The mitochondrial DNA polymerase as a target of oxidative damage

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ABSTRACT

The mitochondrial respiratory chain is a source of reactive oxygen species (ROS) that are responsible for oxidative modification of biomolecules, including proteins. Due to its association with mitochondrial DNA, DNA polymerase γ (pol γ) is in an environment to be oxidized by hydrogen peroxide and hydroxyl radicals that may be generated in the presence of iron ions associated with DNA. We tested whether human pol γ was a possible target of ROS with \( \text{H}_2\text{O}_2 \) and investigated the effect on the polymerase activities and DNA binding efficiency. A 1 h treatment with 250 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) significantly inhibited DNA polymerase activity of the p140 subunit and lowered its DNA binding efficiency. Addition of p55 to the p140 catalytic subunit prior to \( \text{H}_2\text{O}_2 \) treatment offered protection from oxidative inactivation. Oxidatively modified amino acid residues in pol γ resulting from \( \text{H}_2\text{O}_2 \) treatment were observed in vitro as well as in vivo, in SV40-transfected human fibroblasts. Pol γ was detected as one of the major oxidized mitochondrial matrix proteins, with a detectable decline in polymerase activity. These results suggest pol γ as a target of oxidative damage, which may result in a reduction in mitochondrial DNA replication and repair capacities.

INTRODUCTION

Oxidative damage to mitochondrial DNA (mtDNA) has been correlated with the aging process and can produce mutations that lead to degenerative diseases (1,2). Reactive oxygen species (ROS) and other free radicals are generated in the mitochondrial membranes by electron transport during oxidative phosphorylation. As much as 1–5% of the oxygen consumed in the mitochondria is released as superoxide or hydrogen peroxide (3). Hydrogen peroxide is the most stable oxygen species but the toxic effects are partially a result of its ability to form damaging radicals. Hydrogen peroxide can react with transition state metals to form hydroxide ion and hydroxyl radicals via the Fenton reaction. mtDNA is located near the site of free radical generation in the mitochondrial inner membrane and suffers more oxidative damage than nuclear DNA (4–7). Early reports comparing nucleotide substitutions in mtDNA from somatic tissues of different primates revealed a 10-fold higher rate of evolution for mtDNA relative to the nuclear genome (8,9), implying a relatively high mutation rate for mtDNA. Oxidative damage of mtDNA is more extensive and persists when cells are treated with prolonged exposure to hydrogen peroxide as compared to nuclear DNA damage (10).

Besides DNA, hydroxyl radicals also attack lipids and proteins. Protein oxidation results mainly in introduction of carbonyl groups, which can be easily detected (11). Proteins bearing carbonyl groups are generally dysfunctional since these moieties may alter both structure and function of the protein. Hydrogen peroxide, apart from indirectly causing oxidation of proteins by being a source of highly reactive free radicals, can also directly modify several amino acids, such as cysteine, methionine, tryptophan and tyrosine (12–15), leading to significant loss of protein function (16–20).

The DNA backbone is associated with iron ions which are thought to account for oxidation of the DNA via Fenton chemistry which generates hydroxyl radicals (21). Additionally, DNA-binding proteins, such as replication proteins, are also in this environment for oxidation via DNA-associated iron molecules. DNA polymerase γ (pol γ) is the sole DNA polymerase in the mitochondria and is responsible for replication and repair of the mtDNA. Due to its association with mtDNA and the location within the mitochondria, pol γ is exposed to various ROS, including hydrogen peroxide. Oxidation of pol γ may be in part responsible for slower DNA replication or repair. Additionally, oxidative inactivation of pol γ may, at least in part, set forth a cascade of further oxidative stress due to the loss of mtDNA replication and subsequent energy decline.

