Effects of a Mitochondrial Mutator Mutation in Yeast POS5 NADH Kinase on Mitochondrial Nucleotides*

Linda J. Wheeler and Christopher K. Mathews

From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331-7305

Background: A mutation in the yeast gene POS5, which encodes mitochondrial NADH kinase, is a mitochondrial mutator.

Results: Mutant mitochondria display higher dNTP pools and lower NADP(H) pools than normal mitochondria.

Conclusion: Abnormal dNTP pools and antioxidant protection probably contribute to elevated Δpos5 mutagenesis.

Significance: Understanding intramitochondrial dNTP and pyridine nucleotide levels contributes to understanding mitochondrial mutagenesis.

Saccharomyces cerevisiae contains three NADH/NAD$^+$ kinases, one of which is localized in mitochondria and phosphorylates NADH in preference to NAD$^+$. Strand et al. reported that a yeast mutation in POS5, which encodes the mitochondrial NADH kinase, is a mutator, specific for mitochondrial genes (Strand, M. K., Stuart, G. R., Longley, M. J., Graziewicz, M. A., Dominick, O. C., and Copeland, W. C. (2003) Eukaryot. Cell 2, 809–820). Because of the involvement of NADPH in deoxyribonucleotide biosynthesis, we asked whether mitochondria in a pos5 deletion mutant contain abnormal deoxyribonucleoside triphosphate (dNTP) pools. We found the pools of the four dNTPs to be more than doubled in mutant mitochondrial extracts relative to wild-type mitochondrial extracts. This might partly explain the mitochondrial mutator phenotype. However, the loss of antioxidant protection is also likely to be significant. To this end, we measured pyridine nucleotide pools in mutant and wild-type mitochondrial extracts and found NADPH levels to be diminished by ~4-fold in Δpos5 mitochondrial extracts, with NADP$^+$ diminished to a lesser degree. Our data suggest that both dNTP abnormalities and lack of antioxidant protection contribute to elevated mitochondrial gene mutagenesis in cells lacking the mitochondrial NADH kinase. The data also confirm previous reports of the specific function of Pos5p in mitochondrial NADP$^+$ and NADPH biosynthesis.

The spontaneous mutation rate for the mitochondrial genome is 1–2 orders of magnitude higher than the nuclear gene mutation rate (1, 2). In previous investigations with rat tissue mitochondria, we identified aspects of DNA precursor metabolism that probably contribute to this difference: 1) a strikingly asymmetric distribution of deoxyribonucleoside triphosphates (dNTPs)$^2$ within the mitochondrion (3) and 2) the status of the elevated mitochondrial dGTP pool as a target for oxidation, yielding the strongly mutagenic DNA precursor 8-oxodeoxyguanosine triphosphate (4).

In 2003, Strand et al. (5) described in Saccharomyces cerevisiae a mutator mutation specific for the mitochondrial genome. A deletion mutation in the POS5 gene was shown to elevate the mutation rate of a mitochondrial marker by some 50-fold, with no significant effect upon the nuclear marker tested. POS5 was shown to encode an NADH/NAD$^+$ kinase, localized to mitochondria and preferentially active on NADH. Several other laboratories have confirmed the mitochondrial location of Pos5p and its preference for NADH as a substrate (6–8).

NADPH, the product of the NADH kinase reaction, is involved in dNTP biosynthesis in at least two ways: 1) as the ultimate electron donor supporting the ribonucleotide reductase reaction and 2) as the reductant to convert a methylene to a methyl group in the thymidylate synthase reaction. An informative way to determine whether the POS5 mutation affects mutagenesis through dNTP metabolic abnormalities is to measure dNTP pools directly in extracts of isolated yeast mitochondria. We report these measurements here. In addition, Strand et al. (5) presented extensive evidence for oxidative damage to DNA and proteins in mitochondria of the POS5 deletion strain. Because this evident lack of antioxidant protection would most likely result from insufficient NADPH synthesis in pos5 mutant mitochondria, we have tested this premise directly through analysis of pyridine nucleotide pools in isolated yeast mitochondria.

