure 2B). Fractions containing glycosylase activity were pooled and represented fraction IV.

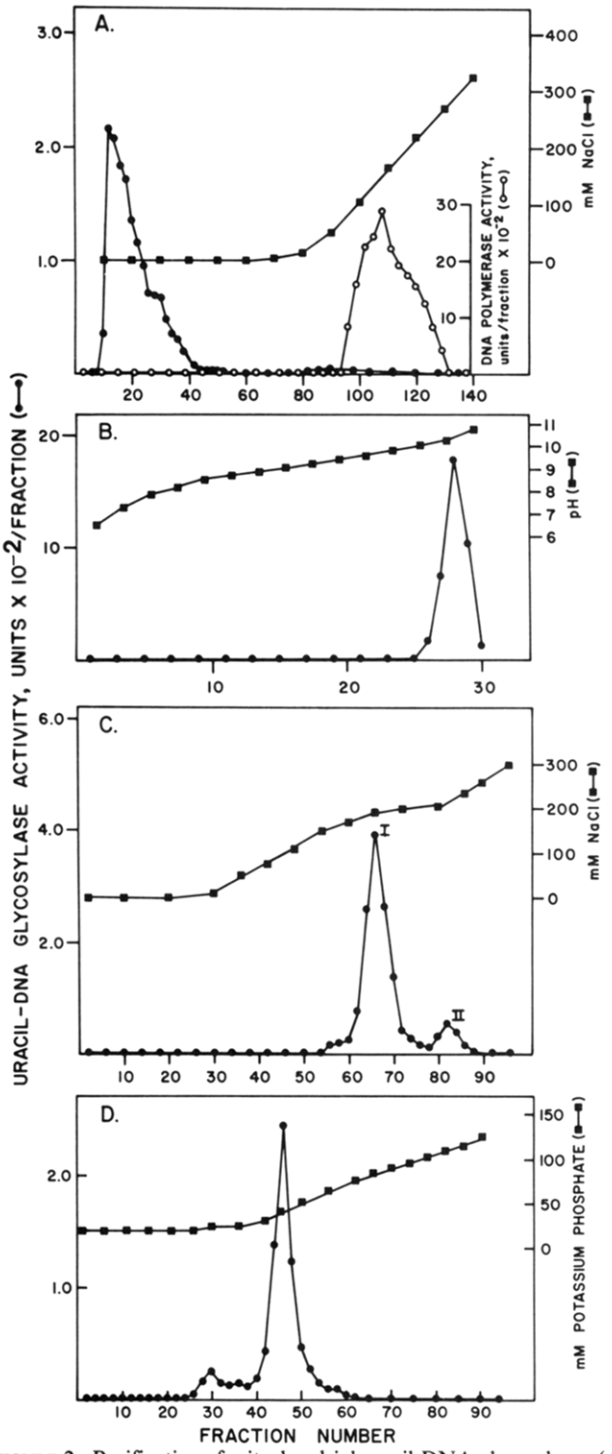


FIGURE 2: Purification of mitochondrial uracil-DNA glycosylase. (A) DEAE-Sephadex A-50 chromatography of fraction II uracil-DNA glycosylase. Fractions containing uracil-DNA glycosylase activity were pooled and concentrated in an Amicon ultrafiltration cell with a Diaflo PM10 membrane. (B) Fraction III uracil-DNA glycosylase was subjected to isoelectric focusing on a flat-bed (LKB) apparatus. The gel bed consisted of Sephadex G-75 (superfine) in a solution of Pharmalyte (pH 7.5–10) ampholytes diluted 1:15 with 10% (w/v) glycerol and was prefocused at 8 W (constant power) for 1 h at 4 °C. The sample (4.4 mL) was applied to the center of the gel bed and focused, as above, for 8 h. After focusing, the gel was fractionated, the pH was measured, the resin was removed by centrifugation, and the enzyme was eluted from the resin with TDEG buffer containing 0.015% Triton X-100. (C) Phosphocellulose chromatography of fraction IV uracil-DNA glycosylase. Two peaks of uracil-DNA glycosylase were eluted and designated form I (~185 mM NaCl) and form II (~220 mM NaCl). The major activity, form I, was pooled as fraction Va and further purified. (D) Hydroxylapatite chromatography of fraction Va uracil-DNA glycosylase. Details of column elution are described under Results. Fractions were assayed for uracil-DNA glycosylase (●) and in some cases DNA polymerase γ (○) as described under Experimental Procedures.