We and others have cloned and overexpressed the human gene encoding the catalytic subunit of pol γ (22–24). The full-length human cDNA for the 55 kDa accessory subunit has been isolated and overexpressed (25–27) and the three-dimensional crystal structure of this protein has been determined (28). When associated with the catalytic subunit, the accessory subunit confers high processivity to pol γ through enhanced DNA binding (25,26). In the course of purification we have noted that pol γ is sensitive to inactivation in the absence of reducing agents. In this paper we examined the effect of

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hydrogen peroxide on pol γ: its activity and DNA binding efficiency as well as the possible impact on the enzyme activity in living cells.

MATERIALS AND METHODS

Reagents

Poly(rA)-oligo(dT)12–18, poly(dA)-oligo(dT)12–18, and unlabelled dTTP (10 mM stock) were purchased from Pharmacia. The radioisotopes [α-32P]dATP and [γ-32P]dCTP were from Amersham. AG 501-X8(D) resin, 20–50 mesh, was from Bio-Rad. Oligonucleotides were purchased from Oligo Etc. Hydrogen peroxide (30%) and the protease inhibitor cocktail were from Sigma. The OxyBlot protein oxidation detection kit was purchased from Intergen. SuperSignal West Pico chemiluminescent substrate, an enhanced chemiluminescent substrate for the detection of horseradish peroxidase, was from Pierce. Autoradiogram films were from Kodak. Minimum essential medium (MEM) with and without L-glutamine, MEM non-essential and essential amino acids solutions, MEM vitamin solution, L-glutamine and trypsin–EDTA were from Life Technologies. Fetal bovine serum was from Gemini Bio-Products.

Cell culture

The simian virus 40 (SV40)-transformed human fibroblast cell line GM00637E (NIGMS Human Genetic Mutant Cell repository, Coriell Institute, Camden, NJ) was cultured in Earle’s modified Eagle’s medium supplemented with 15% fetal bovine serum, MEM essential and non-essential amino acids, 2 mM L-glutamine at 37°C, 5% CO2 in a humidified atmosphere.

Enzymes

The recombinant wild-type histidine-tagged human pol γ and the exonuclease-deficient form (Exo–p140) were purified to homogeneity from baculovirus-infected insect cells as described (23). The accessory subunit (p55) was purified to homogeneity from Escherichia coli and the heterodimeric forms of the polymerase were reconstituted as described (25). Human DNA polymerase α (pol α) was purified from baculovirus-infected insect cells as described (29,30). Human DNA polymerase β (pol β) was purchased from Chimerx. Catalase was obtained from Pharmacia. The DPG polyclonal antibodies against the catalytic subunit of human pol γ has been described (23).

In vitro protein oxidation

The pol γ subunits p140 and p55, as well as pol α and β, were treated with various concentrations of hydrogen peroxide (0.05, 0.1, 0.25, 0.5, 1, 1.5, 2 and 2.5 mM) for 60 min at 37°C, in buffer containing 50 mM Tris–HCl, pH 7.5, 10% glycerol, 1 mM EDTA and 0.01% NP-40. After the treatment the excess H2O2 was removed by 5 min incubation at room temperature with 500 U catalase (1 U catalase decomposes 1 μM H2O2/min at 25°C, pH 7). Oxidized enzymes were then used for activity determination.

Polymerase assays

Polymerase activity on poly(dA)-oligo(dT) was measured in 50 μl reaction mixtures containing 25 mM HEPES–KOH, pH 8.0, 5 μg acetylated BSA, 2.5 mM 2-mercaptoethanol, 2 mM MgCl2, 10 μM [α-32P]dTTP (1000–3000 c.p.m./pmol), 2.5 μg poly(dA)-oligo(dT)12–18 and 75 mM NaCl. Reverse transcriptase activity in mitochondrial extracts was determined in 50 μl reaction mixtures containing 25 mM HEPES–KOH, pH 8.0, 2.5 mM 2-mercaptoethanol, 10 μg acetylated BSA, 0.5 mM MnCl2, 10 μM [α-32P]dTTP (1000–3000 c.p.m./pmol), 2.5 μg poly(rA)-oligo(dT)12–18 and 75 mM NaCl as previously described (23). In both cases, after 15 min incubation at 37°C, TCA-insoluble radioactivity was determined by liquid scintillation counting. One unit is the amount of enzyme required to catalyze the incorporation of 1 pmol dTMP into TCA-precipitable DNA in 1 h at 37°C.

