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

Linda J. Wheeler and Christopher K. Mathews[1]

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

*Running title: *Yeast mitochondrial dNTP and pyridine nucleotide pools*

[1]To whom correspondence should be addressed: Christopher K. Mathews, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331-7305, 2011 Agricultural and Life Sciences Building, Corvallis, OR 97331-7305, USA, Tel.: (541) 737-1865; Fax: (541) 737-0481; E-mail: mathewsc@onid.orst.edu

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Background: Mutation in yeast gene *POS5* encoding 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 Δ*pos5* mutagenesis.
Significance: Understanding intramitochondrial dNTP and pyridine nucleotide levels contributes to understanding mitochondrial mutagenesis.

SUMMARY

***Saccharomyces cerevisiae* contains three NAD^+/NADH kinases, one of which is localized in mitochondria and phosphorylates NADH in preference to NAD^+. Strand et al (*Euk. Cell* 2:809 (2003)) reported that a yeast mutation in *POS5*, which encodes the mitochondrial NADH kinase, is a mutator, specific for mitochondrial genes. Because of the involvement of NADPH in deoxyribonucleotide biosynthesis, we asked whether mitochondria in a *pos5* deletion mutant contain abnormal deoxyribonucleoside triphosphate pools. We found pools of the four dNTPs[2] to be more than doubled in mutant mitochondrial extracts relative to wild-type. 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 levels of NADPH to be diminished about fourfold 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 one to two orders of magnitude higher than the nuclear gene mutation rate (1,2). In previous investigations with rat tissue mitochondria we have identified aspects of DNA precursor metabolism that probably contribute toward this difference—first, a strikingly asymmetric distribution of deoxyribonucleoside triphosphates (dNTPs) within the mitochondrion (3), and second, 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 fiftyfold, with no significant effect upon the nuclear marker tested. *POS5* was shown to encode an NADH/NAD^+ kinase, localized to mitochondria and preferentially active upon NADH. Several other laboratories have confirmed the mitochondrial location of POS5p and its preference for NADH as a substrate (6,7,8).

NADPH, the product of the NADH kinase reaction, is involved in dNTP biosynthesis in at least two ways—first, as the ultimate electron donor supporting the ribonucleotide reductase reaction, and second, 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. *Saccharomyces cerevisiae* strain YPH925 and a YPH925 derivative carrying a *pos5* deletion mutation are described in reference 5. These strains were supplied by Dr. Gregory R. Stuart in the laboratory of Dr. William C. Copeland, National Institute of Environmental Health Sciences. The strains are referred to in this paper as wild-type (WT) and *POS5*, respectively. We thank Dr. Stuart for advice in growing and handling these strains.

Growth of yeast and isolation of mitochondria. A single yeast colony was streaked and transferred to YPD (rich dextrose) medium (5). When grown to saturation, the culture was diluted one hundredfold into lactate medium (3 g/l yeast extract, 0.5 g/l glucose, 0.5 g/l $CaCl_2 \cdot 2H_2O$, 0.5 g/l NaCl, 0.9 g/l $MgCl_2 \cdot 6H_2O$, 1.0 g/l KH_2PO_4, 4 g/l NH_4Cl, 24 ml 85% DL-lactic acid, and 8 g/l NaOH, pH adjusted to 5.5 with NaOH). Growth with aeration was carried out overnight (about 15 hours) at 30^o, 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^o C. Cells were lysed and mitochondria 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^o C, with occasional mixing, for about one hour. The extracts were next heated for 3 minutes in a boiling water bath, then centrifuged at 17,000xg for 15 minutes. 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).

rNTP analysis. Ribonucleoside triphosphate 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 in reference 3.

Pyridine nucleotide analysis. Pyridine nucleotides were analyzed by cycling reactions, as described originally by Passoneau and Lowry (11), 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/1 mM EDTA, 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 minutes at 60^o to destroy the other components (NADPH and NADH in the acid extract, $NADP^+$ and NAD^+ in the alkaline extract). The samples were neutralized with either 25 µl 0.1 M Tris-HCl, pH 8.1, and 25 µl 0.05 M HCl for the alkaline extract or 50 µl 0.4 M Tris base for the acidic extract.