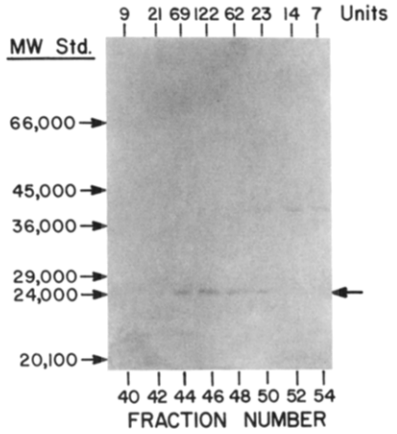


FIGURE 3: SDS–polyacrylamide slab gel electrophoresis of fractions containing uracil-DNA glycosylase activity from the hydroxylapatite column. Portions (750 μL) of fractions from the hydroxylapatite column that contained uracil-DNA glycosylase were concentrated to 50 μL with Centricon 10 concentrators. Each sample was denatured and subjected to electrophoresis on a 12.5% polyacrylamide gel as described under Experimental Procedures. Protein bands were visualized after Rapid-Ag staining (ICN). The molecular weight standards were bovine serum albumin, ovalbumin, glyceraldhyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, and soybean trypsin inhibitor, as indicated from top to bottom, respectively. The arrow on the right indicates the location of the protein band that showed an intensity which correlated with uracil-DNA glycosylase activity. Enzyme units loaded into each lane are given at the top of the gel.

Phosphocellulose Chromatography. Fraction IV was applied to a phosphocellulose column (1.8 cm^2 × 6 cm) equilibrated with TDEG buffer containing 0.015% Triton X-100. At this step, the inclusion of Triton X-100 was absolutely required to maintain activity. The column was washed with 20 mL of equilibration buffer followed by a 150-mL linear gradient of 0–450 mM NaCl in the equilibration buffer. Two peaks of uracil-DNA glycosylase activity were eluted (Figure 2C) and designated form I and form II. Form I and II were pooled separately and identified as fraction Va and Vb, respectively.

Hydroxylapatite Chromatography. A portion of fraction Va (2000 units) was diluted (1:3) with TDEG buffer containing 0.015% Triton X-100 before being loaded onto a hydroxylapatite column (1.8 cm^2 × 3 cm) equilibrated with buffer containing 20 mM potassium phosphate (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, 50 mM NaCl, 10% (w/v) glycerol, and 0.015% Triton X-100. The column was washed with 40 mL of equilibration buffer and enzyme eluted with a 100-mL linear gradient of 0–200 mM potassium phosphate also in the equilibration buffer. The majority of uracil-DNA glycosylase activity eluted as a symmetrical peak at about 40 mM potassium phosphate (Figure 2D). Fractions containing enzyme activity were pooled, adjusted to 150 mM NaCl, and designated fraction VI. A summary of the purification scheme is presented in Table II.

Purity and Molecular Weight

Electrophoresis of individual fractions across the activity peak from the hydroxylapatite column in an SDS–polyacrylamide gel revealed a single silver-stainable polypeptide with an apparent M_r of 24 000 (Figure 3). The staining density of this band correlated with the activity present in each fraction. In sister gels that were sliced, protein extracted, and enzyme renatured, only one peak of uracil-DNA glycosylase activity was recovered which comigrated with the M_r 24 000 band (Figure 4A). We estimate that fraction VI enzyme, which was purified approximately 575 000-fold from the mitochondrial extract, was at least 90% homogeneous. The

Table II: Purification of Rat Liver Mitochondrial Uracil-DNA Glycosylase[a]

fraction	total protein (mg)[b]	total activity (units)	specific activity (units/mg)	purification (x-fold)	yield (%)[c]
(I) mitochondrial extract	2900	12 500	4	1.0	100
(II) ammonium sulfate	920	20 700	23	5.8	166
(III) DEAE-Sephadex	20	9 800	490	123	78
(IV) isoelectric focusing		7 800			62
(Va) phosphocellulose (form I)	0.12	4 100	34 200	8 550	33
(Vb) phosphocellulose (form II)	0.04	400	10 000	2 500	3
(VI) hydroxylapatite[d]	0.001	2 540	2 300 000	575 000	20

[a] Uracil-DNA glycosylase activity was assayed as described under Experimental Procedures. [b] Protein concentrations in fractions I and II were determined with the biuret assay (Gornall et al., 1949), whereas the Bio-Rad assay originally described by Bradford (1976) was used in the other cases. Bovine serum albumin was used as a standard for all determinations. Protein concentration was not reported for fraction IV because of ampholyte interference. [c] In all cases yield was calculated on the basis of the activity observed in the mitochondrial extract. This preparation yielded form I and II activities of 81% and 9%, respectively. [d] Fraction VI was obtained by hydroxylapatite chromatography of form I enzyme.