The processivity of pol γ after treatment with H2O2 was determined as previously described (25). Exonuclease activity of H2O2-treated exonuclease-proficient p140 alone, as well as in the presence of the p55 accessory subunit, was determined as previously described (23).

Gel mobility shift assay

A 38mer oligonucleotide primer (5’-TTA CGT CAC GTA CTG TCA ATA GAC AAT ACA CT-3’) was 5’-end labeled and hybridized to a 1.3 molar excess of a complementary 34mer (5’-GTA TGT TCG CCT GTA ATG AAC GTA GTT GCG A-3’) to generate a primer–template substrate with a 3’-base recessed 3’-end. Prior to DNA binding, Exo p140, p55 and Exo p140/p55 complex (1:1:1 molar ratio) were incubated for 30 min at 37°C with H2O2 (0.05, 0.1, 0.15, 0.2, 0.25, 0.5, 1.0, 1.5, 2 and 2.5 mM) in buffer containing 10 mM HEPES–KOH, pH 8.0, 1.0 mM EDTA and 10% glycerol in a final volume of 10 μl. To remove the remaining H2O2, 1 μl of catalase (25 ng, 20 U/μg) was added to each sample and incubated for 5 min at room temperature. Binding mixtures (20 μl) contained 10 mM HEPES–KOH, pH 8.0, 0.5 mM DTT, 4 μg acetylated BSA (200 μg/ml), 250 fmol end-labeled 38mer–34mer primer–template and 250 fmol either H2O2-treated Exo p140, p55 or Exo p140/p55 complex. After 5 min preincubation at room temperature the mixtures were resolved by electrophoresis on 6% polyacrylamide gels in 20 mM HEPES–KOH, pH 8.0, and 0.1 mM EDTA, pH 8.0. At 3 h at 100 V, 4°C, the gels were dried, exposed to a PhosphorImager screen and analyzed on a Storm 860 PhosphorImager (Molecular Dynamics).

Detection of protein oxidation

As a result of protein oxidation, carbonyl groups are introduced into protein side chains by a site-specific mechanism. We used an OxyBlot kit (Intergen) to immunodetect these carbonyl groups to 2,4-dinitrophenylhydrazine (DNP-hydrazine), in order to derivatize the DNP moiety of the proteins. The next step was incubation with horseradish peroxidase–antibody conjugate directed against the primary antibody. Membranes were then treated with SuperSignal West Pico chemiluminescent substrate and exposed to autoradiography film. The method applied for
detection of the oxidized catalytic subunit of pol γ in mitochondrial lysates from GM00637E cells treated with H$_2$O$_2$ was similar, however, with few modifications enabling immunoprecipitation (see below).

**Hydrogen peroxide treatments**

Hydrogen peroxide stock (30%) was diluted with phosphate-buffered saline (PBS) and the concentration determined by absorbance at 240 nm as described (31). Cells, ~80% confluent, were washed twice with 1× PBS and exposed to 100, 250 or 400 µM H$_2$O$_2$ in serum-free MEM for 2 h. A control cell culture was mock-treated with serum-free medium alone. After treatment, the medium was discarded and cells were immediately harvested by brief treatment with trypsin–EDTA (0.25%) for mitochondria preparation.

