Accumulation of Adenine DNA Glycosylase-sensitive Sites in Human Mitochondrial DNA*

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The mitochondrial respiratory chain inevitably produces reactive oxygen species as byproducts of aerobic ATP synthesis. Mitochondrial DNA (mtDNA), which is located close to the respiratory chain, is reported to contain much more 8-oxoguanine (8-oxoG), an oxidatively modified guanine base, than nuclear DNA. Despite such a high amount of 8-oxoG in mtDNA (1–2 × 10^4 G), mtDNA is barely cleaved by an 8-oxoG DNA glycosylase or MutM, which specifically excises 8-oxoG from a C:8-oxoG pair. We find here that about half of mtDNA molecules are cleaved by another 8-oxoG-recognizing enzyme MutY, which excises adenine from an A:8-oxoG pair. The cleavage sites are mapped to adenines. The calculated number of MutY-sensitive sites in mtDNA is approximately 1.4/10^4 G. This value roughly corresponds with the electrochemically measured amount of 8-oxoG in mtDNA (2.2/10^4 G), raising the possibility that 8-oxoG accumulates as an A:8-oxoG pair.

8-oxoG accumulates as an A:8-oxoG pair but not as a C:8-oxoG pair. We find here that about half of mtDNA molecules are cleaved by another 8-oxoG-recognizing enzyme MutY, which excises adenine from an A:8-oxoG pair. The cleavage sites are mapped to adenines. The calculated number of MutY-sensitive sites in mtDNA is approximately 1.4/10^4 G. This value roughly corresponds with the electrochemically measured amount of 8-oxoG in mtDNA (2.2/10^4 G), raising the possibility that 8-oxoG accumulates as an A:8-oxoG pair.

Experimental Procedures

Materials—Restriction enzymes EcoRI, BamHI, and PvuII were purchased from Takara (Seta, Japan). Phosphocellulose P11 was from...
FIG. 1. Cleavage of oligonucleotides containing C8-oxoG or A:G pair by recombinant MutM and MutY proteins. A, recombinant MutM (left panel) and MutY (right panel) were purified. Proteins were separated on 10% SDS-polyacrylamide gels and visualized by silver staining. B, the 5'-fluorescein isothiocyanate-labeled oligonucleotide substrates, FM and FY (Table I), were cleaved in the presence of 0.5 μg of the total DNA of U937 cells by 30 ng of MutM (shown as M) and 300 ng of MutY (shown as Y), respectively. The DNA was denatured, resolved on 15% polyacrylamide/6 M urea gels, and analyzed with FluorImager 595.
from the mitochondrial fraction by using a Qiagen plasmid mini kit (Hilden, Germany) according to the manufacturer’s instructions. The amount of 8-oxo-dG in mtDNA was measured by an electrochemical method as described previously (25).

RESULTS

Cleavage of Oligonucleotides Containing C:8-oxoG or A:G Pair by Recombinant MutM and MutY—As shown in Fig. 1A, recombinant MutM and MutY proteins were highly purified to homogeneity. 30 ng of MutM and 300 ng of MutY proteins cleaved almost all of 400 fmol of oligonucleotides FM and FY (Table I), respectively, in the presence of 0.5 μg of total DNA (Fig. 1B). The amount of 8-oxoG in total DNA is reported to be, at most, 1/10^7 G or roughly 4 fmol of 8-oxoG in 0.5 μg of DNA (21, 31). Therefore, the MutM or MutY is sufficient for digesting the endogenous 8-oxoG sites in the following experiments.

DNA Digestion by MutM and MutY—After treatment of total DNA with the purified MutM or MutY protein, the amount of uncleaved mtDNA was quantified by Southern blot analysis. As reported by other groups (23, 24), mtDNA remained un-cleaved by the MutM protein both in the nondenaturing and denaturing conditions (Fig. 2A, lanes 2 and 5). In contrast, the amount of uncleaved mtDNA was decreased to approximately one-half by the MutY protein in the glyoxal denaturing conditions (Fig. 2A, lane 4). The MutY-treated mtDNA was apparently uncleaved in the nondenaturing conditions (Fig. 2A, lane 1), indicating that single-strand cleavage occurs by the MutY protein treatment, which is consistent with the nature of adenine DNA glycosylase. In Fig. 2B, changes in the amount of 16-kbp mtDNA on the denaturing gels are shown. The amount of full-length mtDNA of HeLa MRV11 cells was barely decreased by the MutM protein (91.9 ± 26.9, n = 12, mean ± S.D.), whereas the amount was decreased to nearly one-half that of the control by the MutY protein (46.2 ± 11.0, n = 12). In the case of HeLa MR51 cells that over-produce human MTH1 approximately 100-fold (15), the amounts of full-length mtDNA after treatment of the MutM and MutY proteins were 99.6 ± 26.2 and 59.1 ± 17.3 that of the control (mean ± S.D., n = 6), respectively (Fig. 2B).

