THE OXIDATION OF ADDED NADH BY INTACT HEART MITOCHONDRIA

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1. Introduction

The exchange of reducing equivalents between the cytoplasm and the mitochondria involves a very important metabolic control mechanism (e.g. refs. [1,2]). The liver gluconeogeneses require a high rate of NADH formation in the cytoplasm, while in heart cytoplasmic NADH must be reoxidized in order to maintain pyruvate formation from glucose. It is generally accepted that the mitochondrial membrane is virtually impermeable to NADH [3] and this has resulted in the proposal of several shuttle mechanisms suggesting substrate transfer instead of coenzyme transfer across this membrane (e.g. refs. [1,2]).

NADH oxidation catalyzed by mitochondria but stimulated by added cyt. c and not completely inhibited by amytal and antimycin A has often been reported in the literature (e.g. refs. [4,5]). This paper describes, however, a NADH-oxidase which was destroyed upon ageing of the mitochondria and which was highly sensitive to the normal respiratory chain inhibitors. This oxidative system present in pigeon heart mitochondria presumably oxidized added NADH on the mitochondrial surface. It was furthermore characterized by a K_m for NADH below 2 μM and a turnover equal to the turnover of succinate (in the presence of glutamate) in the same mitochondria. The enzyme therefore possesses some of the characteristics which might be expected from a cytoplasmic-mitochondrial NADH-oxidase working *in vivo*. Some preliminary results have been published elsewhere [6].

2. Materials and methods

Mitochondria were prepared from pigeon hearts by a method involving very gentle homogenisation, incubation of the homogenate with a bacterial proteinase (10 min (0°), 2 mg/g tissue (Novo, Copenhagen, Denmark)) and centrifugations at 8700, 350, 7700 and 7700 g, discarding the precipitate of the lowest spin (Sorvall RC-2 centrifuge, rotor SS-34). All light particles were very carefully removed in the washings. The mitochondria were prepared and stored (10–15 mg protein per ml) in a mannitol (225 mM), sucrose (75 mM), Tris (1 mM), EDTA (0.05 mM) medium of pH 7.4. Protein was determined with the biuret method [7] standardized against bovine serum albumin. Oxygen concentration was recorded amperometrically by a Clark type oxygen electrode (e.g. ref. [8]) which was placed in the cuvette of a microfluorometer [9] for simultaneous recording of the NADH fluorescence. The mitocrits were determined by centrifugation of the concentrated mitochondrial suspensions (cf. ref. [10]).

3. Results

NADH added to pigeon heart mitochondria disappeared rapidly with simultaneous consumption of oxygen (fig. 1). The K_m for NADH in this reaction was about 1.8 μM and the stoichiometric relation between NADH oxidized and oxygen atoms consumed was always found to be 1:1 with fresh mitochondria. The NAD formed in this reaction could be reduced by ethanol and alcohol dehydrogenase (EC 1.1.1.1) after removal of the mitochondria by centrifugation and adjustment of the pH of the supernatant to 8.8. The mitochondrial NADH oxidation could furthermore be coupled directly to ethanol oxidation resulting in continuous uptake of all dis-