EXPERIMENTAL PROCEDURES

Yeast Strains—S. cerevisiae strain YPH925 and a YPH925 derivative carrying a pos5 deletion mutation have been described (5). These strains were supplied by Dr. Gregory R. Stuart in the laboratory of Dr. William C. Copeland (NIEHS, National Institutes of Health). The strains are referred to in this paper as WT and POS5, respectively.

Growth of Yeast and Isolation of Mitochondria—A single yeast colony was streaked and transferred to yeast/peptone/dextrose medium (5). When grown to saturation, the culture was diluted 100-fold into lactate medium (3 g/liter yeast extract, 0.5 g/liter glucose, 0.5 g/liter CaCl$_2$·2H$_2$O, 0.5 g/liter NaCl, 0.9 g/liter MgCl$_2$·6H$_2$O, 1.0 g/liter KH$_2$PO$_4$, 4 g/liter NH$_4$Cl, 24 ml of 85% DL-lactic acid, and 8 g/liter NaOH, with pH...
Yeast Mitochondrial dNTP and Pyridine Nucleotide Pools

adjusted to 5.5 with NaOH). Growth with aeration was carried out overnight (∼15 h) at 30 °C, by which time the culture was in late logarithmic phase. The culture was chilled by immersion in crushed ice, following which cells were harvested by centrifugation at 4 °C. Cells were lysed, and mitochondria were prepared by differential centrifugation in sorbitol buffer as described by Glick and Pon (9). A small amount of the initial mitochondrial supernatant was saved for analysis of nucleotides in the cytosolic fraction.

dNTP Analysis—Immediately after isolation, the mitochondrial pellet was mixed with 1 ml of ice-cold sterile water, and 1.5 ml of ice-cold methanol was added. For analysis of cytosolic dNTPs 1.0 ml of cytosolic extract was mixed with 1.5 ml of ice-cold methanol. Samples were vortexed and placed at −20 °C, with occasional mixing, for ∼1 h. The extracts were next heated for 3 min in a boiling water bath and then centrifuged at 17,000 × g for 15 min. The supernatants were transferred to fresh tubes and dried under vacuum. Each residue was dissolved in a minimal volume of water and diluted to an appropriate level for analysis. The dNTPs were analyzed by the DNA polymerase-based assay as described (10).

Ribonucleoside Triphosphate (rNTP) Analysis—rNTP pools in mitochondrial extracts were determined in the same extracts used for dNTP analyses. rNTPs were separated by HPLC and analyzed from peak areas as described (3).

Pyridine Nucleotide Analysis—Pyridine nucleotides were analyzed by cycling reactions as described originally by Pas- soneau and Lowry (11) and later modified by Lin et al. (12) and, for NADPH only, by Minard and McAlister-Henn (13). The mitochondrial pellet was suspended in 250 μl of 0.05 M NaOH and 1 mM EDTA and then split into two 100-μl aliquots. To one aliquot was added 100 μl of 0.1 M HCl (acidic extract for measuring NADP+ and NAD+). The remainder was the alkaline extract (for measuring NADPH and NADH). Both extracts were incubated for 30 min at 60 °C to destroy the other components (NADPH and NADH in the acid extract and NADP+ and NAD+ in the alkaline extract). The samples were neutralized either with 25 μl of 0.1 M Tris-HCl (pH 8.1) and 25 μl of 0.05 M HCl for the alkaline extract or with 50 μl of 0.4 M Tris base for the acidic extract.

The cytosolic fraction (250 μl) was mixed with 250 μl of 0.1 M NaOH and 2 mM EDTA and split into two 200-μl aliquots. To one aliquot was added 200 μl of 0.1 M HCl (acidic extract for measuring NADP+ and NAD+), and the other aliquot was the alkaline extract (for measuring NADPH and NADH). These were incubated for 30 min at 60 °C as described above. Extracts were neutralized either with 50 μl of 0.2 M Tris-HCl (pH 8.1) and 50 μl of 0.1 M HCl for the alkaline extract or with 100 μl of 0.4 M Tris base for the acidic extract.

