Purification and Characterization of a Mitochondrial Thymine Glycol Endonuclease from Rat Liver∗

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Rob H. Stierum, Deborah L. Croteau‡, and Vilhelm A. Bohr§

From the Laboratory of Molecular Genetics, NIA, National Institutes of Health, Baltimore, Maryland 21224-6823

Mitochondrial DNA is exposed to oxygen radicals produced during oxidative phosphorylation. Accumulation of several kinds of oxidative lesions in mitochondrial DNA may lead to structural genomic alterations, mitochondrial dysfunction, and associated degenerative diseases. The pyrimidine hydrate thymine glycol, one of many oxidative lesions, can block DNA and RNA polymerases and thereby exert negative biological effects. Mitochondrial DNA repair of this lesion is important to ensure normal mitochondrial DNA metabolism. Here, we report the purification of a novel rat liver mitochondrial thymine glycol endonuclease (mtTGendo). By using a radiolabeled oligonucleotide duplex containing a single thymine glycol lesion, damage-specific incision at the modified thymine was observed upon incubation with mitochondrial protein extracts. After purification using cation exchange, hydrophobic interaction, and size exclusion chromatography, the most pure active fractions contained a single band of approximately 37 kDa on a silver-stained gel. MtTGendo is active within a broad KCl concentration range and is EDTA-resistant. Furthermore, mtTGendo has an associated apurinic/apyrimidinylase activity. MtTGendo does not incise 8-oxodeoxyguanosine or uracil-containing duplexes or thymine glycol in single-stranded DNA. Based upon functional similarity, we conclude that mtTGendo may be a rat mitochondrial homolog of the Escherichia coli endonuclease III protein.

Reactive oxygen species (ROS) are generated as by-products of cellular respiration and exogenous exposure to chemical and physical agents. Depending upon the site of formation, ROS can interact with intracellular components including proteins, lipids, and DNA. There is evidence that interactions of ROS with these biological macromolecules play a role in the development of cancer and aging. Upon interaction of ROS with DNA, various adducts can be formed. One of these adducts, the pyrimidine hydrate thymine glycol (TG, 5,6-dihydroxydihydro-thymine), is only slightly mutagenic but can block DNA (1–4) and RNA polymerases (5, 6), presumably because TG induces a local structural change in DNA (7). A negative correlation has been found between urinary excretion of TG and lifespan of different mammals (8). In addition, an age-related increase in TG levels was observed in DNA obtained from rat liver (9). Also, increased levels of TG have been observed in DNA obtained from various brain regions from Alzheimer’s patients (10). Treatment of cells with the amyloid β-protein, a protein involved in the pathology of Alzheimer disease, was found to increase pyrimidine hydrates in mitochondrial DNA (11). These studies suggest that due to its possible interference with normal DNA metabolism, the presence of TG in DNA could have biological consequences and might contribute to aging and age-related degenerative diseases.

In Escherichia coli, TG is repaired by the base excision repair enzyme endonuclease III (EndoIII). This enzyme is a DNA glycosylase/AP-lyase that first removes the TG and then incises the DNA at the resulting abasic site. Recently, eukaryotic homologs of this base excision repair enzyme have been cloned and characterized (12–17). Additional DNA repair pathways remove TG in mammalians, and these include nucleotide excision repair (18) and transcription-coupled repair (19). Transcription-coupled repair of TG was reported to be defective in Cockayne syndrome, a rare autosomal recessive disease with characteristics of premature aging (19). Repair of TG was found to be inducible by low doses of ionizing irradiation (20). The fact that a variety of repair mechanisms exist for TG suggests that repair of this DNA lesion is of critical biological importance.

Mitochondrial DNA (mtDNA) consists of a 16.5-kilobase pair circular supercoiled genome that encodes components of the electron transport chain. About 85% of the cellular oxygen consumption is consumed by the mitochondrial electron transport chain (reviewed in Ref. 21). Since mtDNA is localized in close proximity to the electron transport chain, it is more vulnerable to attack by ROS than nuclear DNA. It is conceivable that the presence of oxidative mtDNA lesions that interfere with mtDNA metabolism leads to mtDNA loss or mutations.

Several oxidative DNA lesions have been detected in mtDNA including 8-oxodeoxyguanosine (8-oxoG), 5-hydroxyhydantoin, 5-hydroxymethylhydantoin, 5-hydroxymethylurea, and 5-hydroxycytosine (22, 23). Relatively low levels of EndoIII-sensitive sites have been detected in mtDNA. However, if TG accumulates in mtDNA, mtDNA replication and transcription may be compromised. Consequently, efficient DNA repair of TG may be important for normal mitochondrial function. At present, no study has directly demonstrated the existence of a repair mechanism specific for TG in mitochondria.