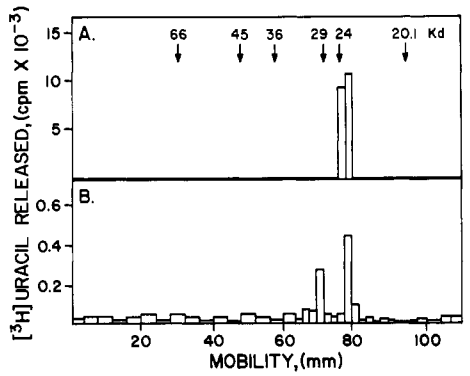


FIGURE 4: Recovery of uracil-DNA glycosylase activity after SDS–polyacrylamide gel electrophoresis. (A) Fraction Va uracil-DNA glycosylase (20 units) or (B) fraction I glycosylase (26 units) was loaded with 10 μg of cytochrome *c* onto a 12.5% SDS–polyacrylamide gel and electrophoresed along side of protein standards as described under Figure 3. The gels were sliced into 2- or 4-mm sections, protein extracted, enzyme renatured, and assayed for uracil-DNA glycosylase activity as described under Experimental Procedures with one modification: the enzyme reaction contained 25 μg of [*uracil*-^{3}H]DNA (72 nM uracil; 18 000 cpm/pmol of uracil) to increase the sensitivity of the assay. Though this reaction measured submaximal velocity, these measurements were still proportional to enzyme concentration.

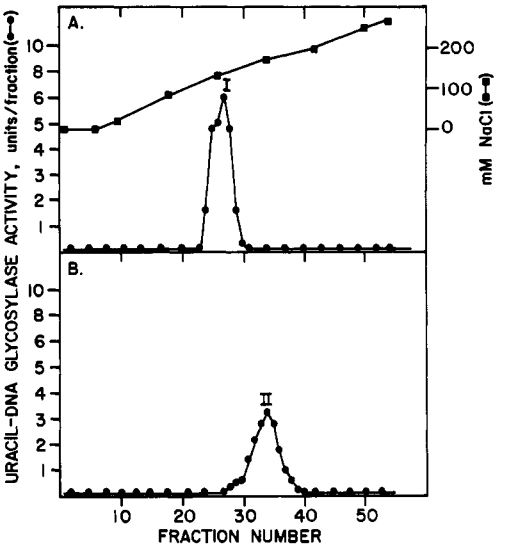


FIGURE 5: Rechromatography of fraction Va and Vb uracil-DNA glycosylase on phosphocellulose columns. (A) Fraction Va uracil-DNA glycosylase form I (12 units) was dialyzed into equilibration buffer (TDEG containing 0.015% Triton X-100) and applied to a phosphocellulose column. The chromatographic parameters were identical with those described for the initial phosphocellulose column (Figure 2C) which are described under Results. (B) Fraction Vb uracil-DNA glycosylase form II (15 units) was treated as above and rechromatographed on another identical phosphocellulose column. Standard uracil-DNA glycosylase assays were as described under Experimental Procedures.

purified enzyme specifically catalyzed the release of uracil from DNA but did not release *5-(hydroxymethyl)uracil* (<10% uracil-DNA glycosylase activity). Furthermore, no contaminating nonspecific or AP endonuclease activity was detected in fraction VI uracil-DNA glycosylase. The limit of detection for endonuclease activity was less than 0.004% of the glycosylase activity.

Characterization of Mitochondrial Uracil-DNA Glycosylase Activity

Reaction Requirements. The general properties of fraction VI uracil-DNA glycosylase are similar to those of the less extensively purified enzyme preparation previously investigated (Domena & Mosbaugh, 1985). In addition, the enzyme was found to be stimulated by NaCl on both single- and double-stranded uracil-DNA with optimum activity occurring between 25 and 50 mM NaCl. In contrast, KCl had no stimulatory effect up to 50 mM and inhibited activity by 50% at 100 mM. No dependence on divalent cations was observed: the enzyme was not inhibited by 1 mM EDTA, and 10 mM $MgCl_2$, $MnCl_2$, and $CaCl_2$ each caused about 50% reduction in enzyme activity.