Enzymes used in the cycling and indicator reactions were obtained from Sigma, with the exception of glutamate-oxaloacetate aminotransferase, which was purchased from Roche Applied Science. Alcohol dehydrogenase was Sigma grade A-3263, offered especially for this purpose.

Measuring NADP+ and NADPH—For the cycling reaction, 5 μl of sample or standard (0−5 pmol of NADP+) was mixed with 100 μl of cycling reagent (10 mM Tris-HCl (pH 7.4), 25 mM ammonium acetate, 0.01% (w/v) BSA, 7.4 mM α-ketoglutarate, 5 mM glucose 6-phosphate, 0.1 mM ADP, 1 unit/reaction glutamate dehydrogenase, and 0.6 unit/reaction glucose-6-phosphate dehydrogenase). Incubation was carried out for 60 min at 37 °C, following which the mixture was heated at 100 °C for 5 min to stop the reaction and then centrifuged to remove precipitated proteins.

For the indicator reaction, 100 μl of the product of each cycling reaction was transferred to a spectrophotometer cuvette and mixed with 1.0 ml of indicator reagent (50 mM imidazole (pH 7.0), 30 mM ammonium acetate, 2 mM MgCl2, 0.1 mM EDTA, 0.3 mM NADP+, and 0.3 unit/reaction 6-phosphogluconate dehydrogenase). Incubation was carried out for 20 min at room temperature, and the NADPH formed was measured at 340 nm.

Measuring NADP+ and NADPH—For the cycling reaction, 5 μl of sample or standard (0−5 pmol of NADP+) was mixed with 100 μl of cycling reagent (100 mM Tris-HCl (pH 8.1), 2 mM β-mercaptoethanol, 0.02% (w/v) BSA, 300 mM ethanol, 2 mM oxaloacetate, 3 units/reaction alcohol dehydrogenase, and 3 units/reaction malate dehydrogenase). Incubation was carried out for 60 min at room temperature, following which the mixture was heated at 100 °C for 5 min to stop the reactions and then centrifuged to remove precipitated proteins.

For the indicator reaction, 100 μl of the product of each reaction was transferred to a spectrophotometer cuvette and mixed with 1.0 ml of indicator reagent (50 mM aminomethyl propanol (pH 9.9), 5 mM potassium glutamate, 0.3 mM NAD+, 6 units/reaction malate dehydrogenase, and 1 unit/reaction glutamate-oxaloacetate aminotransferase). Incubation was carried out for 10 min at room temperature, and the NADH formed was measured at 340 nm.

RESULTS AND DISCUSSION

dNTP Pools—We expected the perturbation of mitochondrial pyridine nucleotide metabolism to affect the pool sizes of dNTPs within the mitochondrion because NADPH is involved directly in the dihydrofolate reductase-thymidylate synthase cycle and more indirectly in ribonucleotide reduction through reduction of oxidized glutaredoxin or thioredoxin. The extent to which these reactions occur within yeast mitochondria is not clear. In mammalian mitochondria, thymidylate synthase and dihydrofolate reductase have recently been shown to exist and to function (14), and preliminary data from our laboratory support the existence of a mitochondrial ribonucleotide reductase (15). Comparable experiments have not been done with yeast.

According to a method described by Glick and Pon (9), a small amount of the initial mitochondrial supernatant was saved for analysis of nucleotides in the cytosolic fraction. When we analyzed yeast mitochondrial extracts, we did see an effect of the pos5 mutation on dNTP pools. As shown in Fig. 1, the dNTP pools were more than twice as high in Δpos5 mitochondrial extracts as in WT mitochondrial extracts (with all four p values <0.02). No such effect was seen upon measurement of the cytosolic dNTP pools (Fig. 2), where the dNTP levels in the post-mitochondrial supernatant were roughly equal when WT and pos5 mutant extracts were compared. The variability of the cytosolic dNTP measurements was considerably higher than that of the mitochondrial measurements recorded in Fig. 1. We do not know the reason for this difference, but the critical mitochondrial measurements do support an approximate doubling of dNTP pools in mutant relative to
WT mitochondria, and the data of Fig. 2 are consistent with the conclusion that cytosolic pools do not differ significantly between mutant and WT cells.