It has been the notion that mitochondria were devoid of DNA repair since repair of pyrimidine dimers was not observed (24). However, more recent reports have documented the removal of...
other types of mtDNA damage including alkylation lesions (25), cisplatin interstrand cross-links (25), damage induced by 4-nitroquinoline (26), and oxidative base damage (27, 28) including endonuclease III-sensitive sites (29). Although these and other studies suggest the existence of mitochondrial base excision repair, little is known about the mechanism of oxidative DNA damage processing in mitochondria. Purification of mtDNA repair enzymes in sufficient amounts to allow detailed characterization of the repair process is difficult, and eukaryotic oxidative DNA damage processing enzymes are generally expressed at low levels. In addition, the isolation of sufficient amounts of pure mitochondria is a limiting factor in the purification procedure. As a result, only a few mitochondrial DNA repair enzymes involved in base excision repair of oxidative DNA damage have been characterized. Tomkinson et al. (30) described two class II AP endonuclease-activity-like enzymes in mitochondria from mouse placemacytoma cells. In our laboratory, a mitochondrial enzymatic activity specific for 8-oxoG (mtODE) was recently partially purified from rat liver mitochondria (31). Finz and Bogenhagen (32) recently reconstituted base excision repair of an AP site with purified Xenopus laevis mitochondrial AP endonuclease, mtDNA ligase, and mtDNA polymerase γ. Tomkinson et al. (33) identified three mitochondrial endonucleases that recognized DNA lesions induced by high levels of UV light. These enzymes were not well characterized, and no specific substrates were reported.

Here, we describe the purification and characterization of a mitochondrial enzyme from rat liver that processes TG and abasic sites. To our knowledge, this is the first direct evidence for the existence of a mitochondrial base excision repair enzyme for TG. The enzyme shares functional similarities with E. coli EndoIII and the mammalian EndoIII homologs.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were, unless otherwise stated, from Sigma. Livers were obtained from 6-month-old male Wistar rats (Animal Colony of Gerontology Research Center, Baltimore). Percoll and chromatography equipment were from Amersham Pharmacia Biotech. Protease inhibitors were from Boehringer Mannheim. [γ-32P]ATP was from NEN Life Science Products and [α-32P]dATP was from Amersham Pharmacia Biotech. NensorbTM-20 nucleic acid purification cartridges. To generate an AP site containing oligo (AP), the uracil-containing oligo (U) was incubated for 30 min at 37 °C with 1 unit of uracil DNA glycosylase. To demonstrate that AP sites were generated, the AP oligo was incubated for 1 h at 37 °C with 0.02–2 units of EndoIII (~0.8 enzyme-sensitive sites/molecule).

Oligonucleotide Substrates—Table I shows the sequences of the 28-mer oligonucleotide substrates used in this study. Important features of the oligonucleotide are underlined. The thymine-containing oligonucleotide (T) and its complementary strand were obtained from Life Technologies, Inc. The 8-oxoG (OG), AP site control (APC) oligonucleotide, uracil-containing oligonucleotide (U), and the complementary strand, uracil-containing oligonucleotide (C), were purchased from Midland Certified Reagent Co. Oligonucleotides were purified on a 20% polyacrylamide gel prior to use. To generate the thymine glycol (TG)-containing substrate, 1 µg of thymine-containing oligo was incubated for 30 min at room temperature in a 100-µl reaction volume containing 15 mM OsO4 and 2% (v/v) pyridine. 3 Oligonucleotides were separated from unreacted OsO4 and pyridine using NensorbTM-20 nucleic acid purification cartridges. To generate an AP site containing oligo (AP), the uracil-containing oligo (U) was incubated for 30 min at 37 °C with 1 unit of uracil DNA glycosylase. To demonstrate that AP sites were generated, the AP oligo was incubated for 1 h at 37 °C with 1 unit of EndoIV. About 99% of the AP oligo was incised.

Plasmid Incision Assay—PKSγ plasmid was modified with osmium tetroxide in a reaction mixture (volume, 250 µl) containing 1.2 mM OsO4, 0.4 mM NaCl, 50 µg of PKSγ and incubated for 60 min at 70 °C. DNA was precipitated by addition of 0.2 volume of ammonium acetate and 2.5 volumes of 100% ethanol, air-dried, and dissolved in 100 µl of TE, pH 8.0. Supercoiled molecules were isolated from nicked molecules on a sucrose gradient. Plasmid incision assays were performed in the following mixture: 20 mM HEPES, pH 7.6, 75 mM KCl, 5% glycerol, 1 mM DTT, 0.1 mg/ml bovine serum albumin, and 2 mM dithiothreitol, 50 ng of PKSγ, and amounts of protein indicated in the legend of Fig. 1. The reaction mixture was incubated for various times as indicated in Fig. 1. Nicked molecules were separated from supercoiled molecules on a 1% neutral agarose gel and visualized by scanning after ethidium bromide staining using a FluorImagerTM. The intensity of bands representing supercoiled and nicked forms were quantified with ImageQuant software. The number of sites per plasmid was calculated using the formula: -ln(1.4 × intensity of supercoiled form)/(1.4 × intensity of supercoiled form + intensity of nicked form). The presence of OsO4-induced lesions was confirmed by incubating 100 ng of damaged plasmid for 1 h at 37 °C with 0.02–2 units of EndoIII (~0.8 enzyme-sensitive sites/molecule).