The cytosolic fraction, 250 µl, was mixed with 250 µl 0.1 M NaOH/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 minutes at 60^o as described in the previous paragraph. Extracts were neutralized with either 50 µl of 0.2 M Tris-HCl, pH 8.1, and 50 µl of 0.1 M HCl for the alkaline extract or 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. The alcohol dehydrogenase was Sigma grade A-3263, offered especially for this purpose.

Measuring $NADP^+$ and NADPH. 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 minutes at 37^o, following which the mixture was heated at 100^o for 5 minutes to stop the reaction, and then centrifuged to remove precipitated proteins.

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, 2mM $MgCl_2$, 0.1 mM EDTA, 0.3 mM $NADP^+$, and 0.3 unit per reaction 6-phosphogluconate dehydrogenase). Incubation was carried out for 20 minutes at room temperature, and the NADPH formed was measured at 340 nm.

Measuring NAD^+ and NADH. Cycling reaction: 5 µl of sample or standard (0–5 pmol of NAD^+) 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 minutes at room temperature, following which the mixture was heated at 100^o for 5 minutes to stop the reactions, then centrifuged to remove precipitated proteins.

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 aminomethylpropanol, pH 9.9, 5 mM potassium glutamate, 0.3 mM NAD^+, 6 units per reaction malate dehydrogenase, and 1 unit per reaction glutamate oxaloacetate aminotransferase). Incubation was carried out for 10 minutes 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 (15) support the existence of a mitochondrial ribonucleotide reductase. Comparable experiments have not been done with yeast.

When we analyzed yeast mitochondrial extracts, we did see an effect of the *pos5* mutation on dNTP pools. As shown in Figure 1, the dNTP pools are more than twice as high in Δ*pos5* mitochondrial extracts as in wild-type mitochondria (all four p-values less than 0.02). No such effect was seen in measurement of the cytosolic dNTP pools (Figure 2), where the dNTP levels in the postmitochondrial supernatant were roughly equal when wild-type and *pos5* mutant extracts were compared. The variability of the cytosolic dNTP measurements was considerably higher than that for the mitochondrial measurements recorded in Figure 1. We don't know the reason for this difference, but the critical mitochondrial measurements do support an approximate doubling of dNTP pools in mutant relative to wild-type mitochondria, and the data of Figure 2 are consistent with the conclusion that cytosolic pools do not differ significantly between mutant and wild-type 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 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 artefact to be a significant source of error only at rNTP/dNTP ratios greater than 100. However, to determine whether our dGTP or dCTP levels were overestimated, we measured ribonucleoside triphosphate pools in extracts of both mutant and wild-type mitochondria (Table I). From these values and from the dNTP pools recorded in Figure 1, we estimate the [GTP]/[dGTP] ratios to be 143 and 105, respectively, for wild-type and mutant mitochondria, and the [CTP]/[dCTP] ratios to be 126 and 89, respectively, for wild-type and mutant mitochondria. 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, while dCTP may be overestimated by about 30 percent in both mitochondrial extracts. Because dATP and dTTP measurements are not affected by this artefact, and dGTP only minimally, the general conclusion that *pos5* mutant mitochondrial dNTP levels are about twice those in wild-type 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 Figure 1 show that in wild-type 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 wild-type 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 (17) that small uniform increases in dNTP concentrations have a disproportionate effect in stimulating mutagenesis. In *Escherichia coli* a twofold expansion of dNTP pools caused by ribonucleotide reductase overexpression was correlated with a thirtyfold increase in frequency of mutation to rifampicin resistance (see Table 3 of reference 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 for extension from a matched 3' terminus. For example, Perrino and Loeb (19) reported K_M for extension 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, while K_M for extension from mismatched termini ranged from 22 to 4760 μM (20). More such data are reviewed in reference 17.

In rat liver our measurements of mitochondrial dNTP pools ranged from 1.5 to 18 pmol/mg protein, corresponding to concentrations of about 2 to 20 μM (3). If the corresponding values for yeast mitochondrial dNTPs are comparable, we can estimate that the mitochondrial replisome *in vivo* 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 proofreading exonuclease is not directly affected by dNTP concentration, then the effect of doubling the mismatch extension rate is to approximately double the replication error rate, with a corresponding increase in mutagenesis. This is illustrated schematically in Figure 3 for a hypothetical replisome with an average K_M of 1 μM for chain extension from a matched terminus and 1 mM for extension from a mismatch–values close to those cited in the previous paragraph. Under these conditions, doubling the dNTP concentration will double the rate of extension from a mismatch, with no effect on correct chain extension, which is already operating at V_{max}.

