SYNTHESIS OF PYRIDINE NUCLEOTIDES BY MITOCHONDRIAL FRACTIONS OF YEAST

by

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SUMMARY

Washed mitochondrial fractions from yeast catalyze the synthesis of nicotinate mononucleotide and nicotinate adenine dinucleotide from nicotinate or nicotinamide. The synthesis is stimulated 10 fold by sonic disruption of the fractions and shows an absolute requirement for ATP, 5-phosphoribosyl-1-pyrophosphate, and $MgCl_2$.

INTRODUCTION

The impermeability of the yeast inner mitochondrial membrane to pyridine nucleotides (1) raises the question of the origin of the mitochondrial pools of pyridine nucleotide. Recently Grunicke et al. (2) presented evidence for the intramitochondrial synthesis of NAD^+ from nicotinamide in isolated, intact preparations of rat liver mitochondria but were unable to detect pyridine nucleotide synthesis in sonically disrupted preparations. We describe here the synthesis of pyridine nucleotides from nicotinate and nicotinamide in sonically disrupted mitochondrial fractions of yeast.

MATERIALS AND METHODS

Saccharomyces cerevisiae was grown, converted to spheroplasts, and a mitochondrial fraction was isolated as described previously (3) with the following modifications: (i) Following Glusulase treatment, the spheroplasts were washed with 20 mM KH_2PO_4-K_2HPO_4, pH 6.8, 1.5 M sorbitol, 1 mM EDTA. (ii) Rather than utilizing a Waring blendor to aid lysis, the spheroplasts were lysed by gentle stirring for 15 to 30 min at 0-4°C in 20 mM KH_2PO_4-K_2HPO_4 pH 6.8, 250 mM sucrose, 1 mM EDTA. We have found that even gentle

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Waring blending converts a sizeable fraction of the total DNA to a form that does not sediment at either 1000g or 10,000g, indicating nuclear fragmentation. With the present methods of lysis, over 95% of the total DNA sediments at 1000g. (iii) The isolated mitochondrial fraction was washed 5 times to minimize cross contamination with soluble cellular components.

For analysis of pyridine nucleotide synthesis, mitochondrial fractions were incubated for 20 min at 30°C in a medium (complete incubation mixture) containing 111 mM Tris (H_3PO_4) pH 8.4, 4.4 mM ATP, 22 mM $MgCl_2$, 1.5 mM PRPP*, an ATP regenerating system containing 22 mM phosphoenol pyruvate, 22 mM KCl, and 100 µg/ml pyruvate kinase, and either 8 µM [^{3}H] nicotinate (2.2 x 10^8 cpm/µmole) or 15 µM [^{14}C] nicotinamide (1.3 x 10^8 cpm/µmole). Portions of the incubation mixtures were fractionated by chromatography on paper or thin-layers of microcrystalline cellulose. Authentic carrier compounds were co-chromatographed with samples containing radioactivity. Development in n-butanol-acetic acid-water (4/1/2) was used to separate pyridine bases (nicotinate, nicotinamide) from pyridine mononucleotides (NMN, NaMN) and pyridine dinucleotides (NAD^+, $NaAD^+$). The two-dimensional system of Kahn and Blum (4) was used to separate nicotinate, nicotinamide, NMN, NaMN, NAD^+, and $NaAD^+$. Radioactivity migrating with authentic carrier compounds was quantified by liquid scintillation counting.