Mitochondrial Thymine Glycol Endonuclease

| Name              | Abbreviation | Sequence                             |
|-------------------|--------------|--------------------------------------|
| Thymine           | T            | 5’ GAA CGA CAG A 5’                  |
| Thymine glycol    | TG           | 5’ GAA CGA CAG ATGG ACA CAG ACA AGC A 3’ |
| Uracil            | U            | 5’ GAA CGA CTG T 3’                 |
| AP-site control   | APC          | 5’ GAA CGA CTG T 3’                 |
| AP-site           | AP           | 5’ GAA CGA CTG T 3’                 |
| Guanine           | G            | 5’ GAA CGA CTG A 5’                 |
| 8-Oxo-G           | OG           | 5’ GAA CGA CTG A 5’                 |

**TABLE I**

| Name          | Abbreviation | Sequence                                    |
|---------------|--------------|---------------------------------------------|
| Thymine       | T            | 5’ GAA CGA CAG A 5’                         |
| Thymine glycol| TG           | 5’ GAA CGA CAG ATGG ACA CAG ACA AGC A 3’    |
| Uracil        | U            | 5’ GAA CGA CTG T 3’                         |
| AP-site control| APC         | 5’ GAA CGA CTG T 3’                         |
| AP-site       | AP           | 5’ GAA CGA CTG T 3’                         |
| Guanine       | G            | 5’ GAA CGA CTG A 5’                         |
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Mitochondrial Thymine Glycol Endonuclease

and 62 µl of 100% ethanol and pelleted by centrifugation. Pellets were washed with 70% ethanol and dissolved in formamide loading dye consisting of 90% formamide, 0.002% bromphenol blue, and 0.002% xylene cyanol. After heating for 2 min at 80 °C, samples were electrophoresed on a denaturing 20% polyacrylamide, 7 M urea, TBE gel. Because of the heat lability of the AP site, samples containing the AP site oligo were heated to 55 °C prior to loading, instead of 80 °C. Gels were first subjected to autoradiography at −80 °C after electrophoresis.

PhosphorImages were exposed to the frozen gel and quantified using a Molecular Dynamics PhosphorImager combined with ImageQuant software. 1 unit of mtTendo activity is defined as 1 fmol of TG-containing oligonucleotide incised during a 4-h incubation at 37 °C.

Isolation and Lysis of Rat Liver Mitochondria—Mitochondria were purified from rat liver as described (31). All procedures were carried out at 4 °C, unless otherwise indicated. Mitochondria obtained from 4 rat livers (∼8 g) were pooled and resuspended in 15 ml of buffer A with 300 mM KCl. Buffer A consisted of 20 mM HEPES, pH 7.6, 1 mM EDTA, 5% glycerol, 0.015% Triton X-100, 5 mM diithiothreitol. The following protease inhibitors were added prior to use: 1 µg/ml aprotinin, 1 µg/ml pepstatin A, 1 µg/ml chymostatin A, 2 µg/ml leupeptin, 2 µM benzamide hydrochloride, 1 µM phenylmethylsulfonyl fluoride, and 1 µM E-64.

Mitochondria were lysed by slowly adding 10% Triton X-100 to a final concentration of 0.5%. The mitochondrial lysate was subsequently clarified by centrifugation for 1 h at 150,000 × g in a SW-50.1 rotor (Fraction I).