Each of the four dNTP pools is doubled, or somewhat more, as a result of the *pos5* mutation, and the effects on replication error frequency should be additive, because error-prone extension from the mismatches results from independent events. Hence, as a rough approximation, doubling each of the four dNTP pools could account for an eightfold increase in mutation rate. The data of Strand et al (5) indicate that the *pos5* mutation stimulated mutagenesis by fiftyfold, suggesting that the dNTP pool size changes shown here can account for some, but not all, of the mutagenesis observed.

However, the above scenario accounts for substitution mutations, whereas the *ARG8* reversion assay used by Strand et al (5) scored for –1 frameshifts. On the other hand Bebenek *et al* (21) have shown that a misincorporated nucleotide can stimulate –1 frameshift mutations when a mismatched nucleotide recognizes a correct complementary nucleotide to the 5' side in the template DNA, for example, as shown in scheme 1, where ***G*** represents an initially mismatched dGMP residue. Such a process could be favored by dNTP imbalances, and Bebenek *et al* showed that in vitro dNTP pool imbalances promoted –1 frameshifts. For example in the Scheme 1 scenario, dCTP excess would promote chain

extension from the initially mismatched ***G***. Whether such events are forced by proportional dNTP accumulations is not known, and to this end it would be valuable to know whether the *pos5* deletion promotes base substitution mutagenesis as well as frameshifting.

Pyridine nucleotide pools. In a semi-quantitative 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 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 Figure 4, that is what we observed. Wild-type mitochondria contained about four times as much NADPH as *pos5* mutant mitochondria, and about 2.5 times as much $NADP^+$.

The standard deviation for the NADPH data in wild-type mitochondria was much higher than the data for mutant mitochondria. The p-value from comparing the two measurements was 0.12, which doesn't provide strong assurance that the two average values are statistically different. However, in five separate experiments the wild-type 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 in wild-type mitochondria. The data for $NADP^+$ measurements were more robust, with a p-value <0.01 indicating that the difference between the *POS5* and wild-type values is significant.

Standard deviations were higher in the NADH and NAD^+ assays than in those for NADPH and $NADP^+$. NADH might well be expected to accumulate when NADH kinase activity is missing, and the NADH measurements in Figure 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 standard deviations for the NADH and NAD^+ assays were unacceptable and those values are not shown here, but the data of Figure 5 suggest that the *pos5* mutation does not affect pyridine nucleotide levels in 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, nor are cytosolic NAD^+/NADH kinases 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 depeletion. 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 deoxyribonucleoside triphosphates.

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FOOTNOTES

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[1] To whom correspondence should be addressed: 2011 Agricultural and Life Sciences Building, Oregon State University, Corvallis, OR 97331-7305. Fax: 541-737-0481; E-mail: mathewsc@onid.orst.edu

[2] The abbreviations used are: dNTP, deoxyribonucleoside 5'-triphosphate; rNTP, ribonucleoside 5'-triphosphate.

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FIGURE LEGENDS

Figure 1. Yeast mitochondrial dNTP pools. Data represent averages of six independent experiments ± standard deviation.

Figure 2. Yeast cytosolic dNTP pools. Data represent averages from six independent experiments ± standard deviation.

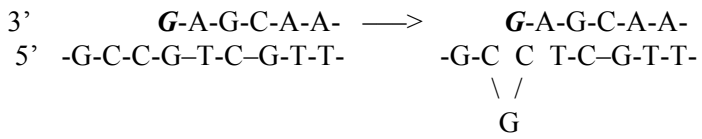
Figure 3. Substrate saturation curves for a hypothetical, but realistic DNA polymerase with a K_M of 1 μM for chain extension from a properly base-paired 3' terminus and 1000 μM for extension from a mismatched 3' terminus. Kinetic data for extension from properly and improperly matched termini are compiled in reference 17.

