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OXIDATION OF REDUCED NICOTINAMIDE HYPOXANTHINE DINUCLEOTIDE PHOSPHATE BY INTACT RAT LIVER MITOCHONDRIA

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SUMMARY

1. While intact rat liver mitochondria oxidize external NADPH at an extremely low rate (0.1–0.4 nmole per mg protein per min), reduced nicotinamide hypoxanthine dinucleotide phosphate (NHDPH) is relatively rapidly oxidized (12–13 nmoles per mg protein per min).

2. After sonication, the rate of mitochondrial NHDPH oxidation is increased by 2-fold, and the NADPH oxidation rate is double that of NHDPH.

3. NADPH and NHDPH oxidation is neither coupled to ATP synthesis nor inhibited by rotenone or CN^-, but is inhibited under anaerobic conditions. These data indicate that, although molecular oxygen is the ultimate electron acceptor, the respiratory chain is not functional in the oxidation reaction.

4. The NADPH oxidase activity resides in the submitochondrial particle fraction isolated from sonicated mitochondrial preparations.

5. These results are interpreted to indicate the presence of an NADPH oxidase system in rat liver mitochondria which is bound to the inner surface of the inner membrane and independent of the respiratory chain. Further, it is concluded that the inner membrane may be significantly more permeable to NHDPH than to NADPH.

INTRODUCTION

We recently reported the rapid oxidation of external reduced nicotinamide hypoxanthine dinucleotide (NHDH) by rat liver mitochondria which oxidized NADH at a very low rate[1]. The slow rate of mitochondrial NADH oxidation is generally believed to result from the low permeability of the inner membrane to pyridine dinucleotides[2–6]. Thus, the intramitochondrial respiratory chain-linked NADH dehydrogenase is effectively separated from external NADH. NHDH oxidation was inhibited by rotenone as well as by CN^- and coupled to ATP synthesis ($P:2e^-$ ratio = 3)[1]. It was concluded that the primary electron acceptor for NHDH is the intramitochondrial NADH dehydrogenase. The much more facile oxidation

Abbreviations: NHD^+, oxidized nicotinamide hypoxanthine dinucleotide; NHDH, reduced nicotinamide hypoxanthine dinucleotide; $NHDP^+$, oxidized nicotinamide hypoxanthine dinucleotide phosphate; NHDPH, reduced nicotinamide hypoxanthine dinucleotide phosphate.

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of NHDH than NADH may be attributed to a greater permeability of the inner membrane to the former coenzyme. NADPH and NADH inhibited, while $NADP^+$ stimulated NHDPH oxidation by intact mitochondria. Hence, a carrier-mediated mechanism for NHDH transport into the matrix was proposed[1]. As an extension of these studies on NHDH, we report here on the oxidation of NADPH and reduced nicotinamide hypoxanthine dinucleotide phosphate (NHDPH) by intact and disrupted rat liver mitochondria.

MATERIALS AND METHODS

Mitochondria were prepared from the livers of Sprague–Dawley rats, weighing 375–400 g, by the procedure of Johnson and Lardy[7]. Disrupted mitochondria were prepared by sonication as previously described[1]. To prepare the submitochondrial particles, sonicated mitochondria were centrifuged 7 min at $25000 \times g$. Being careful to omit the white surface layer, the supernatant solution was decanted and centrifuged at $165000 \times g$ for 30 min. The supernatant solution, *i.e.* the soluble fraction, was decanted, and the submitochondrial pellet was suspended in 0.25 M sucrose. Protein concentration was determined by the biuret method[8] and standardized with bovine serum albumin.

Mitochondrial oxidation of NADPH or NHDPH was performed in an incubation medium (1.4 ml) at 25 °C containing: 57 mM potassium phosphate buffer (pH 7.5), 171 mM KCl, 2.3 mM EDTA, 0.86 mM $MgCl_2$ and given concentrations of pyridine dinucleotides. Reactions were initiated by the addition of mitochondria. The reactions were terminated by addition of 0.2 ml of 1 M KOH, placed on ice for 5 min and then adjusted to pH 8 with 0.16 ml of 1 M acetic acid. Precipitated protein was removed by centrifugation at $105000 \times g$ for 10 min and an appropriate aliquot of the supernatant solution was diluted to 1 ml with water. The NADPH or NHDPH content of the diluted aliquots was determined by the addition of 0.01 ml of 100 mM oxidized glutathione and 0.4 units of glutathione reductase (EC 1.6.4.2) contained in 0.002 ml. The change in the absorbance of the sample at 340 nm was used to calculate NADPH or NHDPH concentrations assuming an extinction coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$ (ref. 9).