Evidence for Two Distinct Mitochondrial Forms of Uracil-DNA Glycosylase. As described above, phosphocellulose chromatography of fraction IV uracil-DNA glycosylase resolved two enzyme species (form I and II) which constituted about 85% and 15% of the total activity, respectively (Figure 2C). When individually rechromatographed on phosphocellulose, neither exhibited any interconversion between forms (Figure 5). Thus, these two species were not found to be in equilibrium with one another. In addition, form I and II did not seem to be generated during purification. Both activities were also resolved from an earlier step in the purification scheme. For example, phosphocellulose chromatography of fraction III uracil-DNA glycosylase also resulted in two peaks with approximately the same ratio as described above (data not shown). Since neither activity shared chromatographic properties with the nuclear enzyme isolated from the same tissue (Domena & Mosbaugh 1985), we attempted to determine if form I and form II were both mitochondrial in origin. To do this, isolated mitochondria were extracted and subjected to SDS-polyacrylamide gel electrophoresis. Two peaks of uracil-DNA glycosylase were recovered after renaturing the enzyme from the gel (Figure 4B). The major activity (~66%) migrated with a mobility corresponding to a polypeptide of about M_r 24 000, identical with that of form I. The minor activity (~34%) had a molecular weight of about 29 000; however, attempts to correlate this activity with form II have been equivocal. Nevertheless, these results suggest that two

Table III: Effect of Apyrimidinic Sites on Uracil-DNA Glycosylase Activity[a]

kinetic parameter	nuclear	mitochondrial form I	mitochondrial form II
K_m (μM uracil residues)	0.5	1.1	0.9
K_i (μM AP sites)	0.3	1.2	0.8
K_{cat} (uracils released/min)	8	1000	
inhibition	competitive	competitive	competitive

[a] Inhibition of uracil-DNA glycosylase activity by apyrimidinic sites was determined as described in Figure 6. The K_i values were determined from a Dixon (1953) replot.

forms exist and that the lower molecular weight form I is present in intact mitochondria.

Inhibition of Uracil-DNA Glycosylase by Reaction Products. To assess the relationship between form I and form II uracil-DNA glycosylase, several catalytic properties were compared. Like other uracil-DNA glycosylases (Lindahl et al., 1977; Mosbaugh, 1988), both mitochondrial activities were inhibited by free uracil. When 0.5 mM uracil was added to standard reactions containing form I or II, 44% and 42% inhibition was observed, respectively. Using [*uracil*-^{3}H]-poly(dA)·poly(dT) as substrate, we determined that uracil acted as a noncompetitive inhibitor with a K_i value of about 0.6 mM as determined for fraction VI uracil-DNA glycosylase. Several uracil analogues were also tested, but none of the following compounds caused significant inhibition (<10% inhibition) at 1 mM: 5-(hydroxymethyl)uracil, 5-aminouracil, 5-fluorouracil, 6-azauracil, and 2-thiouracil. Similarly, preincubation of enzyme with 1 mM 5-fluorouracil at 4 °C for 30 min, which has been reported to inhibit uracil-DNA glycosylase from human placenta (Seal et al., 1987), had no effect on either mitochondrial activity.

The other reaction product, the AP site, was also examined as a potential inhibitor. For comparative purposes, we established the kinetic parameters using both mitochondrial species and a nuclear uracil-DNA glycosylase also from rat liver. When [*uracil*-^{3}H]poly(dA)·poly(dT) was used as substrate, both forms of the mitochondrial enzyme exhibited similar apparent K_m values for uracil residues in DNA which equaled 1.1 μM (form I) and 0.9 μM (form II) (Figure 6; Table III). The nuclear enzyme was found to have a slightly lower K_m of approximately 0.4 μM uracil-DNA. The turnover numbers for the nuclear and form I mitochondrial uracil-DNA glycosylases, which are both apparently homogeneous preparations, were determined to be about 8 and 1000 uracil residues released per minute, respectively. Addition of poly(dA)·poly(dT+AP) to these reactions resulted in competitive inhibition of all three uracil-DNA glycosylases (Figure 6). This inhibition was dependent only on AP sites, not on polymer, since all reactions contained an equal amount of poly(dA)·poly(dT) nucleotide and uracil residues. The apparent K_i values for AP sites in DNA were calculated to be 0.3 μM (nuclear), 1.2 μM (mitochondrial form I), and 0.8 μM (mitochondrial form II) AP sites (Table III). A comparison of these K_i values with those for free uracil indicated a 500-fold difference favoring inhibition by AP sites, in the case of fraction VI uracil-DNA glycosylase. A somewhat greater difference (1330-fold) was observed for the nuclear enzyme. On the other hand, AP site analogues such as deoxyribose and deoxyribose 5′-phosphate were not found to inhibit (<10% inhibition) either nuclear or mitochondrial (form I and II) uracil-DNA glycosylase at concentrations up to 1 mM. Thus, inhibition seemed to require that the sugar phosphate be located in DNA.