**Mitochondrial preparation**

Harvested SV40-transformed human fibroblasts were washed twice with PBS, centrifuged (5000 r.p.m., 5 min) and supernatants were discarded. The pellets were weighed and gently resuspended in HDB buffer (5 mM KPO$_4$, pH 7.5, 2 mM MgCl$_2$, 1 mM 2-mercaptoethanol), at 9× volume of the wet weight of the cells. Protease inhibitor cocktail (1:100 v/v) was added and the cells were left on ice for 1 h. Once the cells were swollen (as judged with 5 µl on a slide with a 20/40 objective lens) they were broken by Dounce homogenization for 20–30 strokes until only 0–2 intact cells/ microscope field remained. Mannitol–sucrose buffer (2.5×: 0.525 M mannitol, 0.175 M sucrose, 5 mM Tris–HCl, 5 mM EDTA, 5 mM MgCl$_2$, pH 7.5) was added to a 1× concentration. To resuspend the nuclei the cells were centrifuged for 5 min at 2000 r.p.m. and 4°C and the supernatants with remaining mitochondria were collected. They were then centrifuged for 30 min at 15 000 r.p.m. The pellets containing mitochondria were washed three times with 1× mannitol–sucrose buffer, in order to remove remaining nuclear DNA and proteins. Mitochondria pellets were resuspended in mitochondrial lysis buffer containing 1% NP-40, 0.3 M NaCl, 10% glycerol, 20 mM Tris–HCl, pH 8.0, 14 mM 2-mercaptoethanol and proteinase inhibitors and left on ice for 10 min. The lysates were then centrifuged for 2 h at 14 000 r.p.m. Supernatants containing pol γ were frozen in 25 µl aliquots in liquid nitrogen and stored at −80°C. These mitochondrial lysates were used for determination of either reverse transcriptase activity or level of pol γ catalytic subunit oxidation.

**Immunoprecipitation of DNA polymerase γ from crude mitochondrial lysates**

Two milliliters of Dpg polyclonal antiserum (~10 ng/ml) were mixed with 1 ml of protein A–Sepharose for 1 h in 100 mM Tris–HCl, pH 8.0, at 4°C. The Sepharose beads were then extensively washed with 100 mM Tris–HCl, pH 8.0, followed by 10 mM Tris–HCl, pH 8.0. To detect oxidatively modified proteins in mitochondrial lysates we used an OxyBlot kit (Intergen) as described above. Briefly, protein extracts (~950 µg/sample) were treated with DNP-hydrazine, in order to derivatize the carbonyl groups to DNP-hydrazine. Then the buffer was exchanged for 10 mM Tris–HCl, pH 8.0, (40–50 min centrifugation, 4°C, Microcon 30) to enable immunoprecipitation; final sample volumes were 50 µl. Fifty microliters of antibody–protein A–Sepharose resin were mixed for 1 h at 4°C, end over end, with DNP-hydrazine-modified proteins in 10 mM Tris–HCl, pH 8.0, buffer. The Sepharose beads were then washed extensively with the same buffer and 25 µl samples were resolved on ~20% polyacrylamide gels and proteins electrotransferred to an Immobilon-P membrane (Millipore). Anti-DNP antibodies from the OxyBlot kit were used to detect DNP moieties of the oxidatively modified pol γ catalytic subunit as described above.

**Other methods**

Deionized water used for all buffer preparations was purified on AG 501-X8(D) resin in order to remove any remaining metal ions. Protein concentration was determined according to Bradford (32), with BSA as the standard. For immunoblots, mitochondrial lysates were resolved by SDS–PAGE, electrotransferred to Immobilon-P membranes (Millipore) in 0.025 M Tris, 0.192 M glycine, probed with anti-Dpg polyclonal antiserum (23) and visualized with alkaline phosphatase-conjugated second antibody and Western Blue reagent (Promega).

**RESULTS**

**In vitro oxidation of pol γ and effect on activities**

To address whether pol γ can be modified by ROS and what effect oxidative modification has on the activity of the polymerase we measured the modification and inactivation of the polymerase by treatment with hydrogen peroxide. Reducing agents such as 2-mercaptoethanol or dithiothreitol and BSA were omitted from the H$_2$O$_2$ reaction, thereby enhancing the accessibility of enzyme treatment. After incubation with H$_2$O$_2$ the remaining hydrogen peroxide was removed from reactions by incubation with catalase for 5 min. Oxidatively modified enzymes were then characterized for activity and oxidation.