NADPH and $NADP^+$ were obtained from P–L Biochemicals. Sigma Chemical Co. provided the rotenone, oxidized glutathione, glucose 6-phosphate and enzymes. All other chemicals were reagent grade of commercial origin. $NHDP^+$ was prepared from $NADP^+$ by the method of Kaplan *et al.*[10], and reduced using glucose 6-phosphate and Torula yeast glucose 6-phosphate dehydrogenase (EC 1.1.1.49). NHDPH

$$NADPH + NAD^+ \rightleftharpoons NADP^+ + NADH \quad (1)$$

was separated from the dehydrogenase by column chromatography on Sephadex G-25, lyophilized to dryness and stored at −20 °C.

RESULTS AND DISCUSSION

Mammalian mitochondrial pyridine dinucleotide transhydrogenase catalyzes a reversible oxidation–reduction reaction between intramitochondrial NAD^+ and NADPH (Eqn 1)[11,12]. Since NADH dehydrogenase does not oxidize NADPH

directly[13], this transhydrogenase reaction provides a means by which endogenous NADPH may be oxidized by the respiratory chain. Having shown that intact rat liver mitochondria rapidly oxidize external NHDH but not NADH[1], it was of interest to examine whether the inner membrane may also be more permeable to NHDPH than to NADPH. If this were the case, it was anticipated that mitochondrial oxidation of external NHDPH would be more rapid than NADPH oxidation and that this oxidation would be coupled to the respiratory chain by the transhydrogenase catalyzed reduction of endogenous NAD^+. In Table I (Expt 1), the rates of NADPH and NHDPH oxidation by intact and sonicated mitochondria are compared. As

TABLE I

EFFECT OF RESPIRATORY INHIBITORS ON NADPH AND NHDPH OXIDATION

In Expt 1, 0.5 mM NADPH or NHDPH was incubated with intact mitochondria (3.9 mg protein) or sonicated mitochondria (3.9 mg protein) as described under Materials and Methods. When indicated, 0.1 mM rotenone or 5 mM KCN was added. In Expt 2, 0.67 mM NADPH was incubated with sonicated mitochondria (1.44 mg protein) in the presence and absence of air. Anaerobic conditions were obtained in a standard Warburg tube by bubbling nitrogen gas through the reaction mixture for 7 min followed by four evacuations of the tube. The reaction was initiated by addition of mitochondria from the side arm.

Additions	*NADPH or NHDPH oxidation* ($nmoles \cdot mg^{-1}$ *protein* $\cdot min^{-1}$)	
	Intact	*Sonicated*
Expt 1		
NADPH	0.4	44.5
NADPH + rotenone	1.0	44.5
NADPH + CN^-	0.6	45.5
NHDPH	12.5	28.9
NHDPH + rotenone	13.1	27.0
NHDPH + CN^-	11.0	26.3
Expt 2		
NADPH (aerobic)	—	19.2
NADPH (anaerobic)	—	0.4

indicated, the rate of NHDPH oxidation by intact mitochondria is over 30-fold more rapid than NADPH oxidation. As shown in Fig. 1, half-maximal NHDPH oxidation velocity was attained at about 0.15 mM NHDPH. The maximal velocity of NHDPH oxidation, in this experiment, was about 13 nmoles per mg protein per min at 25 °C, although with various other mitochondrial preparations the rate varied from between 6 and 15 nmoles per mg protein per min. These data compare to maximal rates of NHDH oxidation, under identical conditions, in the range of 60–65 nmoles per mg protein per min, with half-maximal velocities obtained at 0.3 mM NHDH[1]. The rate of NHDPH oxidation was a linear function of mitochondrial protein in the range investigated (1–5 mg).