Figure 4. Yeast mitochondrial pyridine nucleotide pools. Data represent averages from five independent experiments ± standard deviation.

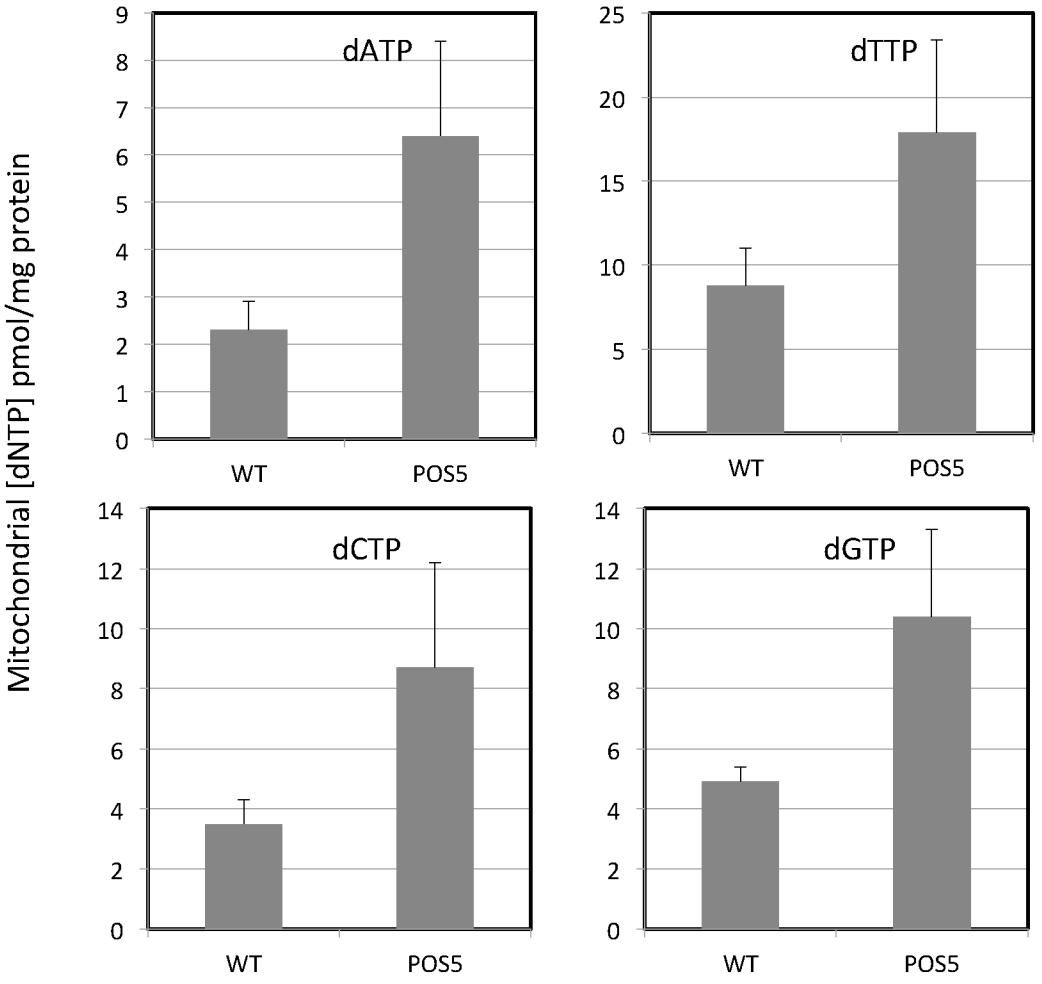
Figure 5. Yeast cytosolic NADPH and $NADP^+$ pools. Data represent averages from four independent experiments ± standard deviation.