Substrate Specificity. Since the general catalytic properties

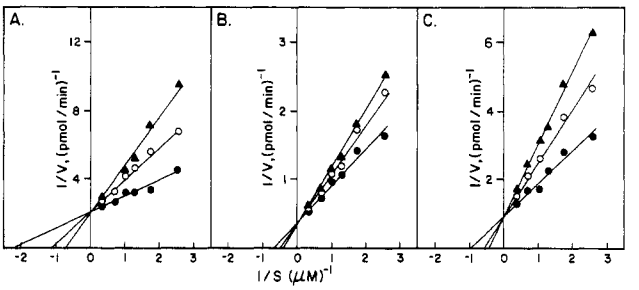
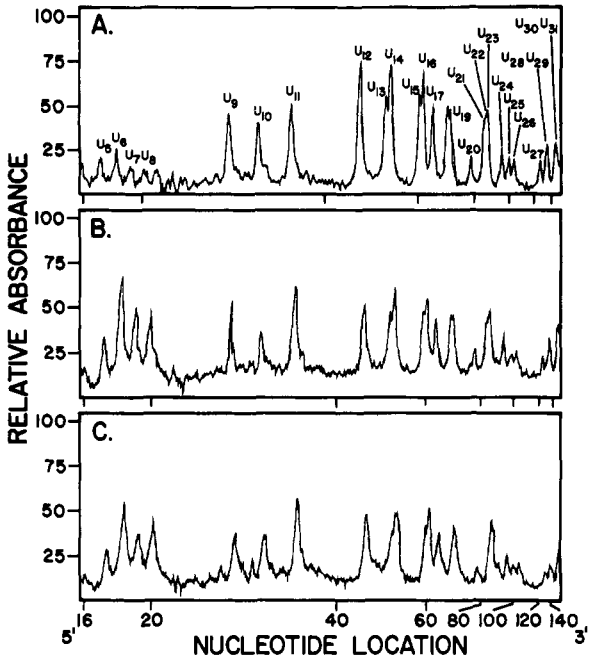


FIGURE 6: Inhibition of rat liver nuclear and mitochondrial forms of uracil-DNA glycosylase by apyrimidinic sites in DNA. Standard uracil-DNA glycosylase assays were performed as described under Experimental Procedures except that [*uracil*-^{3}H]poly(dA)·poly(dT) was used as substrate. The [*uracil*-^{3}H]poly(dA)·poly(dT) concentration (●) was varied as indicated, but the total nucleotide concentration was held constant at 40 μM by addition of poly(dA)·poly(dT). Each reaction also contained either (A) fraction V mitochondrial form I (1.3 units/mL), (B) mitochondrial form II (0.4 units/mL), or (C) fraction VI nuclear (0.2 unit/mL) uracil-DNA glycosylase. In some reactions poly(dA)·poly(dT+AP) containing 0.5 (○) or 1.0 μM (▲) apyrimidinic sites was added as an inhibitor. Incubation was for 15 min at 37 °C, and kinetic parameters were determined by Lineweaver–Burk analysis.

of form I and II uracil-DNA glycosylase appeared very similar, we investigated whether differences existed in substrate specificity. Both mitochondrial forms were found to hydrolyze uracil from single-stranded DNA at approximately twice the rate observed for duplex DNA. Under standard reaction conditions, form I and II released 1.9 and 2.1 uracil residues, respectively, from heat-denatured DNA for every uracil released from the standard (duplex) uracil-DNA substrate. A similar substrate preference was also observed at various NaCl concentrations between 25 and 75 mM. When NaCl was omitted from the reactions, the preference of both enzymes for the single-stranded substrate was somewhat reduced (data not shown).