Purification of Mitochondrial Thymine Glycol Recognizing Enzymes—The supernatant obtained after ultracentrifugation was applied to a 25-ml DEAE-Sepharose Fast Flow column, equilibrated with buffer A containing 300 mM KCl. After application of Fraction I to the matrix, the column was washed with 125 ml of the same buffer. 5-ml fractions were collected, and fractions with an absorbance at 280 nm higher than 0.2 absorption units were pooled, and the salt concentration was adjusted to 100 mM KCl (Fraction II). Fraction II was loaded onto a fast protein liquid chromatography HR 10/10 Mono S column (equilibrated with buffer A containing 100 mM KCl). The column was washed with 25 ml of the same buffer and then eluted with a 40-ml linear gradient from 100 mM to 1 M KCl. 1-ml fractions were collected and dialyzed overnight against buffer A containing 100 mM KCl and dialyzed against buffer A containing 300 mM KCl without 0.015% Triton X-100. 4 M ammonium sulfate (pH 7.6 adjusted with KOH) was slowly added to a final concentration of 0.15% Triton X-100. 4 M ammonium sulfate was dialyzed against 10% Triton X-100 (to adjust each fraction to a final concentration of 0.015%). Fractions were dialyzed for 4 h against buffer A containing 100 mM KCl and then dialyzed against fresh buffer overnight. mtTendo activity was assayed, and the peak activity was found to elute at ~230 mM ammonium sulfate. Active fractions were pooled and the buffer was exchanged in a Centricon-10 concentrator for buffer A containing 100 mM KCl without 0.015% Triton X-100. 4 M ammonium sulfate was adjusted with KOH to 0.6 M. Active fractions were pooled and the buffer was exchanged in a Centricon-10 concentrator for buffer A containing 100 mM KCl. The column was calibrated with blue dextran 2000, albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A (low molecular weight standards; Amersham Pharmacia Biotech). The column was eluted with 30 ml of the same buffer, and 400-µl fractions were collected. mtTendo activity was directly assayed, and active fractions 17 and 18 were pooled and dialyzed against 40 mM HEPES, pH 7.6, 100 mM KCl, 1 mM EDTA, 50% glycerol, 0.015% Triton X-100, 5 mM diithiothreitol, and stored at −20 °C (Fraction III).

RESULTS

Rat Liver Mitochondria Contain Enzymatic Activities That Recognize Osmium Tetroxide-induced DNA Damage—By using differential centrifugation and Percoll gradient centrifugation purified mitochondria, we identified mitochondrial enzymatic activities that recognize OsO4-induced DNA damage. Initially, we observed nicking of OsO4-modified plasmid upon incubation with a DEAE-fractionated mitochondrial extract. Fig. 1A shows increased conversion of OsO4-modified supercoiled plasmid molecules toward nicked molecules with increasing time of incubation with the DEAE-fractionated extract. In Fig. 1B quantification of the number of sites/plasmids recognized by the DEAE-fractionated mitochondrial extract is shown. A small amount of the observed nicking (∼0.2 sites/plasmid) was non-specific as observed in the lanes in which unmodified plasmid was incubated with extract. After 24 h of incubation, damage-specific incision was ∼0.4 sites/plasmid (∼50% of the EndoIII-sensitive sites). Incubation of unfraccionated extract with plasmid DNA resulted in complete degradation of the substrate, presumably because of mitochondrial endonucleases (34, 35). Since OsO4 mainly introduces TG (36), the enzymatic activity was followed using a radiolabeled TG-containing oligonucleotide.

Osmium Tetroxide Modification of a Single Thymine-containing Oligonucleotide Generates One E. coli Endonuclease III-sensitive Site per Oligonucleotide—Upon 32P-labeling and digestion of the OsO4-modified thymine-containing oligonucleotide with EndoIII, >80% of the modified oligonucleotide was found to be incised (Fig 2, lane 6). Migration of the incision product of EndoIII-digested OsO4-modified oligonucleotide was similar to the incision product produced by EndoIII digestion of

![Fig. 1. OsO4-treated PKS+ is nicked upon incubation with fractionated mitochondrial extract. A, specific incision of supercoiled (SC) to nicked (N) form of plasmid damaged with osmium tetroxide. 16.5 µg of DEAE-fractionated mitochondrial extract or dialysis buffer was added to 50 ng of untreated or OsO4-treated plasmid and incubated as described under “Experimental Procedures” for different times at 37 °C. B, sites per plasmid sensitive to digestion by fractionated extract in untreated and OsO4-treated plasmid. Open squares, undamaged plasmid; closed circles, OsO4-treated plasmid. Results represent average sites/plasmid ± S.D. of duplicate experiments corrected for background sites/plasmid, with fractionated extract from pooled mitochondria obtained from two rat livers.](image)
an oligonucleotide containing a single AP site at the identical position (11th nucleotide relative to the 5'-end of the oligonucleotide, see Fig. 8, A and B, lanes 3 and 5). No other incision products indicative of cytosine modification were observed when the OsO4-modified thymine-containing oligonucleotide was extensively incubated with *E. coli* EndoIII. This strongly suggests that, in our hands, only the single thymine was converted to TG. No incision was observed upon digestion of the TG oligo with either EndoIV (Fig. 2, lane 7) or Fpg protein (Fig. 2, lane 8) demonstrating that no AP sites or Fpg-sensitive sites were generated during the preparation of the oligonucleotides.