To examine in vitro hydrogen peroxide influence on human mtDNA pol γ activity we treated both the catalytic (p140) and accessory (p55) subunits separately, as well as in complex, with increasing H$_2$O$_2$ concentrations. Reduction in polymerase activity by this treatment was measured on poly(dA)·oligo(dT)$_{12–18}$ as substrate. Polymerase activity of the pol γ p140 catalytic subunit (Exo–p140) proved to be moderately sensitive to hydrogen peroxide (Fig. 1). We observed 50% activity loss following its preincubation with 250 µM H$_2$O$_2$ for 60 min. When in complex with the p55 accessory subunit the polymerase activity was preserved and the complex remained 50% active even when the H$_2$O$_2$ concentration exceeded 1.5 mM. However, when p140 was oxidized prior to complex formation, p55 addition did not prevent significant activity loss. When p55 alone was subjected to hydrogen peroxide prior to association with p140, the polymerase activity in the complex was affected to a much lesser extent than treatment of p140 alone and this activity was only slightly lower than the p140/p55 complex subjected to oxidation after complex formation. This result indicates that the oxidized p55 did not significantly lower polymerase activity. These results also suggest that the p55 accessory subunit, when in complex with p140, may protect the catalytic subunit from oxidative modification by H$_2$O$_2$.

We also tested the effect of oxidizing the accessory subunit on the processivity of p140/p55 complex. Treatment of p55 with up to 1 mM H$_2$O$_2$ prior to association with p140 had no effect on the processivity of the complex when compared to the
untreated p140/p55 complex and demonstrated DNA synthesis in excess of several thousand nucleotides in length (data not shown) and was similar to what was previously observed for the complex (25).

Hydrogen peroxide treatment reduced DNA binding activity in pol γ

In order to compare the sensitivity to hydrogen peroxide among DNA polymerases from different polymerase classes, we treated two nuclear polymerases, human pol α (B class) and human pol β (X class), with H₂O₂ in exactly the same manner as we did with both subunits of pol γ, and compared their activities using the same substrate, poly(dA)-oligo(dT) (Fig. 2). The catalytic subunit of pol γ was more susceptible to hydrogen peroxide than the two nuclear DNA polymerases. Pol γ activity was reduced by 50% in 250 µM H₂O₂, whereas at the same H₂O₂ concentration pol α and pol β retained ~80% and almost 100% of their polymerase activity, respectively. Pol α showed significant (>50%) activity loss at H₂O₂ concentrations >1 mM. To observe similar activity loss for pol β we had to preincubate the polymerase with 5 mM H₂O₂.

The effect of H₂O₂ on the intrinsic exonucleolytic activity of pol γ was also examined. Preincubation with hydrogen peroxide at concentrations up to 1 mM had little effect on exonuclease activity of the wild-type p140 and almost no effect on exonucleolytic activity of the wild-type p140/p55 complex (data not shown).

We also examined the influence of H₂O₂ on p140 activity over time (Fig. 3). For that purpose the enzyme was preincubated at 37°C in the presence of 250 µM H₂O₂. At selected time intervals (0–120 min) portions of p140 (H₂O₂-treated and an untreated control) were taken and incubated for 5 min with catalase to remove remaining H₂O₂. Polymerase activity in all collected samples was then determined. Samples of p140 that were not treated with H₂O₂ were used as relative controls for corresponding time point samples of H₂O₂-treated enzyme. When exposed to 250 µM H₂O₂ over time p140 lost 50% of its activity during the first 30–40 min; after that time activity loss was slower and 30% activity remained after 2 h of treatment. After the 2 h incubation the hydrogen peroxide concentration decreased by only 12% as compared to 0 min (Fig. 3).