Table I. Mitochondrial Ribonucleoside Triphosphate Pools

Yeast strain	Mitochondrial rNTP pool, nmol/mg protein			
	ATP	CTP	GTP	UTP
Wild-type	1.52	0.44	0.70	0.77
pos5 mutant	2.33	0.66	1.09	1.40



Scheme 1


Mitochondrial [dNTP] pmol/mg protein
dATP
dTTP
dCTP
dGTP
WT
POS5
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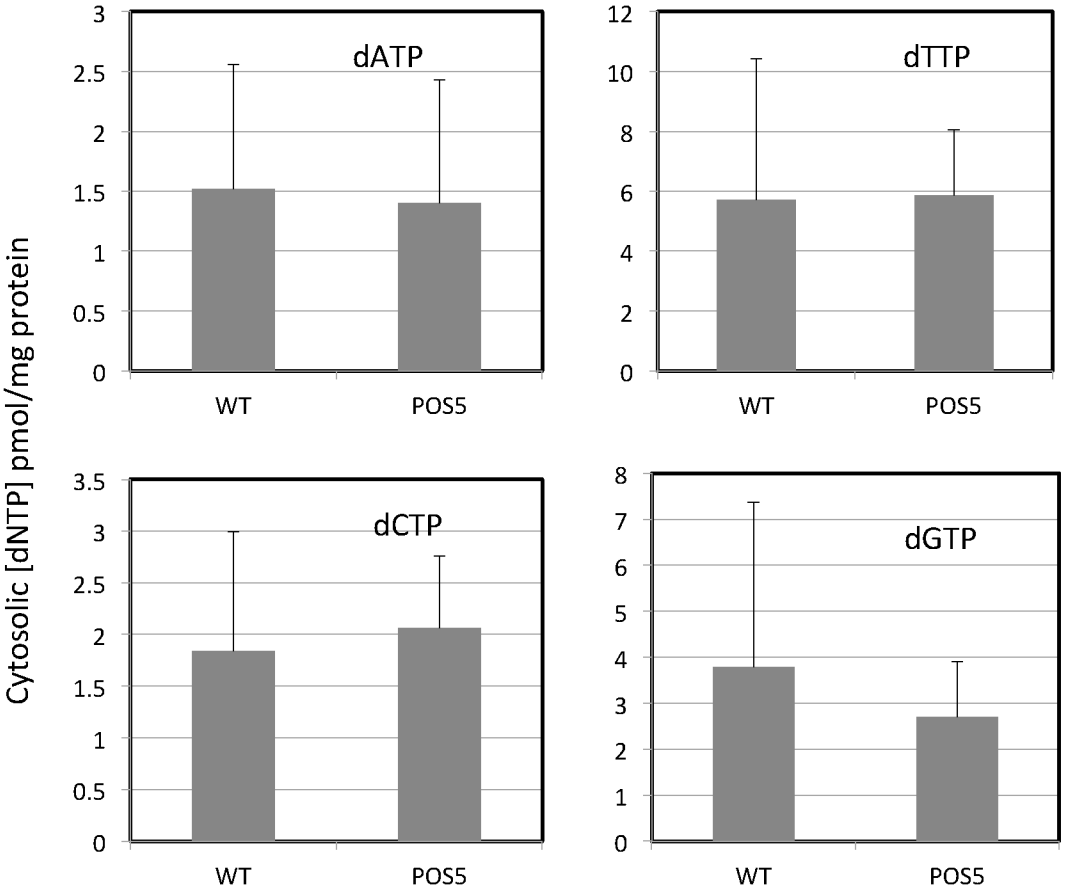
1
2
3
4
5
6
7
8
9
10
12
14
15
20
25