In order to more specifically assess substrate specificity, an in vitro assay was developed to determine whether uracil residues located within various nucleotide sequences were recognized equally by different uracil-DNA glycosylases. The substrate used was a defined restriction fragment (152 nucleotides) obtained from M13mp2 phage DNA that was produced by infecting an *E. coli* CJ236(*ung,dut*) strain. Therefore, some of the thymine residues in the phage DNA were replaced by uracil. The nucleotide sequence of the natural DNA is shown in Figure 7. This single-stranded DNA fragment was 5′-end labeled with ^{32}P and treated with approximately equal amounts of either nuclear or the two mitochondrial forms of uracil-DNA glycosylase. After a partial digest, the products were treated with piperidine to cause strand scission specifically at apyrimidinic sites generated by uracil-DNA glycosylase. The resulting DNA fragments were resolved by electrophoresis and identified relative to other fragments generated by Maxam and Gilbert sequencing reactions. The results of this experiment are shown in Figure 7, which we refer to as the catalytic fingerprint of the uracil-DNA glycosylase preparation. Treatment of the substrate with piperidine without exposure to uracil-DNA glycosylase did not produce any fragments smaller than 152 nucleotides (data not shown). Hence, the uracil-DNA glycosylases did not contain any contaminating exonuclease activity. Several points can be made from these results: (1) All three uracil-DNA glycosylase preparations cleaved only at sites where uracil could be substituted in the DNA fragment. (2) Each enzyme recognized all 32 potential uracil residues within the DNA fragment. However, only the fifth to the thirty first



5′AATTCACTGGCCGTCGTTTT$_{20}$ ACAACGTCGTGACTGGGAAA$_{40}$ ACCCTGGCGTTACCCAACTT$_{60}$
AATCGCCTTGCAGCACATCC$_{80}$ CCCTTTCGCCAGCTGGCGTA$_{100}$ ATAGCGAAGAGGCCCGCACC$_{120}$
GATCGCCCTTCCCAACAGTT$_{140}$ GCGCAGCCTGAA 3′

FIGURE 7: Substrate specificity of rat liver nuclear and mitochondrial forms of uracil-DNA glycosylase. Reaction mixtures (10 μL) contained 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 mM dithiothreatol, 0.1 μg of bovine serum albumin, [^{32}P]DNA (152-nucleotide single-stranded DNA fragment), and either (A) 0.07 unit of nuclear, (B) 0.06 unit of mitochondrial form I, or (C) 0.04 unit of mitochondrial form II uracil-DNA glycosylase. After incubation for 30 min at 37 °C, the reactions were adjusted to 100 μg/mL calf thymus DNA and precipitated with ethanol. DNA was resuspended in 100 μL of 1 M piperidine and heated at 90 °C for 30 min. After the piperidine was removed by repeated lyophilization, the DNA fragments were prepared for electrophoresis as described under Experimental Procedures. The DNA sequence of the 152-nucleotide fragment was determined with base-specific modification/cleavage reactions (G, G+A, T+C, C) as described by Maxam and Gilbert (1980). Samples (5 μL) were loaded onto a 12% polyacrylamide–7 M urea gel and electrophoresed, and the gel was prepared for autoradiography. The relative absorbence of each band was determined on a Bio-Rad (Model 620) scanning densitometer.

uracil residues of the sequence are shown in Figure 7 due to our inability to reproducibly resolve and accurately quantitate the terminal fragments. (3) Assuming that all potential uracil sites are equally substituted, the results suggest that the different enzyme preparations exhibit a distinct preference for certain sites over others within the oligonucleotide fragment. (4) The overall pattern of the catalytic fingerprint produced by the nuclear uracil-DNA glycosylase preparation differs significantly from either generated by the mitochondrial preparations. (5) In contrast, the catalytic fingerprints obtained for mitochondrial form I and II are very similar. Thus, the two mitochondrial enzymes appear to share very similar substrate specificity. Taken together, these results suggest that mitochondrial form I and II are at least very similar enzymes and could be derived from the same polypeptide.

Discussion

The major mitochondrial uracil-DNA glycosylase from rat liver was purified to apparent homogeneity, characterized, and localized as an inner membrane-associated protein. This represents not only the first report on an extensively purified mitochondrial uracil-DNA glycosylase but the only comparative study to date between these homogeneous nuclear and mitochondrial enzymes. We also described the existence of two submitochondrial forms of this enzyme which exhibit very similar catalytic properties.

Mitochondrial and nuclear activities have been jointly isolated but only partially purified from other sources, including human fibroblasts (Gupta & Sirover, 1981), rat hepatoma H4 cells (Lavel et al., 1981), HeLa S3 cells (Wittwer & Krokan, 1985), and *Zea mays* seedlings (Benson & Warner, 1987). In all cases where the native molecular weight of the mitochondrial enzyme was determined by gel filtration chromatography, a M_r 18 000–20 000 enzyme was observed. The rat liver mitochondrial enzyme (M_r 20 000) was no exception (Domena & Mosbaugh, 1985). The polypeptide molecular weight was determined by SDS–polyacrylamide gel electrophoresis to be 24 000, indicating the enzyme exists as a monomer. In contrast, rat liver nuclear uracil-DNA glycosylase has a native molecular weight of approximately 33 000 (Domena & Mosbaugh, 1985). Several other reports suggest a similar high molecular weight for the nuclear enzyme in other mammalian systems (Caradonna & Cheng, 1980; Leblanc & Laval, 1982; Seal et al., 1987). In vitro translation of human placental polyadenylated RNA yields a M_r 37 000 protein product recognized by anti-uracil-DNA glycosylase monoclonal antibody (Vollberg et al., 1987) which most likely corresponds to the nuclear enzyme. Thus, we conclude that at least two subcellular forms exist which are physically distinct in rat liver and most likely in other tissues.