RESULTS AND DISCUSSION

Washed mitochondrial fractions from yeast were incubated either intact or after sonic disruption in the complete incubation mixture containing 8 µM [^{3}H] nicotinate and portions were examined by paper chromatography for conversion into pyridine mononucleotides or dinucleotides (Table I). Incubation of intact mitochondrial fractions resulted in detectable conversion of nicotinate into both pyridine mononucleotides and dinucleotides. However, sonic disruption of the fractions resulted in an approximately 10 fold increase of incorporation into both mononucleotides and dinucleotides. The lower activity in the non-disrupted fraction is probably the result of permeability barriers to one or more of the substrates which suggests that the activity observed is indeed associated with the particulate fractions and does not represent adhering cytoplasmic contamination. Analysis of the pyridine mononucleotides and dinucleotides by two-dimensional thin-layer chromatography revealed that the radioactivity was located exclusively in the nicotinate derivatives (NaMN and $NaAD^+$). The incorporation observed in the sonically disrupted fractions represented the formation of 2.6 nmol/mg protein of NaMN and 0.5

*Nonstandard abbreviations used: PRPP, 5-phosphoribosyl-1-pyrophosphate; NMN, nicotinamide mononucleotide; NaMN, nicotinate mononucleotide, $NaAD^+$, nicotinate adenine dinucleotide, oxidized form.

Table I

Pyridine Nucleotide Synthesis in Mitochondrial Fractions

Incubation Conditions	Conversion of [^{3}H] nicotinate into Pyridine Mononucleotides	Pyridine Dinucleotides
	CPM	CPM
No incubation	104	73
20 min, 30°C		
minus sonic disruption	912	253
plus sonic disruption	11,399	2,102

The incubation mixtures (100 µl, 1.0 mg protein/ml), containing mitochondrial fractions, were incubated in the complete incubation mixture and 20 µl portions containing approximately 35,000 total cpm were subjected to chromatographic analysis.

nmol/mg protein of $NaAD^+$. The requirements for the *in vitro* synthesis of pyridine nucleotides are shown in Table II. The formation of both NaMN and $NaAD^+$ from nicotinate showed an absolute requirement for 5-phosphoribosyl-1-pyrophosphate, ATP, and $MgCl_2$, and the synthesis was stimulated by the presence of the ATP regenerating system. These data strongly suggest that the conversion of radioactivity represented net pyridine nucleotide synthesis rather than exchange reactions. Results similar to those shown in Table I and II were observed when [^{3}H] nicotinate was replaced by [^{14}C] nicotinamide, and it was observed that the extracts rapidly converted nicotinamide into nicotinate.

These observations represent the first reported evidence for pyridine nucleotide biosynthesis from pyridine ring precursors in yeast mitochondria. Previously Griffiths and Bernofsky (5) have demonstrated that yeast mitochondria contain a unique isozyme of NAD kinase that catalyzes the synthesis

Table II

Requirements for Pyridine Nucleotide Synthesis

Incubation Conditions	Incubation Mixture	Conversion of [^{3}H] nicotinate into Pyridine Mononucleotides	Pyridine Dinucleotides
		CPM	CPM
No incubation	Complete	227	79
20 min, 30°C	Complete	10,564	1,992
	Complete minus ATP	208	63
	Complete minus PRPP	177	95
	Complete minus $MgCl_2$	78	61
	Complete minus ATP regenerating system	6,255	679

The incubation mixtures (100 µl, 1.0 mg protein/ml) contained sonically-disrupted mitochondrial fractions and 20 µl portions containing approximately 35,000 total cpm were subjected to chromatographic analysis.

of NADPH from NADH. The results obtained here with yeast mitochondrial fractions differ from the previously reported synthesis of pyridine nucleotides in rat liver mitochondria (2). The rat liver preparations utilized nicotinamide but not nicotinate for NAD^+ formation, and activity was not detected in sonically disrupted fractions. In contrast, the yeast preparations utilized either nicotinamide or nicotinate and the synthesis of nicotinate pyridine nucleotides was observed. In two experiments in which glutamine was included in the incubation mixture, the formation of NAD^+ was not detected.

Yeast mitochondria are known to contain many metabolic capabilities distinct from those of the cytoplasm, and these studies suggest that this may be the case for the synthesis of pyridine nucleotides. However, additional studies will be needed to assess the significance of the activities observed here in mitochondrial pyridine nucleotide metabolism.

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