Figure 1


Cytosolic [dNTP] pmol/mg protein
dATP
dTTP
dCTP
dGTP
WT
POS5
0
0.5
1
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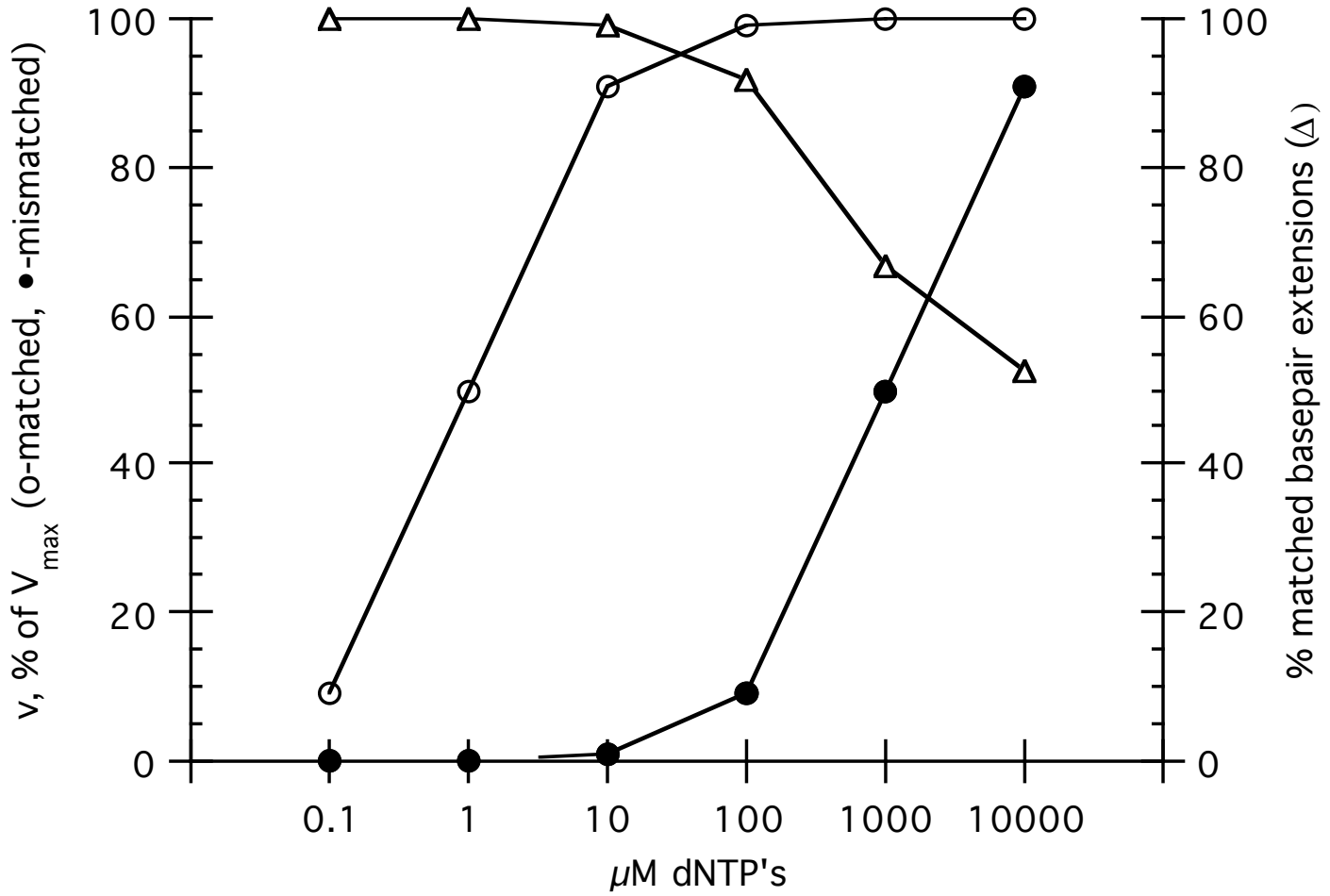
2
2.5
3
3.5
4
5
6
7
8
10
12

Figure 2

Figure 3


v, % of V_{max} (o-matched, •-mismatched)
% matched basepair extensions (Δ)
100
80
60
40
20
0
0.1
1
10
100
1000
10000
μM dNTP's