**Purification of a Mitochondrial Endonuclease Recognizing Thymine Glycol**—The purification scheme for the mitochondrial TG endonuclease is summarized in Table II. The mitochondrial lysate (Fraction I) was fractionated on a DEAE-Sepharose Fast Flow column (Fraction II). Fraction II was subjected to fractionation on a Mono S column with a 100–1000 mM KCl linear gradient, and 1-ml fractions were collected. All Mono-S fractions were assayed for incision activity on a TG-containing oligonucleotide. Incision was only observed within Mono-S fractions 18–33. The extent of incision observed in these fractions was quantified and plotted along with the KCl concentration against the fraction number (Fig. 3B). Three separable incision activities that recognize TG were found and the peaks from these activities eluted at ~410, ~550, and ~650 mM KCl. The incision observed was specific for TG, because the oligo does not contain EndoIV or Fpg-sensitive sites (Fig. 2). The most abundant TG-incising fraction eluted at ~410 mM KCl. The active fractions were pooled, and the KCl concentration was adjusted to 100 mM KCl (Fraction III) and further purified on a phenyl-Superose column (Fraction IV) and a Superdex 75 column (Fraction V). Fraction V was entitled mitochondrial TG endonuclease (mtTGendo). Total and specific activities for each fraction are shown in Table II. We achieved roughly a 450-fold purification.

To exclude that prolonged incubation might induce any additional oxidative DNA damage in the substrate, we incubated a control oligonucleotide containing a single thymine for up to 18 h with protein extracts obtained at different stages of the purification. No incision was observed in the thymine-containing control substrate but only in the TG-containing oligonucleotide (data not shown). Thus, no potential additional oxidative lesions are formed during the incubation period that could be substrates for mtTGendo and protein fractions obtained earlier during the purification.

**SDS-polyacrylamide gel electrophoresis**: The purified mtTGendo from rat liver mitochondria was separated by SDS-PAGE analysis with a silver-stained gel (Fig. 4A). Therefore, utilizing this purification scheme it seems that we have highly enriched mtTGendo.

**mtTGendo Is Associated with the Mitochondrial Inner Membrane or Matrix**—Fractionation of the purified mitochondria into submitochondrial fractions was used to demonstrate that mtTGendo was localized within the mitochondria. Fractionation by digitonin (according to Ref. 37) produces two submitochondrial fractions, an outer membrane and a mitoplast fraction. The mitoplast fraction contains both the inner membrane and matrix components. As shown in Table III, the outer membrane fraction was associated with low cytochrome *c* oxidase activity (a marker enzyme for the inner membrane fraction) and high monoamine oxidase activity (a marker enzyme for the outer membrane fraction). Conversely, the mitoplast fraction was associated with high cytochrome *c* oxidase activity and low monoamine oxidase activity. The majority of the TG incising activity was co-localized with the mitoplast fraction (Fig. 5 and Table III). This indicates that mtTGendo is associated with the mitochondrial inner membrane or matrix space. In addition, after incubating the TG-containing oligonucleotide with Pronase-digested mitochondria, incision activity was retained (data not shown), again demonstrating that the observed activity is not of extramitochondrial origin. Thus, we conclude that mtTGendo is localized within the mitochondria.

**Molecular Weight and Catalytic Properties of mtTGendo**—Based on the calibration of the gel filtration column, mtTGendo eluted at a position corresponding to a molecular mass range of

| Fraction | Volume | Protein | total activity (10^6 units/mg) | specific activity (10^6 units/mg) | Purification |
|----------|--------|---------|--------------------------------|----------------------------------|--------------|
| I. Lysate | 29.5   | 490     | 137                            | 0.28                             | ~147         |
| II. DEAE | 185    | 408     | ~147                           | ~0.36                            | ~1.3         |
| III. Mono S | 0.78 | 3.3     | 39.6                           | 12                               | ~43          |
| IV. Phenyl-Superose | 0.23 | 0.073  | 5.5                            | 75                               | 270          |
| V. Superdex 75 | 0.3  | 0.026  | 3.2                            | 123                              | 440          |

* One unit of activity is defined as 1 fmol of 28-mer TG-containing oligonucleotide incised per 4-h incubation.
25,000–30,000 Da (Fig. 6). Further fractionation on Superdex 75 of the two other activities detected after Mono-S fractionation, from which the peaks eluted at 550 and 650 mM KCl, showed predicted mass of 36 and 42 kDa, respectively. As shown in Fig. 4, the most pure fractions contained a single band corresponding to a mass of 37 kDa, which may represent mtTGendo. In preliminary experiments, an antibody raised against native E. coli endonuclease III cross-reacted with the Superdex 75 fractions that contained mtTGendo activity. The antibody cross-reacted with our putative mtTGendo band.

Further characterization of mtTGendo showed that it is active within a broad KCl concentration range from 50 to 100 mM. The enzyme does not require Mg\(^{2+}\) and is resistant to 10 mM EDTA. Incubation with increasing protein concentrations (0–400 ng of Fraction V) resulted in increased incision of the TG-containing oligonucleotide. With 400 ng of protein (Fraction V), maximum incision (∼70% of the oligonucleotide incised) was achieved during 4 h incubation (data not shown).