In considering the dNTP measurements recorded in this study, we must take into account a source of error in the DNA polymerase-based dNTP assay. Ferraro et al. (16) reported that the Klenow fragment, which we used as our polymerase source, has relaxed specificity and can incorporate CTP and GTP at high concentrations, leading to possible overestimation of dCTP and dGTP, respectively. We confirmed this finding (3) but found the artifact to be a significant source of error only at rNTP/dNTP ratios >100. However, to determine whether our dGTP or dCTP levels were overestimated, we measured rNTP pools in extracts of both mutant and WT mitochondria (Table 1). From these values and from the dNTP pools recorded in Fig. 1, we estimated the [GTP]/[dGTP] ratios to be 143 and 105 for WT and mutant mitochondria, respectively, and the [CTP]/[dCTP] ratios to be 126 and 89 for WT and mutant mitochondria, respectively. In our hands (3), the dCTP assay is more sensitive than the dGTP assay to this source of error. With all four rNTP/dNTP ratios lying near 100, we conclude that the dGTP levels we have determined are not significantly affected by GTP in the extracts, whereas dCTP may be overestimated by ~30% in both mitochondrial extracts. Because dATP and dTTP measurements are not affected by this artifact and dGTP only minimally, the general conclusion that dNTP levels in pos5 mutant mitochondria are about twice those in WT mitochondria appears justified.

An interesting feature of the mitochondrial dNTP pools is that we did not observe a pronounced asymmetry, with dGTP in great excess, as we have observed for mitochondrial pools in rat tissues (3). The data of Fig. 1 show that, in WT yeast mitochondria, dTTP is the most abundant dNTP, with a pool size about double that of dGTP, the second most abundant nucleotide.

At present, we cannot explain why the pos5 mutation causes uniform expansion of mitochondrial dNTP pools; with an expected defect in NADPH biosynthesis, we might have expected the opposite result. However, we note that Strand et al. (5) reported pos5 mutant cells to contain about three times as much mitochondrial DNA as WT cells. It would be of interest to know whether the dNTP accumulation reported here plays any role in the DNA accumulation in mutant mitochondria. Again, this observation seems somewhat counterintuitive.

To what extent might a doubling of the four dNTP pools explain the mutator phenotype resulting from the pos5 mutation? Previously, we showed that small uniform increases in dNTP concentrations have a disproportionate effect in stimulating mutagenesis (17). In Escherichia coli, a 2-fold expansion of dNTP pools caused by ribonucleotide reductase overexpression was correlated with a 30-fold increase in frequency of mutation to rifampicin resistance (see Table 3 of Ref. 17). We ascribed this phenomenon to a next-nucleotide effect, with increased dNTP levels globally favoring chain extension over proofreading of replication errors. Similar findings were reported by Gon et al. (18), who found that proportional dNTP accumulation during the DNA damage response in E. coli promoted both spontaneous and induced mutagenesis.

Consistent with the idea that proportional dNTP accumulations inhibit proofreading, several investigators have reported that $K_m$ values for chain extension from a mismatched 3’ terminus by eukaryotic DNA polymerases are orders of magnitude higher than those for extension from a matched 3’ terminus. For example, Perrino and Loeb (19) reported the $K_m$ for exten-
sion from a matched terminus for calf thymus DNA polymerase α to be 0.6 μM and $K_m$ values for various mismatches to range from 250 to 5700 μM. For Drosophila polymerase α, $K_m$ values for extension from matched termini ranged from 0.2 to 2.2 μM, whereas $K_m$ values for extension from mismatched termini ranged from 22 to 4760 μM (20). More such data are reviewed in Ref. 17.

In rat liver, our measurements of mitochondrial dNTP pools ranged from 1.5 to 18 pmol/mg of protein, corresponding to concentrations of ~2–20 μM (3). If the corresponding values for yeast mitochondrial dNTPs are comparable, we can estimate that the mitochondrial replisome in vitro is operating in the zero-order range for correct chain extension and in the first-order range for extension from a mismatch. If so, doubling the concentration of one dNTP would double the ratio of mismatched to matched extension rates. If the activity of the proof-reading exonuclease is not directly affected by dNTP concentrations, this would be valuable to know whether the pos5 mutation promotes base substitution mutagenesis as well as frameshifting.