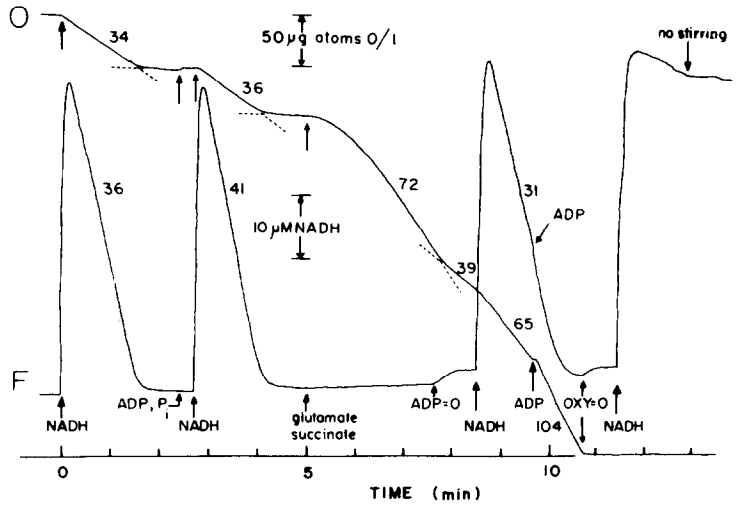

Fig. 1. Mitochondrial respiration of NADH measured by the simultaneous recording of oxygen concentration (O) and fluorescence (F, 366 nm/450 nm). The numbers at the curves represent consumed µg atoms oxygen/l X min and oxidized µM NADH/min. The mitochondria were suspended in a medium (450 µg protein/ml) containing mannitol (225 mM), sucrose (75 mM), Tris (20 mM), and EDTA (0.5 mM), pH 7.35. All additions are indicated on both curves: 4 X 49 µM NADH, 225 µM ADP + 6.4 mM inorganic phosphate, 7 mM glutamate + 6 mM succinate, and 300 µM ADP. The increased fluorescence at the point marked ADP = 0 represents the total reduction of the endogenous pyridine nucleotides.

solved oxygen (230 µM O_2) in the presence of a few µM NADH. The mitochondria were completely unable to catalyze NADPH oxidation either directly or coupled with glucose-6-phosphate dehydrogenase (EC 1.1.1.49). The NADH-oxidase activity appeared to be almost constant for our standard preparation of mitochondria when compared with the maximal respiratory capacity, i.e., the rate of oxygen measured when the mitochondria were supplemented with excess succinate, glutamate, ADP and inorganic phosphate. The NADH oxidase activity amounted to 32% ± 3 (s.e.m. 6 preparations) of this maximal capacity or about 100% of the maximal respiratory capacity under non-phosphorylating conditions (i.e., succinate + glutamate + inorganic phosphate). This amounts to 100 ng atoms oxygen/min X mg protein. This oxidase activity was about an order of magnitude larger than the one published for rabbit heart mitochondria [11].

Pigeon liver mitochondria showed less than 2% of the NADH oxidase activity of the heart mitochondria prepared simultaneously. The only difference between the procedures for preparation of the liver and heart mitochondria was the incubation of the heart homogenate with proteinase. Heart mitochondria prepared without this incubation did, however, exhibit NADH-oxidase activity as well.

The possibility that the NADH oxidase activity was due to damaged or somehow modified mitochondria seemed unlikely considering the effects of ageing of the oxidase (cf. table 1 and fig. 2). The NADH-oxidase was found to be far more sensitive to ageing at 35° than the other respiratory activities (e.g. succinate + glutamate) although not as sensitive as the energy-coupled

Table 1
Half times of some mitochondrial reactions during ageing of the concentrated suspensions at 35°.

NAD reduction by succinate	3	min
Oxidative phosphorylation *	7–10	min
Oxidation of added NADH	70	min
Respiration of succinate ± glutamate	>400	min

* Judged by the ability of ADP to cause oxidation of endogenous NADH.