Substrate Specificity of mtTGendo—To address the question of whether incision by mtTGendo was specific for TG, a set of other substrates (see Table I) was tested (Fig. 7). Reactions were carried out as described under “Experimental Procedures.” A single-stranded TG-containing oligo incubated with mtTGendo was not incised (lane 3). Thus, mtTGendo requires double-stranded DNA for catalytic activity. To generate an AP site (dsAP), a double-stranded uracil-containing oligonucleotide (dsU) was first digested with uracil DNA glycosylase. The double-stranded oligonucleotide containing a single AP site (dsAP) was a substrate for mtTGendo (lane 7). Partial hydrolysis of this oligonucleotide to smaller products was observed during the incubation period (lane 10). However, these products were present in small amounts, and their migration was different than the main incision product generated by mtTGendo digestion of the AP-containing oligonucleotide. Therefore, mtTGendo has an associated AP endonuclease activity. A very small amount of incision was observed when the uracil-containing oligo (dsU) was incubated with mtTGendo (lane 6). This could be due to incision by the associated AP endonuclease activity on AP sites generated by contaminating trace amounts of mitochondrial uracil DNA glycosylase.

Reaction Products Generated by mtTGendo—To determine the type of incision product generated by mtTGendo, the migration of the incision product was compared with the migration of incision products generated by some bacterial repair enzymes on a TG or AP site containing oligo (Fig. 8). EndoIII

![Fig. 3. Rat liver mitochondria contain three enzymatic activities that recognize thymine glycol. A, 4 µl of each Mono S fraction was assayed for incision activity on a TG-containing oligonucleotide, and the reaction products were separated on a 20% polyacrylamide, 7 M urea gel. No incision was observed upon incubation of the Mono S fractions with the thymine-containing oligonucleotide. B, percent incision of TG-containing oligonucleotide and KCl concentration of the corresponding fraction plotted against fraction number.](image)

![Fig. 4. The Superdex 75 column fractions that contain mtTGendo activity are highly enriched for a protein with molecular mass of ∼37 kDa. 10 µl of Superdex 75 column fractions were electrophoresed on a 12% polyacrylamide Tris glycine gel and silver-stained (A) and 4 µl of each fraction were assayed for mtTGendo activity (B).](image)

**Table III**

| Enzyme activities in fractionated mitochondria |
|-----------------------------------------------|
| Fraction | Monoamine oxidase\(^a\) | Cytochrome c oxidase\(^a\) | mtTGendo\(^b\) |
|----------|--------------------------|--------------------------|-------------------|
|          | nmol/mg/min              | µmol/mg/min              | fmol/100 µg protein |
| Mitochondria | 8.6 (2.8)               | 16.3 (2.4)              | 58.7 (6.4)        |
| Mitoplasts   | 8.3 (1.0)               | 12.1 (0.7)              | 59.4 (13.1)       |
| Outer membrane | 61.7 (7.6)             | 5.2 (2.0)               | 14.6 (7.8)        |

\(^a\) Monoamine oxidase and cytochrome c oxidase data from Croteau et al. (31). Identical submitochondrial fractions were used for assaying mtTGendo activity.

\(^b\) mtTGendo activity was measured during an 18-h incubation period as described under “Experimental Procedures.” Data are presented as the mean ± S.E.
incises 3′ to the lesion through β-elimination and generates a 3′-unsaturated aldehyde and a 5′-phosphate group. EndoIV incises 5′ to the lesion and generates a 3′-hydroxyl group and a 5′-phosphate deoxyribose residue. Fpg protein incises both 3′ (through β-elimination) and 5′ (through δ-elimination) to the lesion, releasing the sugar residue and generating 5′- and 3′-phosphate groups. The chemical structures of the 3′ termini created by these different types of incisions are displayed in Fig. 8.

MtTGendo produces an incision product on TG or AP-containing oligonucleotides that migrates like that produced by EndoIII (compare lanes 2 and 3, and lanes 4 and 5). (The additional smaller band seen in Fig. 8A and B when both TG and AP oligo were digested with EndoIII migrates similarly to the incision product generated by Fpg protein (lane 7), suggesting that part of the incision product underwent additional δ-elimination.)

We then characterized the 5′-end generated by mtTGendo. The thymine glycol-containing oligo was 3′-labeled with terminal nucleotidyltransferase and [α-32P]ddATP, annealed to its complementary oligo, and incubated with mtTGendo or EndoIII. Both proteins showed a band consistent with a 17-mer oligonucleotide containing a 5′-phosphate residue (Fig. 8C, lanes 2 and 6). Together with the incision data obtained from the 5′-radiolabeled oligo, this also supports that mtTGendo is an incision activity and not a 3′-5′-exonuclease stalling at the TG. Thus, like E. coli EndoIII mtTGendo incises 3′ to the lesion and generates a 3′-unsaturated aldehyde and a 5′-phosphate group, suggesting a β-elimination reaction mechanism.