In order to examine H₂O₂ influence on p55, p140 and p140/p55 DNA binding efficiency we performed gel mobility shift assays with a single-stranded, 32P-labeled oligonucleotide annealed to its complementary sequence (Fig. 4A and B). Hydrogen peroxide affected the ability of p140, p55 and p140/p55 complex to bind to double-stranded (ds)DNA template, however to a lesser extent than it affected the activity of these proteins; again p140 proved to be most sensitive, losing ~50% of its dsDNA ‘binding capacity’ when preincubated for 30 min with >0.5 mM H₂O₂. Also, the ability of p140/p55 complex to bind dsDNA was affected by preincubation with H₂O₂, but to a lesser extent than that of p140; even when preincubated with...
2.5 mM H$_2$O$_2$ the complex retained ~60% ‘binding capacity’ (Fig. 4B).

Detection of oxidized amino acids in pol $\gamma$

The next step was to examine whether the inhibitory effect of hydrogen peroxide on activity and DNA binding efficiency of the proteins analyzed is a result of oxidative modifications introduced into the protein molecule itself. We used an OxyBlot kit (Intergen), specially designed for the detection of oxidatively modified proteins. Protein oxidation mainly results in introduction of carbonyl groups into protein side chains by a site-specific mechanism. After treatment with DNP-hydrazine these carbonyl groups are derivatized to DNP-hydrazone, which can be detected with specific antibodies against the DNP moiety of the protein. We treated both the pol $\gamma$ catalytic and accessory subunits as well as pol $\alpha$ and pol $\beta$ with increasing concentrations of hydrogen peroxide (0.1, 0.5, 1 and 2.5 mM) and developed DNP-hydrazone groups as described in Materials and Methods. Oxidation of all four proteins was detected with the anti-DNP moiety antibodies (Fig. 5A and B), but only in samples preincubated with the higher concentrations of H$_2$O$_2$ (1 and 2.5 mM). These results demonstrate oxidative modification of the proteins by treatment with hydrogen peroxide.

Pol $\gamma$ is oxidized in vivo

In order to examine the in vivo effects of H$_2$O$_2$ on pol $\gamma$ we treated SV40-transformed human fibroblasts with hydrogen peroxide and assayed mitochondrial lysates for reverse transcriptase activity and protein oxidation assessment. The reverse transcriptase assay allowed us to distinguish between pol $\gamma$ and other polymerase activities that may be contaminating the mitochondrial lysates. Mitochondria were isolated and
mitochondrial lysates made after 2 h treatment of SV40-transfected human fibroblasts with 100 and 250 μM H₂O₂. Oxidized proteins in these lysates and in pol γ immunoprecipitates were detected by western blot using the anti-DNP antibodies (Fig. 6). We were able to detect oxidized proteins in the soluble mitochondrial lysates (Fig. 6, lanes 2–4) and in p140 immunoprecipitates from mitochondrial lysates (Fig. 6, lanes 5–7). One of the more prominent oxidized polypeptides observed in the mitochondrial lysate co-migrated with the p140 catalytic subunit of pol γ and represented ~11% of all the oxidized polypeptides above 30 kDa (Fig. 6, lanes 2–4). This polypeptide was confirmed to be the pol γ catalytic subunit by enrichment of the DNP-positive polypeptide in the immunoprecipitate with polyclonal antibodies against the catalytic subunit (Fig. 6, lanes 5–7). This enriched 140 kDa polypeptide was also positive in a western blot using polyclonal antibodies against the catalytic subunit of human pol γ (data not shown). Lane 2 displays the oxidized proteins from untreated mitochondria and represents the endogenous level of oxidized mitochondrial proteins. The 140 kDa polypeptide is barely visible as part of the five DNP-positive polypeptides in the untreated control.