Differences between the nuclear and mitochondrial uracil-DNA glycosylases were also revealed by their catalytic properties. In general, the kinetic parameters differed only slightly between the subcellular forms; however, striking differences existed in the turnover number and catalytic fingerprint. Whereas, the K_{cat} for the major mitochondrial form was ~1000 uracil residues released per minute, the nuclear uracil-DNA glycosylase catalyzed the reaction almost 100 times slower. However, other factors may influence these values in vivo. The mitochondrial enzyme (form I) turnover number approximately equaled that described for *E. coli* uracil-DNA glycosylase (Lindahl et al., 1977). Whether or not the mitochondrial enzyme might be related to bacterial uracil-DNA glycosylases remains to be determined; however, the molecular weights of these two enzymes are very similar and comparable to several other prokaryotic uracil-DNA glycosylases (Lindahl et al., 1977; Cone et al., 1977; Leblanc et al., 1982). Significant differences also exist in the catalytic fingerprints produced by nuclear and mitochondrial uracil-DNA glycosylase preparations. However, we detected no significant variance between the catalytic specificity of form I and II uracil-DNA glycosylase. Thus, we suggest that the two mitochondrial enzymes are very similar or perhaps even related proteins. We do not wish to necessarily interpret these data to indicate absolute differences between various uracil-containing sites for any given enzyme but only to describe relative changes between various enzymes. The former interpretation would require that we know the degree of uracil substitution introduced at each location.

Although mitochondria are semiautonomous organelles, only a few proteins are coded by mitochondrial DNA. Since several mitochondrial genomes have been completely sequenced and potential open-reading frames assigned to proteins other than uracil-DNA glycosylase (Chomyn et al., 1986), we conclude that this mitochondrial enzyme is probably coded by a nuclear gene. In most cases, proteins destined for mitochondria are made in the cytoplasm as higher molecular weight precursors, containing amino-terminal extensions of 20–60 amino acids

(Hay et al., 1984) which are removed upon transport into the mitochondria. We have observed two distinct molecular weight forms of mitochondrial uracil-DNA glycosylase which are separated by SDS–polyacrylamide gel electrophoresis. When mitochondrial extracts were directly applied to these gels, the activity was recovered as two species corresponding to M_r 29 000 and 24 000, the predominant form being the smaller polypeptide. Futhermore, two chromatographically distinct forms were also resolved during purification by phosphocellulose. The major activity (form I) corresponded to the M_r 24 000 protein, and preliminary experiments suggest that form II might represent that M_r 29 000 protein. A conclusive result for form II was hampered by the low abundance, impure nature, and inability to consistently resolve these molecular weight forms by electrophoresis. Whether these two forms result from mitochondrial import, protein modification, proteolysis, or separate gene products will require additional investigation at the protein or genetic level.

We have systematically examined inhibition of nuclear and mitochondrial uracil-DNA glycosylase by both uracil and apyrimidinic sites. Our finding that AP sites competitively inhibit these enzymes at a concentration about 3 orders of magnitude lower than that observed for free uracil and at a level approximately equivalent to the K_m value suggest that AP site inhibition may have physiological significance. Similar results have also been described for the calf thymus uracil-DNA glycosylase (Talpaert-Borle et al., 1982). Assuming that AP sites inhibit by binding the enzyme, then our results infer that uracil-DNA glycosylase might remain associated at the baseless site for some time after uracil has been removed. This need not impair the function of the enzyme in DNA repair but only transiently modulate its activity. Such an interaction with DNA might serve to couple subsequent steps of base-excision repair or to block RNA or DNA polymerases from traversing the baseless site. The latter process has been shown to be mutagenic, since misincorporation of nucleotides frequently occurs opposite the AP site (Kunkel et al., 1983).