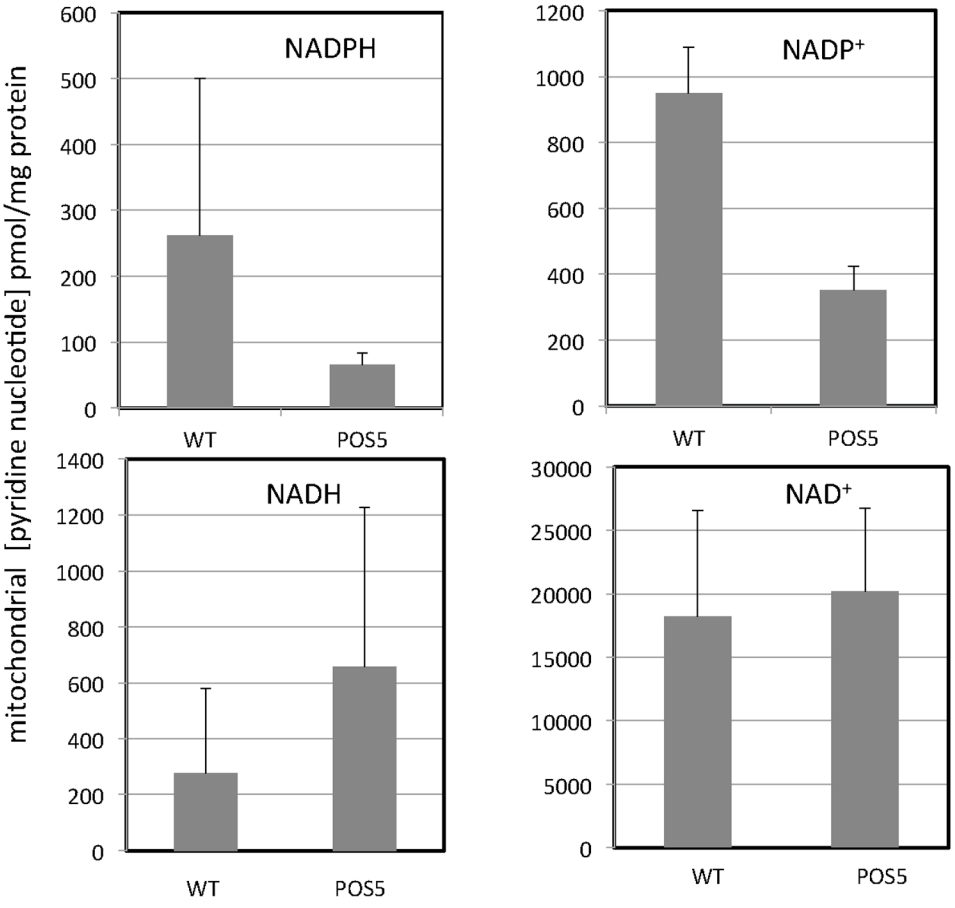
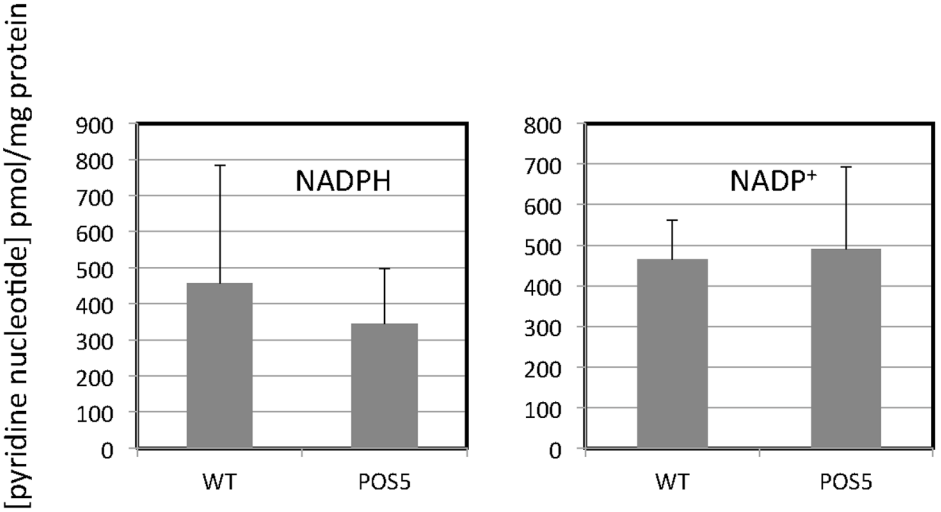

mitochondrial [pyridine nucleotide] pmol/mg protein
NADPH
600
500
400
300
200
100
0
WT
POS5
NADP+
1200
1000
800
600
400
200
0
WT
POS5
NADH
1400
1200
1000
800
600
400
200
0
WT
POS5
NAD+
30000
25000
20000
15000
10000
5000
0
WT
POS5

Figure 4

Figure 5


[pyridine nucleotide] pmol/mg protein
900
800
700
600
500
400
300
200
100
0
NADPH
WT
POS5
800
700
600
500
400
300
200
100
0
NADP+
WT
POS5