Although we readily detected oxidation of the pol γ catalytic subunit in H₂O₂-treated cells, pol γ activity in these mitochondrial lysates was reduced by only 10 and 15% on treatment with 250 and 400 μM H₂O₂, respectively. This is most likely due to the fact that in mitochondria in vivo the majority of the pol γ catalytic subunit may be present in complex with the accessory subunit, and is at least partially protected from oxidative modification and activity loss. The protection by p55 probably only blocks the oxidation of a minority of amino acids, some of which are involved in polymerase function or substrate binding, while leaving the rest of the solvent-accessible amino acids in p140 available for oxidative modification. These latter amino acids, although readily oxidized, do not result in inactivation of polymerase activity. This would explain that while we readily detected p140 oxidation in vivo, most of the pol γ was still active. The observed slight activity loss may correspond to inactivation of this p140 fraction that remains unbound to p55.

**DISCUSSION**

Electron leakage from the electron transport chain during oxidative phosphorylation in the mitochondria is known to generate ROS and is believed to be the largest source of endogenous oxidative damage (3). Because of the proximity of the intra-mitochondrial matrix to the electron transport complexes, the possibility arises that pol γ is a target for oxidative modification. We tested this hypothesis by studying the ability of pol γ to be oxidized in vitro and assessed the effect on DNA polymerase activity and DNA binding. We also observed in mitochondrial lysates from cells treated with H₂O₂ that pol γ was one of the more abundant proteins to be oxidized.

**In vitro** treatment of the single catalytic subunit of pol γ readily inactivated polymerase activity without a significant effect on exonuclease activity. The same treatment of the nuclear polymerases, human pol α and β, had little effect on activity. Western blot analysis of these three polymerases with antibodies specific for DNP-hydrazine-derivatized carbonyl groups verified that all three polymerases were modified at similar levels. Thus, the sensitivity of the pol γ catalytic subunit to H₂O₂ relative to that of pol α and β indicates that a solvent-accessible amino acid(s) in pol γ becomes modified and that this amino acid plays a key role in the polymerase activity, either directly in catalysis or in binding of the substrates. Due to the strong conservation of amino acids in the active site between polymerase families A (pol γ) and B (pol α), direct oxidation of a catalytic residue seems less likely in pol γ. Thus, human pol γ may contain sensitive sites that participate in substrate binding directly or indirectly which when oxidized inhibit normal substrate binding. The decrease in DNA binding activity upon oxidation further suggests a role of these oxidized amino acids in substrate binding.

Interestingly, addition of p55 prior to H₂O₂ treatment protected the p140 catalytic subunit from this oxidation. Treatment of the accessory subunit alone had little effect on the polymerase activity or processivity of the complex, suggesting
that the function of the accessory subunit is not compromised when oxidized. We have observed a similar scenario for N-ethylmaleimide (NEM) inhibition of pol γ where the Hp55 accessory subunit protected the catalytic subunit from inhibition (25). NEM is known to modify solvent-accessible sulfhydryl groups such as cysteine residues. Since no cysteine residue is known to participate in DNA polymerase catalysis among family A polymerases, the modification of a cysteine by NEM may reflect the function of this residue in substrate binding. It is interesting that a similar protection by Hp55 from H₂O₂ oxidation was observed in this study and raises the possibility that the same cysteine residue protected by Hp55 from NEM treatment is oxidized by H₂O₂ but also protected by Hp55. Further experiments need to be carried out to address this possibility.

The modification of pol γ by H₂O₂ was also observed in vivo in cells treated with H₂O₂. Oxidized protein residues became detectable at much lower hydrogen peroxide concentrations than were required to detect in vitro oxidation of purified proteins (see Fig. 5A and B). This can be explained by the fact that all possible factors (e.g. metal ions) that would participate in hydrogen peroxide conversion to other, potentially more reactive, ROS (OH⁻) were mostly eliminated in the in vitro assay. When in living cells, hydrogen peroxide interacts with cellular biomolecules contributing to an increase in the steady-state concentrations of various reactive species within the cell. The final observed oxidation effect is most probably due to those secondary events. Mitochondrial lysates showed several proteins modified by H₂O₂ treatment and specifically a polypeptide that migrates with the p140 catalytic subunit of pol γ. This polypeptide appeared to be one of the more abundant proteins modified. Immunoprecipitation with polyclonal antibodies against pol γ confirmed that this band was indeed the pol γ catalytic subunit. Thus, the pol γ catalytic subunit is oxidized in vivo and may represent one of the major targets in the mitochondrial matrix. This oxidation was accompanied by a small but significant decline in activity, suggesting that the accessory subunit was protecting the polymerase from most of the deleterious effects on DNA replication activity.