Pyridine Nucleotide Pools—In a semiquantitative sense, the data on dNTP pools suggest that the uniform pool doubling that we see can account for some (but not all) of the mutagenesis stimulated by the pos5 mutation. This is hardly surprising because the data of Strand et al. (5) indicated a pronounced lack of oxidant protection in the pos5 deletion mutant, and oxidative damage to DNA is mutagenic. Because the Pos5 protein is an NADH/NAD$^+$ kinase with a preference for NADH, we might expect pos5 mutant mitochondria to contain low pools of NADPH and possibly of NADP$^+$ as well. We tested this directly by analysis of mitochondrial extracts for pyridine nucleotides. As shown in Fig. 4, that is what we observed. WT mitochondria contained about four times as much NADPH as pos5 mutant mitochondria and ~2.5 times as much NADP$^+$.

The S.D. for the NADPH data in WT mitochondria was much higher than that for the data in mutant mitochondria. The $p$ value obtained by comparing the two measurements was 0.12, which does not provide strong assurance that the two average values are statistically different. However, in five separate experiments, the WT NADPH value exceeded the pos5 mutant value by factors of 9.1, 1.9, 5.4, 1.3, and 2.2, respectively, with an average of 4.0. Hence, we conclude that NADPH levels in pos5 mutant mitochondria are considerably lower than those in WT mitochondria. The data for NADP$^+$ measurements were more robust, with $p < 0.01$, indicating that the difference between the pos5 and WT values is significant.

The S.D. values were higher in the NADH and NAD$^+$ assays than those in the NADPH and NADP$^+$ assays. NADH might well be expected to accumulate when NADH kinase activity is missing, and the NADH measurements in Fig. 4 are consistent with this expectation. However, this conclusion is not as strong as our conclusion that NADPH and NADP$^+$ pools are significantly depleted in mutant mitochondria.

We also analyzed pyridine nucleotides in cytosolic extracts. The S.D. values for the NADH and NAD$^+$ assays were unacceptable, and those values are not shown here, but the data of Fig. 5 suggest that the pos5 mutation does not affect pyridine
nucleotide levels in the cytosol. These findings are consistent with the mitochondrial localization of the POS5 NADH/NAD\(^+\) kinase. However, our findings leave open the question of the source of the mitochondrial pools of NADP\(^+\) and NADPH in pos5 mutant mitochondria. The data of Bieganowski et al. (8) indicate that cytosolic pools of NADP\(^+\) and NADPH do not exchange with mitochondrial pools and that cytosolic NADH/NAD\(^+\) kinases are not transported into mitochondria. Hence, the origins of the NADPH and NADP\(^+\) pools in mutant mitochondria are not known.

In summary, our data confirm the role of Pos5p as a kinase capable of phosphorylating both NADH and NADP\(^+\), with action confined to mitochondria. These data are fully in accord with the conclusion that the mitochondrial mutator phenotype results from oxidative damage to DNA, caused by loss of oxidant protection resulting from the NADPH depletion. However, our data on mitochondrial dNTP pools support the hypothesis that at least some of the enhanced mutagenesis in the pos5 mutant is caused by a small but significant uniform accumulation of dNTPs.

Acknowledgments—We thank Drs. William C. Copeland and Gregory R. Stuart for yeast strains and other help and advice.