Identification of the Cleavage Sites by MutY—To determine the cleavage sites, we amplified the cleaved strands by LPMPCR. Consistent with the results in the Southern blot experiments, there were few signals detected in the control and LMPCR. Consistent with the results in the Southern blot experiments, there were few signals detected in the control and LMPCR. In the case of HeLa MRV11 cells, we examined a human monocytic cell line, U937. The ratio of the 18 S rRNA gene to mtDNA in the MutY

![Fig. 2. Southern blotting of mtDNA cleaved with MutM or MutY. A, the total DNA was extracted from HeLa MRV11 cells and digested with BamHI. The BamHI-digested DNA was treated with 30 ng of MutM or 300 ng of MutY. After the native (left panel) or denatured (right panel) DNA was electrophoresed, mtDNA was probed with the 32P-labeled 1.9-kbp fragment of mtDNA (nt 2573–4431) and quantified. C, control; M, MutM; Y, MutY. B, the amount of mtDNA was quantified and is shown as a percent of the control. The values represent means of twelve and six independent experiments (HeLa MRV11 and MR51 cells, respectively) with standard deviations.](http://www.jbc.org/)

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**TABLE I**

| Name | Sequence | Pair by Recombinant MutM and MutY |
|------|----------|----------------------------------|
| FM   | 5’ FITC-GGAATTCCTCGAGGGTAGACGTCGCGA 3’ |
| FY   | 5’ FITC-GGAATTCCTCGAGGGTAGACGTCGCGA 3’ |

8-Oxoguanine is shown in bold character. The cleavage site is marked by an underline. FITC, fluorescein isothiocyanate.
protein-treated sample is reduced to approximately 50% of that in the control (Fig. 4B). The 18 S rRNA gene was barely cleaved by either the MutM or MutY protein. Similar results were also obtained when using Jurkat cells (results not shown).

**DISCUSSION**

Preparation of mtDNA from purified mitochondria raises a possibility that intact mtDNA is preferentially collected from intact mitochondria. This is considered to be one of the reasons why mtDNA is resistant to MutM (21, 33). Although we used total cellular DNA to avoid any possible bias resulting from the mtDNA preparation, we could hardly detect MutM-dependent cleavage of mtDNA by Southern blot analysis. The collective number of MutM-cleavable lesions such as C:8-oxoG, C:formamidepyrimidine (Fapy), and an abasic site is below 1 per 10 molecules of mtDNA as long as we observe the amount of 16-kbp mtDNA. It has been recently reported that the oxidized bases exist predominantly in fragmented mtDNA (33). We, however, did not observe such fragmented bands of mtDNA by Southern blot analysis (Fig. 2A).

In contrast, approximately one-half of mtDNA molecules were sensitive to the MutY protein, i.e. there is one MutY-sensitive site per two mtDNA molecules. The cleavage of mtDNA is not due to nuclease(s) remaining in the purified MutY sample for the following reasons: 1) the MutY protein was highly purified (Fig. 1A); 2) the cleavage was a single-strand break in nature (Fig. 2); 3) essentially all of the cleavage sites were mapped to adenines (Fig. 3); 4) a nuclearly encoded 18 S rRNA gene was barely cleaved (Fig. 4); and 5) the oligonucleotide FY was specifically cleaved at an A:G site (Fig. 1B). Accordingly, we conclude that mtDNA is cleaved by an adenine DNA glycosylase activity of the MutY.

MutY removes adenine residues from A:G and A:8-oxoG pairs with similar efficiency and also removes the residue from an A:C pair, but with 20-fold lower efficiency (27). In general, MutY-sensitive sites are generated by the following three ways: misincorporation of normal nucleotides (A:G and A:C pairs); misincorporation of dAMP opposite 8-oxo-dG in DNA (A:8-oxoG pair); and misincorporation of 8-oxo-dGMP opposite template adenine (A:8-oxoG pair). The MutY cleavage sites in mtDNA were adenines of A:T on the mtDNA sequence (Fig. 3), suggesting that dGMP, dCMP, or 8-oxo-GMP is misincorporated opposite template adenine. Chick embryo DNA polymerase γ exhibits a fidelity of less than one error for every 260,000 bases polymerized when normal dNTPs are used as substrates (34). It is therefore unlikely that human DNA polymerase γ misincorporates dGMP or dCMP opposite template adenine as frequently as observed here. On the other hand, an A:T to C:G transversion rate for the chick embryo DNA polymerase γ is increased more than 1000-fold for synthesis in the presence of 8-oxo-dGTP at a concentration equal to that of normal dGTP (35). We electrochemically measured 8-oxo-dG in mtDNA that is prepared from mitochondria by an alkaline SDS method for extraction of closed circular DNA. The amount of 8-oxo-dG was approximately 2.2×10^4 G. There are about 3,500 G per single strand of mtDNA. One MutY-sensitive site per two mtDNA molecules means that human mtDNA contains approximately one MutY-sensitive site per 7,000 G or 1.4 per 10^4 G, roughly corresponding to electrochemically measured 8-oxo-dG of 2.2/10^4 G.

