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$NADP^+$ PHOSPHATASE: A NOVEL MITOCHONDRIAL ENZYME

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Mitochondria contain a $NADP^+$ phosphatase in the matrix space. This is shown by both incubation of mitochondria and subfractions derived thereof with added $NADP^+$ and by analysis of endogenous pyridine nucleotides after enzymatic oxidation in Ca^{2+}-loaded mitochondria. The apparent K_M for $NADP^+$ is about 1.2 mM. NADPH is not a substrate. The enzyme may be important for modulation of posttranslational modification of macromolecules in mitochondria.
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Pyridine nucleotides are very versatile coenzymes. NAD serves not only as a prosthetic group of redox enzymes but also as an ADP-ribosylating (1) or adenylylating (2) agent in covalent modification of macromolecules. It also participates in the urocanase reaction with no apparent redox change (3). Unlike NAD, NADP serves mainly redox reactions. The only exceptions are the recently described covalent protein modification reactions by NADP-dependent phospho-ADP-ribosylation and 2'-phosphoadenylylation (4, 5).

In mitochondria, pyridine nucleotides are indispensable for fatty acid β-oxidation, dehydrogenation, the citric acid cycle, hydroperoxide reduction, and the respiratory chain. Recently, they received new attention since NAD^+ serves as substrate for mitochondrial protein ADP-ribosylation (4, 6, 7) which may regulate Ca^{2+} efflux from mitochondria (8).

Since the cytosolic and mitochondrial pools of pyridine nucleo-

Abbreviations: EGTA, ethylene glycol bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid
Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
MSH, 210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.4
SMP, submitochondrial particles.

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tides are not readily exchangeable it is generally assumed that mitochondria possess independent metabolic pathways for pyridine nucleotides. Whereas the metabolism of cytosolic pyridine nucleotides has been investigated in detail, very little is known about the biosynthesis of the mitochondrial coenzymes. Intramitochondrial hydrolysis of pyridine nucleotides at the β-N-glycosidic bond linking nicotinamide and ADP-ribose(phosphate) has been reported (9) and a NAD^+ glycohydrolase has been isolated from mitochondria and characterized (10). We now report the existence of a $NADP^+$ phosphatase in the mitochondrial matrix.

MATERIALS AND METHODS

Rat liver mitochondria, mitoplasts, SMP and the mitochondrial matrix fraction were isolated by conventional procedures as described by Loetscher et al. (9). In some experiments mitochondria were isolated by Percoll gradient centrifugation (11). When indicated, the matrix fraction was also obtained by ultracentrifugation of frozen and thawed mitoplasts. The content of mitochondrial pyridine nucleotides was determined according to Frei et al. (12). O_2 uptake was measured in a Clark-type electrode at 37°C with 2 mg protein/ml in MSH buffer. $NADP^+$ phosphatase activity was determined with 3-6 mg protein/ml in MSH at 37°C and 3 mM $NADP^+$, unless indicated otherwise. At time intervals aliquots were withdrawn and assayed for NAD^+ as described for intramitochondrial pyridine nucleotides (12). Inorganic phosphate was determined according to Lanzetta et al. (13). For the determination of intramitochondrial pyridine nucleotides in the presence of Ca^{2+} and t-butylhydroperoxide, the standard incubation procedure outlined in ref. 12 was used. Protein was determined by the biuret method with bovine serum albumin as the standard. Reagents were of the highest quality commercially available.

RESULTS

Rat liver mitochondria or mitoplasts (mitochondria freed of the outer membrane by digitonin treatment) hydrolyze $NADP^+$ to inorganic phosphate (not shown) and NAD^+. With 3 mM $NADP^+$ at 37° C mitochondria and intact mitoplasts (see below) produce 1.46 and 0.95 nmol NAD^+/min per mg of protein, respectively. NAD^+ production is linear for at least 10 min, dependent on the presence of $NADP^+$, slower at 25°C than at 37°C, and sensitive to boiling of mitochondria or mitoplasts. Fractionation of sonicated or frozen/thawed mitoplasts by ultracentrifugation into inner membrane and matrix fraction leads to a complete recovery of $NADP^+$ phosphatase activity in the matrix (specific activity: 1.37 nmol NAD^+/min per mg of protein). The same results were obtained with mitochondria purified on a Percoll gradient.