The apparent absence of a mechanism in mammalian mitochondria for repairing ultraviolet light induced DNA damage (Clayton et al., 1974) cannot be extrapolated to mean that mitochondria generally lack DNA repair pathways. Models that suggest that mitochondria process DNA damage by destroying the genome could only suffice for DNA adducts that are infrequently introduced into DNA. Lesions that occur frequently would prompt more significant destruction of mitochondrial DNA and might jeopardize cell survival, if not repaired. On the basis of estimated spontaneous deamination and depurination/depyrimidination rates (Lindahl & Nyberg, 1972; Lindahl, 1979), we calculate that roughly 2 uracils and 100 AP sites would be expected to form in the mitochondrial DNA of each cell per day. [The cellular concentration of mitochondrial DNA is about 100 times less than that of nuclear DNA (Bestwick et al., 1982)]. If the accumulative frequency of these two lesions reached an unacceptable level, then one could argue that DNA repair might be necessary for both, since uracil residues are converted to AP sites via uracil-DNA glycosylase. The presence of a mitochondrial uracil-DNA glycosylase and two AP endonuclease isozymes,[2] which would be required for the first two steps in repair, suggests that base-excision repair functions in these organelles. Alternatively, these two enzymes might be used to initiate degradation of uracil-containing mitochondrial DNA. The physiological role of uracil-DNA glycosylase in mitochondrial DNA metabolism must await further experimentation.

[2] A. E. Tomkinson, R. T. Bonk, and S. Linn, personal communication.

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Monoclonal Antibodies to Human Fibroblast Procollagenase. Inhibition of Enzymatic Activity, Affinity Purification of the Enzyme, and Evidence for Clustering of Epitopes in the NH_2-Terminal End of the Activated Enzyme†

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Received February 22, 1988; Revised Manuscript Received May 2, 1988

ABSTRACT: This study describes 11 monoclonal antibodies (Mabs) against human fibroblast collagenase that (i) inhibit the specific catalytic activity of the enzyme and/or (ii) react with one or more forms of the enzyme on Western blots. Each of the Mabs specifically immunoprecipitated the M_r 57 000/52 000 procollagenase from [^{35}S]methionine-labeled culture medium. Five Mabs, designated VI-3, VI-4, 2C5, 4A2, and 7C2, inhibited the activity of fibroblast-type collagenase against soluble monomeric collagen and against reconstituted collagen fibrils but did not inhibit the genetically distinct human PMN leukocyte collagenase. The interstitial collagenase produced by human mucosal keratinocytes (SCC-25) was also inhibited, whereas the corresponding enzyme from rat was not. Assignment of epitopes to structural domains within the molecule based on immunoperoxidase staining of Western blots of collagenase and its autocatalytic fragments revealed that 9 of 11 epitopes, including those recognized by 4 inhibitory Mabs, were clustered in a 169-residue domain, which constitutes the NH_2-terminal part of the M_r 46 000/42 000 active enzyme. One Mab (X-2a) specifically recognized the M_r 57 000/52 000 zymogen species and failed to react with the active M_r 46 000/42 000 form. The inhibitory Mab VI-3 was used for immunoaffinity purification of procollagenase from culture media with a recovery better than 80% and a yield of ~1.4 mg of enzyme/L of medium.

Fibroblast-type collagenase, a neutral secretory metalloproteinase capable of cleaving interstitial types I–III collagens at a specific site in the helical domain, is expressed either constitutively or after TPA-induction by a number of different cell types including fibroblasts (Birkedal-Hansen et al., 1976, Aggeler et al., 1984), macrophages (Welgus et al., 1985, Wahl et al., 1977), osteoblasts (Otsuka et al., 1984), and keratinocytes (Lin et al., 1987). The enzyme is secreted as a major M_r 52 000 unglycosylated and a minor M_r 52 000 glycosylated form (Wilhelm et al., 1987). Both of these are inactive collagenase precursors that are subsequently converted to catalytic form by a poorly understood mechanism. Activation may be achieved either by preincubation with proteolytic enzymes such as trypsin (Stricklin et al., 1983), plasmin (Eekhout & Vaes, 1977), and kallikrein (Nagase et al., 1982) or by exposure to dissociative agents such as KSCN (Abe & Nagai, 1972), NaI (Abe et al., 1973), and SDS (Birkedal-Hansen & Taylor, 1982). Activation is often, but not necessarily, associated with loss of an 81-residue propeptide (Stricklin et al., 1977, 1983; Grant et al., 1987), which results in formation of a single-chain

† This work was supported by grants from the National Institute of Dental Research (DE 05817, DE 06028, DE 02670, DE 08228, and AR 20614) and by a gift from Procter and Gamble Co.
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0006-2960/88/0427-6751$01.50/0