Although the p55 subunit offered protection from H₂O₂ during DNA synthesis and its function in DNA replication necessitates the high processivity that p55 offers, the role of p55 in DNA repair is unknown. We previously observed two forms of pol γ isolated from HeLa cells, the single catalytic subunit alone and in complex with the accessory subunit (23,25). We previously proposed that although DNA replication would require high processive synthesis characterized by the two subunit complex, the role of pol γ in base excision repair would only require the single catalytic subunit. The pol γ catalytic subunit has been observed throughout mitochondria (non-replicating and replicating) of the cell whereas active replication of mtDNA has only been observed in the perinuclear mitochondria (33,34). The non-replicating form of pol γ presumably functions in base excision repair, which does not require the accessory subunit in vitro (35). We observed that although the accessory subunit appears to protect much of the pol γ activity from oxidative damage, the activity of the single catalytic subunit of pol γ, possibly functioning alone during mtDNA repair, may be compromised by oxidative damage.

Although many studies have focused on the oxidation of DNA, oxidation of proteins is well documented (12,36–38). Oxidative stress in yeast has been shown to modify mitochondrial proteins, including Hsp60, pyruvate dehydrogenase, α-keto-glutarate dehydrogenase, aconitase, cytosolic fatty acid synthase and glyceraldehyde 3-phosphate dehydrogenase (20). Oxidative stress in yeast appears to arrest many of the mitochondrial functions, such as the tricarboxylic acid cycle, thereby shifting glucose metabolism to the pentose phosphate pathway, which generates the NADPH needed by antioxidant enzymes (20). Protein oxidation in mitochondria from Drosophila appears specific to a few proteins. Investigation of oxidized carbonyl groups from Drosophila mitochondrial proteins appears to be limited to the adenine translocator (ANT) in the membrane (39) and to aconitase in the mitochondrial matrix (40). The oxidation of ANT and aconitase in Drosophila appeared to occur as an age-related process with a decrease in the activity of these proteins. The age-specific oxidation of aconitase may be part of a larger biological mechanism by which oxidative stress causes an age-specific decline in mitochondrial function (41). In addition to protein oxidation, the age-associated accumulation of point mutations in mtDNA cannot be ignored as a parallel process (2). In human cells, the detection of carbonyls from the mitochondrial matrix, indicative of oxidized proteins, did not limit the oxidation to a single protein as observed in Drosophila. We observed at least 10 different polypeptides oxidized to varying degrees in the mitochondrial lysate. The pol γ catalytic subunit along with two other proteins represented one of the more abundant proteins oxidized. Given the fact that pol γ is a rare protein, representing ~0.008% of mitochondrial proteins (23), the oxidation we observed may be specific for a subset of mitochondrial proteins including pol γ. Oxidative inactivation of pol γ could slow mtDNA replication and eventually lead to inhibition of oxidative phosphorylation. The specific inactivation of pol γ may be part of a more general mechanism in response to oxidative stress. This could also explain why a 60 min treatment of cells with hydrogen peroxide leads to less efficient repair of mtDNA (10).

In summary, we have determined that pol γ is a target for oxidative stress and that this oxidation can inactivate the catalytic subunit. The accessory subunit, however, offered significant protection from this oxidation, suggesting that a key oxidizable residue is masked by the binding of p55 to the catalytic subunit. Although much research has focused on the oxidation of mtDNA, the oxidation of mitochondrial proteins and, specifically, pol γ should now be considered as targets of ROS. Damage to pol γ and other mtDNA replication and repair proteins can have a consequence on the overall function of the mitochondria as well as the cell.

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