TABLE I

Content of Mitochondrial Pyridine Nucleotides in the Presence of Ca^{2+} and t-butylhydroperoxide[a]

	time	
	0 min	9 min
pyridine nucleotide	nmol/mg of protein	
NADH	1.97	0.12
NADPH	3.57	1.12
NAD^+	0.91	3.50
$NADP^+$	0.00	1.86
NAD^+ + NADH	2.88	3.62
$NADP^+$ + NADPH	3.57	2.98
total content	6.45	6.60

[a]Mitochondria were incubated according to the standard procedure (12) and loaded with 63 nmol of Ca^{2+}/mg of protein. Immediately before (time 0 min), and 9 min after addition of 100 μM *t*-butylhydroperoxide pyridine nucleotides were extracted and analyzed as described in the *Methods* section.

t-Butylhydroperoxide causes oxidation and hydrolysis of pyridine nucleotides in Ca^{2+}-loaded mitochondria (9, 12, 14). With moderate Ca^{2+} loads hydrolysis is virtually complete after 8 min as judged from nicotinamide release whereas at this time the total content of intramitochondrial pyridine nucleotides amounts to about 80% of the initial value indicating rapid resynthesis (12). At lower Ca^{2+} loads (63 nmol of Ca^{2+}/mg of protein) no net loss of pyridine nucleotides is observed after completion of Ca^{2+} release (Table I). However, there is an increase in the amount of NAD(H) at the expense of NAD(P)(H).

When mitochondria or mitoplasts are incubated at 2 mg of protein/ml and 37°C, addition of 3 mM NADH causes consumption of 9 nmol O_2/min per mg of protein. O_2 consumption increases upon subsequent addition of 5 mM succinate and is further stimulated transiently by 200 μM Ca^{2+} demonstrating intactness of the organelles.

The dependence on $NADP^+$ concentration of the phosphatase was studied. The apparent K_M values are 3 mM for intact and 1.5 mM for frozen/thawed mitoplasts or the matrix fraction. V_{max} values of mitoplasts and matrix are 1.1 and 2.4 nmol NAD^+/min and mg of protein, respectively. At $NADP^+$ concentrations below 1 mM intact mitoplasts have a lower activity than broken mitoplasts or the matrix fraction indicating restricted substrate availability in the former situation.

DISCUSSION

Here we report the existence of a $NADP^+$ phosphatase in the mitochondrial matrix fraction. The localization is inferred from three observations: i) The activity of mitoplasts is completely recovered in the supernatant after freezing and thawing or sonication and subsequent ultracentrifugation. ii) At low $NADP^+$ concentration the activity is higher with frozen/thawed than with intact mitoplasts. iii) Intramitochondrial NAD(P)(H) is converted to NAD(H) upon exposure of mitochondria to Ca^{2+} and t-butylhydroperoxide.

Mitochondria isolated by conventional centrifugation or purified by gradient centrifugation have a higher $NADP^+$ phosphatase activity than mitoplasts. This may be due to contamination by extramitochondrial enzyme(s) or the presence of another mitochondrial enzyme in the intermembrane space or outer membrane. We favour the second possibility since mitochondria isolated by either method have the same specific activity and mitoplasts have a lower specific activity than mitochondria.

Added pyridine nucleotides do not easily enter mitochondria or mitoplasts. Since the phosphatase assays were routinely done with 3 mM $NADP^+$ we measured O_2 consumption with 3 mM NADH. We calculate that at least about 4.5 nmol NADH/min and mg of protein have access to the mitochondrial interior. Assuming a similar penetration rate for $NADP^+$ as for NADH the accessibility of $NADP^+$ does not limit phosphatase activity at this concentration in intact coupled mitoplasts. However, as shown above, at low $NADP^+$ concentrations accessibility can become rate limiting.

The function of the mitochondrial $NADP^+$ phosphatase is presently not known. It may simply participate in the catabolism of NADP and/or in more complex processes like protein ADP-ribosylation.

Further work is necessary to obtain detailed information about this novel mitochondrial enzyme.

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