Neither rotenone nor CN^- inhibited NHDPH oxidation (Table I), indicating

that NHDPH is not oxidized by intramitochondrial NAD^+ and that a pathway other than transhydrogenation must be present in the mitochondria for its oxidation.

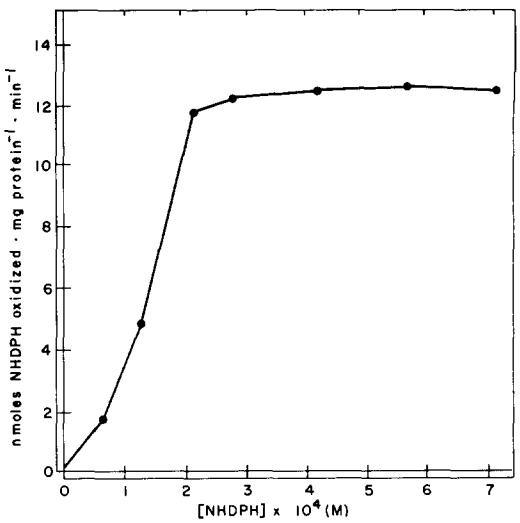

Fig. 1. Mitochondrial NHDPH oxidation as a function of its concentration. Various concentrations of NHDPH were incubated with intact mitochondria (3.8 mg protein) for 1 min. NHDPH oxidation was assayed as described under Materials and Methods.

This conclusion is supported by the observation (Table I, Expt 1) that even though external NADPH is very slowly oxidized by intact mitochondria, sonicated mitochondria oxidized NADPH at a rapid rate by a mechanism also insensitive to inhibition by rotenone and CN^-. The presence of Mg^{2+} in the reaction medium prevents the oxidation of NADPH by transhydrogenation to NAD^+ liberated on mitochondrial lysis[14]. NADPH oxidation was inhibited by 98% under anaerobic conditions (Table I, Expt 2). Therefore, the terminal electron acceptor during NADPH or NHDPH oxidation is concluded to be molecular oxygen.

Further evidence indicating that NHDPH is not oxidized by the respiratory chain of intact mitochondria is presented in Table II. It can be seen that while 3 moles of ATP are formed per mole of NHDH oxidized, the oxidation of NHDPH is not coupled to phosphorylation. Kaplan *et al.*[15] have previously reported the presence of a non-phosphorylating pathway for NADPH oxidation in pyridine dinucleotide depleted liver mitochondria. While the function of this enzyme is not known, recent evidence suggests that mitochondrial NADPH oxidation may be coupled to the degradation of membrane polyunsaturated fatty acids[16].

The lack of sensitivity of NHDPH and NADPH oxidation to respiratory inhibitors and the absence of coupling to ATP synthesis[17] could have been explained if the mitochondria were heavily contaminated with microsomal membranes which contain NADPH oxidase activity[17,18]. To test this, the microsomal fraction[22] as well as the mitochondrial fraction were isolated from the same rat liver. The maximal velocity of NHDPH oxidation by the mitochondria was 12.4 nmoles per mg protein per min, while that of the microsomes, assayed under identical conditions (see

TABLE II

DETERMINATION OF THE P:$2e^-$ RATIO FOR NHDPH AND NHDH OXIDATION

The reaction mixtures at 25 °C contained in 1.4 ml: 57 mM potassium phosphate buffer (pH 7.5), 171 mM KCl, 2.3 mM EDTA, 0.86 mM $MgCl_2$, 2.9 mg mitochondrial protein, 100 mM glucose, 5 mg yeast hexokinase, 10 mM ADP and 0.93 mM NHDPH or 0.37 mM NHDH. In each experiment two identical reaction mixtures were incubated for 2 min (NHDH) or 5 min (NHDPH). One reaction was terminated by addition of 0.2 ml 1 M KOH and used for the determination of NHDH or NHDPH content relative to zero time controls. The other reaction was terminated by addition of 0.16 ml 1 M acetic acid and used for the determination of glucose 6-phosphate content. After centrifugation and neutralization NHDPH was assayed as described under Materials and Methods. NHDH and glucose 6-phosphate were analyzed as described previously[1]. Glucose 6-phosphate formed in the absence of NHDH or NHDPH was subtracted from that formed during pyridine dinucleotide oxidation in the P:$2e^-$ ratio calculation.