**DISCUSSION**

Here, we describe the purification and characterization of a mitochondrial enzyme that specifically excises TG. We name this activity mitochondrial TG endonuclease (mtTGendo) because it recognizes TG and incises the DNA. TG lesions can block mitochondrial DNA and RNA polymerases, and if not repaired, this can lead to permanent alterations in the mitochondrial genomes. Consequently, mitochondrial function and thereby cellular energy metabolism may be hampered, leading to biological changes associated with aging. Therefore, efficient removal of TG from the mitochondrial genomes is very important.

The activity described here is purely of mitochondrial origin and is not due to extramitochondrial contamination. We demonstrate that the majority of the activity was co-localized to mitochondria (Fig. 5 and Table III), and after Pronase digestion of the mitochondria, incision activity specific for the TG was retained.

Tomkinson et al. (33) characterized three mitochondrial enzymatic activities from mouse plasmacytoma cells, which recognized UV-C-irradiated plasmid DNA. Since mitochondria lack pyrimidine dimer repair (24), they suggested that the

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**Fig. 5.** Mitochondrial localization of thymine glycol recognizing enzymatic activities. Lanes 1 and 2 represent control incubations of reaction buffer with thymine or thymine glycol oligonucleotide duplexes. Each lane contains 100 μg of protein with T duplex (lanes 3, 5, and 7) or TG duplex (lanes 4, 6, and 8) as indicated under “Experimental Procedures.” Lanes 3 and 4 contain protein from Percoll gradient purified mitochondria (PG). Lanes 5 and 6 represent the mitoplast protein fraction (MP). Lanes 7 and 8 were incubated with the outer membrane fraction (OM). The (small amount of) incision product produced by the outer membrane fraction has a slightly slower mobility than the incision products in other lanes due to loading of the sample in the most outer lane of the gel.

**Fig. 6.** Determination of the molecular weight of mtTGendo using Superdex 75 column gel filtration. A, incision activity of each Superdex 75 column fraction was determined as described under “Experimental Procedures” and plotted against the elution volumes of proteins used to calibrate the column. B, extent of incision in each fraction (10-mer incision product is shown).
activities recognized TG and other pyrimidine hydrates. However, no specific substrates were described for these enzymes. At the UV-C dose employed by these authors (525 J/m²), several other lesions in addition to TG may be introduced into the DNA, including 8-oxodeoxyguanosine (38–40), formamidopyrimidine-guanine (41) and other lesions in addition to TG may be introduced into the DNA, including 8-oxodeoxyguanosine (38–40), formamidopyrimidine-guanine (41) and heat-stable dipyrimidine adduct (42). The enzyme activities described by these authors may be specific for any of these substrates. Thus, it is unclear whether these activities are similar to the three mitochondrial TG endonuclease activities that we detect (Fig. 3).

Recently, human (12, 13), Saccharomyces cerevisiae (14, 15), and Schizosaccharomyces pombe (16) homologs of EndoIII have been cloned. An amino acid sequence was obtained from a bovine pyrimidine hydrate/thymine glycol DNA glycosylase/thymine glycol AP-lyase (17). In addition, a predicted amino acid sequence with homology to EndoIII was obtained from Rattus sp. (17). MtTGendo shares substrate specificity with these eukaryotic enzymes (TG and other pyrimidine hydrates), and all enzymes appear to have an associated AP-lyase function. The molecular mass of mtTGendo (~37 kDa) is comparable to the reported molecular masses for the EndoIII homologs from higher eukaryotes (human, 33.6–36 kDa and bovine, 29–31 kDa). Like mtTGendo, all of the of the eukaryotic homologs are resistant to EDTA. In addition, all of these enzymes are active within a broad salt concentration range. The observed mtTGendo activity is not due to bacterial contamination during the course of isolating the mitochondria, since the E. coli enzyme is much smaller (23.5 kDa) than mtTGendo (~37 kDa).

Most of the amino acid sequences obtained from eukaryotic homologs of EndoIII share a conserved iron-sulfur cluster characteristic of the amino acid sequence Cys-X₇-Cys-X₂-Cys-X₅-Cys and a helix-hairpin-helix domain, which is thought to be involved in catalytic activity. Interestingly, S. cerevisiae has two EndoIII homologs, and one of these, Scr1, encoded by the FUN33 gene (also called Scr1 (15); NTTG1 (14)) lacks the highly conserved iron-sulfur cluster. Instead, Scr1 was proposed to have a putative mitochondrial localization signal (15). Scr2 lacks this sequence but has the iron-sulfur cluster and is thought to be the nuclear counterpart of Scr1 (15, 43). Although the mass of Scr1 (~45 kDa) is larger than mtTGendo (~37 kDa), mtTGendo may be a mammalian homolog of Scr1.