**FIG. 3.** Determination of cleavage sites. After the treatment with MutM or MutY as in Fig. 2, the cleaved strands of mtDNA were amplified by LMPCR using the primer set D8 (30).

**FIG. 4.** Cleavage of nuclear DNA and mtDNA with the MutY. A, the PvuII-digested total DNA was used. The total DNA (1 μg) of HeLa MRV11 cells was treated with various amounts of MutY. mtDNA (16 kbp) and an 18 S rRNA gene (12 kbp) were simultaneously detected and quantified by Southern blotting using alkaline phosphatase-labeled probes for mtDNA and the 18 S rRNA gene as described under “Experimental Procedures.” B, the PvuII-digested total DNA of U937 cells was treated with MutM or MutY. mtDNA was first probed. After deprobing, the 18 S rRNA gene was reprobed. The detection and quantification were performed as in A.
10^4G. Taken together, although the nature of the MutY-sensitive sites is not completely understood at present, it is plausible that a certain part of the MutY-sensitive sites are A:8-oxoG. The over-expression of human MTH1, however, did not much reduce the MutY-sensitive sites (Fig. 2B). \( K_m \) of human MTH1 for 8-oxo-dGTP is 12.5 \( \mu \)M (36), the value of which is level with intracellular concentration of normal dGTP (\(-30 \mu\)M) (37). The inability of human MTH1 in reducing the MutY-sensitive sites might rather suggest that additional factor(s) may be required to eliminate 8-oxo-dGTP.

8-Oxo-dGMP is incorporated opposite C and A with essentially the same efficiency (4). Given that a certain part of the MutY-sensitive sites are A:8-oxoG, the fewer MutM-sensitive sites than the MutY-sensitive sites suggest that C:8-oxoG is more efficiently repaired in mitochondria than is A:8-oxoG that is formed by misincorporation of 8-oxo-dGMP opposite adenine. C and A are incorporated opposite 8-oxoG with almost the same efficiency after removal of A from A:8-oxoG, whereas G is predominantly incorporated opposite C after removal of 8-oxoG from C:8-oxoG. Thus a MutY-like activity is intrinsically less efficient in eliminating A:8-oxoG than is a MutM-like activity in eliminating C:8-oxoG. In addition, a MutY-like activity itself might be insufficient for repair of A:8-oxoG in mitochondria. The activity of 8-oxoG DNA glycosylase is actually detected in human mitochondria (16). On the other hand, the activity of adenine DNA glycosylase has not been detected in human mitochondria to date, although recombinant human MYH is targeted into mitochondria (18). This might reflect the fact that the human MYH activity is low relative to the human OGG1 activity in mitochondria. Another possibility is that human MYH might be less effective in repair of A:8-oxoG formed by misincorporation of dAMP than that formed by misincorporation of dGMP. Although hydrolysis of 8-oxo-dGTP is proposed for prevention of 8-oxo-dGMP misincorporation in the current MutM, Y, and T systems (6, 7), the repair of A:8-oxoG once formed by 8-oxo-dGMP misincorporation is not well elucidated. In the case that 8-oxo-dGMP is misincorporated opposite adenine, a strong MutY-like activity would enhance A:T to C:G transversion (6, 7). In this light, it is conceivable that human MYH is regulated in mitochondria to act mainly on A:8-oxoG formed by misincorporation of dAMP. If so, A:8-oxoG formed by misincorporation of 8-oxo-dGMP might be repaired at later stages by other unknown mechanism(s), e.g. in a transcription-coupled way. We might also need to consider mechanism(s) other than base excision repair, such as selective degradation of mtDNA containing 8-oxoG.

Despite presumed strong oxidative damage of mtDNA, A:T to C:G or C:G to A:T transversion does not frequently occur as is evident from reported mutations (38). It is yet unclear to what extent 8-oxoG contributes to oxidative damage-induced mutations in mitochondria. The mutual roles of MutT-, MutM-, and MutY-like activities are not completely understood in mitochondrion, either. Examination of pairing partners with 8-oxoG in a steady state is important both for understanding of mutagenesis by 8-oxoG and for estimation of relative activities among the three repair enzymes in vivo. To this purpose, the combination of DNA cleavage by DNA glycosylases and LMPCR, as used here, appears to be useful.

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