Substrate	*Δnmoles substrate oxidized*	*Δnmoles glucose 6-P synthesized*	*P:$2e^-$ ratio*
NHDPH	152.9	2.9	0.02
NHDH	318.7	943.2	2.96

Materials and Methods), was 1.25 nmoles per mg protein per min. The NHDPH: NADPH oxidation rate ratio with microsomes was 0.83. This contrasts to a ratio with the mitochondrial fraction of 20–30 (Table I). It is evident, therefore, that microsomal contamination does not contribute significantly to either NHDPH or NADPH oxidation by our mitochondrial preparations.

Three explanations for the lack of influence of respiratory inhibitors on NHDPH oxidation by intact mitochondria may be considered, (a) NHDPH does not traverse the inner membrane and therefore cannot interact with the transhydrogenase active site, (b) NHDPH traverses the inner membrane and is oxidized so rapidly by the non-respiratory chain linked pathway that little NHDPH is available for transhydrogenation and (c) NHDPH traverses the inner membrane, but is not utilized as a transhydrogenase substrate.

While no direct evidence for NHDPH translocation to the matrix is presently available, the latency of NADPH oxidation revealed on sonication (Table I, Expt 1) indicates that the NADPH oxidase is located in a mitochondrial compartment inaccessible to exogenous NADPH, *i.e.* an area bounded by the inner membrane[6]. Even though NHDPH oxidation by intact mitochondria is relatively rapid, its oxidation rate increases after sonication. This result is suggestive that NHDPH translocation to the NADPH oxidase site is rate limiting in its oxidation. Hydrogen donor specificity studies on the transhydrogenase enzyme of rat liver submitochondrial particles show the maximal velocity of NAD^+ reduction by NHDPH, in the absence of Mg^{2+}, to be less than 20% of that found with NADPH (Tischler, M. E. and Fisher, R. R., unpublished). Further, in the presence of the Mg^{2+} concentration normally used for the assay of NHDPH oxidation the transhydrogenase reaction is completely inhibited. The low inherent reactivity of the transhydrogenase towards NHDPH as well as a possible competitive inhibition of NHDPH oxidation by

TABLE III

LOCALIZATION OF NADPH OXIDASE ACTIVITY IN RAT LIVER MITOCHONDRIA

NADPH (0.6 mM) was incubated as described under Materials and Methods with 1.37 mg sonicated mitochondria, 0.21 mg submitochondrial particles or 0.36 mg of the soluble mitochondrial fraction.

Preparation added	*NADPH oxidation*		
	Protein (*mg*)	*Units* (*nmoles·min*$^{-1}$)	*Spec. act.* (*nmoles·mg*$^{-1}$·*min*$^{-1}$)
Sonicated mitochondria	255.0	5036	19.8
Submitochondrial particles	30.1	4133	137.3
Soluble fraction	51.6	248	4.8

intramitochondrial $NADP^+$ (ref. 19) and Mg^{2+} (ref. 14) may favor the NADPH oxidase pathway.

In order to determine the intramitochondrial locale of the CN^- insensitive NADPH oxidase, mitochondria were sonicated and separated by differential centrifugation (see Materials and Methods) into inner membrane submitochondrial particles and a soluble fraction, *i.e.* non-membrane bound enzymes located in the intramembrane space and matrix[6]. As indicated in Table III, over 80% of the total NADPH oxidase units of the crude mitochondrial sonicate were recovered with a 7-fold increase in specific activity in the submitochondrial particle fraction. A total loss of oxidase activity was noted on storage of the particles for 12 h at 4 °C or after freezing and thawing. The soluble fraction was nearly devoid of NADPH oxidase, which in this fraction was of lower purity than that of the crude sonicate. This experiment provides evidence that the NADPH oxidase is bound to the inner mitochondrial membrane. These data taken together with the observed latency[6] of oxidase activity and the fact that the outer surface of submitochondrial particles is derived from the inner surface of the cristal membrane[20,21] leads to our conclusion that the enzyme is bound to the side of the inner membrane facing the matrix.

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