REFERENCES

1. Pesole, G., Gissi, C., De Chirico, A., and Saccone, C. (1999) Nucleotide substitution rates of mammalian mitochondrial genomes. J. Mol. Evol. 48, 427–434
2. Marcelino, L. A., and Thilly, W. G. (1999) Mitochondrial mutagenesis in human cells and tissues. Mutat. Res. 434, 177–203
3. Wheeler, L. J., and Mathews, C. K. (2011) Nucleoside triphosphate pool asymmetry in mammalian mitochondria. J. Biol. Chem. 286, 16992–16996
4. Pursell, Z. F., McDonald, J. T., Mathews, C. K., and Kunkel, T. A. (2008) Trace amounts of 8-oxo-dGTP in mitochondrial dNTP pools reduce DNA polymerase \(\gamma\) replication fidelity. Nucleic Acids Res. 36, 2174–2181
5. Strand, M. K., Stuart, G. R., Longley, M. J., Graziewicz, M. A., Dominick, O. C., and Copeland, W. C. (2003) POS5 gene of Saccharomyces cerevisiae encodes a mitochondrial NAD kinase required for stability of mitochondrial DNA. Eukaryot. Cell 2, 809–820
6. Outten, C. E., and Culotta, V. C. (2003) A novel NAD kinase is the mitochondrial source of NADPH in Saccharomyces cerevisiae. EMBO J. 22, 2015–2024
7. Shi, F., Kawai, S., Mori, S., Kono, E., and Murata, K. (2005) Identification of ATP-NAD kinase isozymes and their contribution to supply of NADPH in Saccharomyces cerevisiae. FEBS J. 272, 3337–3349
8. Bieganowski, P., Seidle, H. F., Wojcik, M., and Brenner, C. (2006) Synthetic lethal and biochemical analyses of NAD and NAD kinase genes in Saccharomyces cerevisiae establish separation of cellular functions. J. Biol. Chem. 281, 22439–22445
9. Glick, B. S., and Pon, L. (1995) Isolation of highly purified mitochondria from Saccharomyces cerevisiae. Methods Enzymol. 260, 213–223
10. Mathews, C. K., and Wheeler, L. J. (2009) Measuring DNA precursor pools in mitochondria. Methods Mol. Biol. 554, 371–381
11. Passoneau, J. V., and Lowry, O. H. (1993) Enzymatic Analysis: A Practical Guide, Humana Press, Totowa, NJ
12. Lin, S. S., Manchester, J. K., and Gordon, J. I. (2001) Enhanced gluconeogenesis and increased energy storage as hallmarks of aging in Saccharomyces cerevisiae. J. Biol. Chem. 276, 36000–36007
13. Minard, K. I., and McAlister-Henn, L. (2005) Sources of NADPH in yeast vary with carbon source. J. Biol. Chem. 280, 39890–39896
14. Anderson, D. D., Quintero, C. M., and Stover, P. J. (2011) Identification of a de novo thymidylate biosynthesis pathway in mammalian mitochondria. Proc. Natl. Acad. Sci. U.S.A. 108, 15163–15168
15. Mathews, C. K., and Song, S. (2007) Maintaining precursor pools for mitochondrial DNA replication. PASEB J. 21, 2294–2303
16. Ferraro, P., Franzolin, E., Pontarin, G., Reichard, P., and Bianchi, V. (2010) Quantitation of cellular deoxyxynucleoside triphosphates. Nucleic Acids Res. 38, e85
17. Wheeler, L. J., Rajagopal, L., and Mathews, C. K. (2005) Stimulation of mutagenesis by proportional deoxyribonucleoside triphosphate accumulation in Escherichia coli. DNA Repair 4, 1450–1456
18. Gon, S., Napolitano, R., Rocha, W., Coulon, S., and Fuchs, R. P. (2011) Increase in dNTP pool size during the DNA damage response plays a key role in spontaneous and induced mutagenesis in Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 108, 19311–19316
19. Perrino, F. W., and Loeb, L. A. (1989) Differential extension of 3’ mispairs is a major contributor to the high fidelity of calf thymus DNA polymerase \(\alpha\). J. Biol. Chem. 264, 2898–2905
20. Mendelman, L. V., Petruska, J., and Goodman, M. F. (1990) Base mispair extension kinetics: comparison of DNA polymerase \(\alpha\) and reverse transcriptase. J. Biol. Chem. 265, 2388–2396
21. Bebenek, K., Roberts, J. D., and Kunkel, T. A. (1992) The effects of dNTP pool imbalance on frameshift fidelity during DNA replication. J. Biol. Chem. 267, 3589–3596