Recently, hNTH1 protein, the human homolog of EndoIII (12, 13), was shown to be localized to both the nucleus and mitochondria (44). This suggests that the hNTH1 protein may facilitate TG repair in nuclear and mitochondrial DNA. MtTGendo may be a rat homolog of the mitochondrial form of hNTH1. Protein microsequencing will be employed to determine whether mtTGendo shares sequence homology with EndoIII and its eukaryotic homologs.

Previously, an enzymatic activity from rat liver mitochondria recognizing 8-oxodG was partially purified and characterized in this laboratory (31). In contrast to the ability of that enzyme, mtTGendo does not recognize a double-stranded oligonucleotide containing one 8-oxodeoxyguanosine (dsOG) (Fig. 7, lane 9). MtODE does not recognize TG-containing oligonucleotide. Also, mtODE is different from mtTGendo since these enzymes elute at a different KCl concentration on Mono S column chromatography. Neither of these mitochondrial base excision repair enzymes require co-factors. In addition, mtODE has and mtTGendo also seems to have (Fig. 8) an associated AP-lyase function.

Recently, Pinz and Bogenhagen (32) reconstituted mitochondrial base excision repair in vitro using an AP site-containing substrate and purified X. laevis mitochondrial proteins. The AP endonuclease they described is a class II enzyme, cutting 5' to the abasic site and generating 3'-hydroxyl and 5'-deoxyribose-phosphate termini. Both the mtDNA ligase and mtDNA polymerase γ were found to be candidates for the β-elimination reaction required to remove the remaining 5'-deoxyribose phosphate residue. The resulting one-nucleotide gap was filled in by mtDNA polymerase γ and sealed by mtDNA ligase. If a similar mechanism exists in mammalian mitochondria, the associated AP-lyase function of mtTGendo and mtODE would not be required in vitro. Mitochondrial class II AP endonucleases have been isolated from mouse cells (30), suggesting the possibility for the concerted action of mitochondrial DNA glycosylases and AP endonucleases in mammalian mitochondria. Alternatively, the AP-lyase function of mtTGendo and mtODE may be required, after which a class II mitochondrial AP endonuclease, mitochondrial ligase, or polymerase γ could process the 3'-unsaturated aldehyde residue further to create a DNA end suitable for elongation by polymerase γ.

Mitochondrial DNA exists in a covalently closed circular supercoiled form. We find that extracts from mitochondria in

Fig. 7. MtTGendo recognizes thymine glycol and has an associated AP-lyase activity. Substrates containing either a single thymine glycol,apurinic site, uracil, or 8-oxoG were incubated with 650 ng of mtTGendo for 4 h at 37 °C as described under "Experimental Procedures." ss T, single-stranded oligonucleotide containing one single thymine; ss TG, single-stranded oligo containing thymine glycol; ds T, double-stranded oligo containing thymine; ds TG, double-stranded oligo containing thymine glycol; ds APC, double-stranded AP control oligo; ds AP, double-stranded AP-containing oligo; ds G, double-stranded G-containing oligo; ds OG, double-stranded 8-oxoG-containing oligo; ds U, double-stranded uracil-containing oligo.
cise an OsO₄-modified supercoiled DNA substrate (Fig. 1), suggesting that mtTGendo (as well as the two other TG activities) exists in vivo and acts on TG present in mitochondrial DNA. As discussed before, processing of AP sites originating from oxidative damage in mtDNA leads to single-strand breaks. The resulting relaxation of the supercoiled mitochondrial genome may represent a signal for the recruitment of other DNA repair enzymes or for the initiation of complete degradation of the oxidized mitochondrial genomes.

We find three separable mitochondrial enzymatic activities for TG (Fig. 3). Although we cannot exclude that these activities may be closely related forms of the same protein, this finding could implicate that several mitochondrial mechanisms exist for repair of TG. Therefore, repair of this lesion from mtDNA could be of critical biological importance.

In conclusion, we have purified a rat liver mitochondrial enzyme (mitochondrial TG endonuclease) to apparent homogeneity. The enzyme recognizes TG and shares significant functional homology with other pyrimidine hydrate-processing base excision repair enzymes. MtTGendo may help to prevent the accumulation of persistent alterations in mtDNA. In turn, this may prevent biological alterations associated with aging and degenerative diseases. Future research will involve the determination of other substrates for mtTGendo and whether alterations in the activity of mtTGendo are associated with aging and age-related diseases.

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