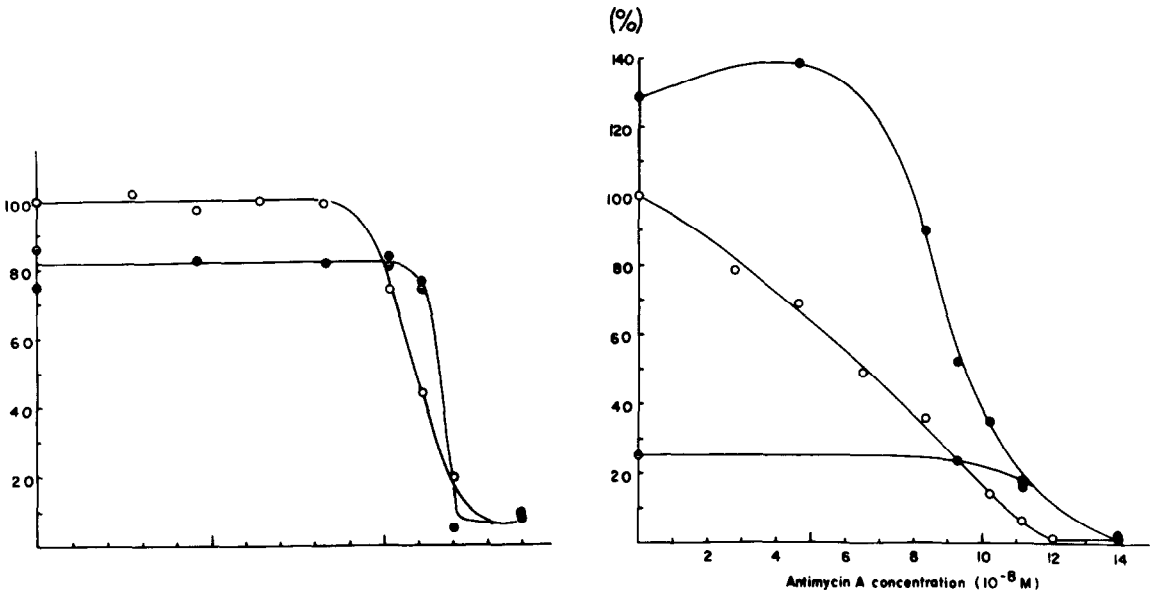

Fig. 2. Antimycin A inhibition of succinate + glutamate respiration (6 + 7 mM, upper part) and of NADH oxidation (30 μM, lower part) catalyzed by fresh mitochondria (open circles), mitochondria aged for 30 min at 35° (filled circles) and for 90 min at 35° (half filled circles). The concentrated mitochondrial suspension was after the ageing diluted to 830 μg protein/ml for the experiments. The rates in the control experiment amounted to 97 μg atoms O/l X min (= 100% for succinate + glutamate respiration) and 97 μM NADH/min (= 100% for NADH oxidation).

Table 2
NADH-oxidase activity of subfractions of the mitochondrial preparation.

A	B	C	D	E	F	G	H	I	J	K
Fraction	rpm	% protein	mitocrit % v/v	suc+glut oxidase	suc-NAD reductase	$\omega^2 t$	r_{limit}	r_{mean}	NADH-oxidase activity	
									per mg	per r^2
1	1575–1750	15.4	4.0	227	103	13.9	9.35–8.48	8.92	22.7	202
2	1750–2010	15.8	4.0	230	92	18.7	8.48–7.31	7.90	30.2	239
3	2010–2275	13.6	3.8	221	95	23.9	7.31–6.46	6.89	30.5	210
4	2275–2335	14.6	4.2	237	66	29.7	6.46–5.80	6.13	44.2	271
5	2335–2800	10.6	3.9	230	82	36.1	5.80–5.26	5.53	46.2	255
6	2800–3235	8.9	4.2	202	94	47.3	5.26–4.59	4.93	62.7	309
7	3235–4375	10.4	4.4	209	92	88.0	4.59–3.37	3.98	79.5	316
8	4375–7875	10.6	3.6	167	93	287.1	3.37–1.86	2.62	113.5	298

The last centrifugation of the normal preparation procedure was replaced by 8 successive centrifugations of the same suspension. The protein recovered in each pellet (column C) was expressed as % of the total protein recovered. The mitocrit (D) of the concentrated suspensions was corrected for differences in protein concentration and expressed as % v/v at 10 mg protein per ml. The succinate + glutamate respiration (mμg atoms O/min X mg protein (E)) was measured in the presence of ADP and inorganic phosphate, and the succinate-NAD-reductase activity (F) was expressed as the % increase per min of the total fluorescence increase. The $\omega^2 t$ (G) was obtained in arbitrary units by integration over the total centrifugation cycle. The ratemeter of the centrifuge was controlled with a stroboscope. The limiting radius (H) was calculated in arbitrary units as $1/\sqrt{(\omega^2 t)}$ because the sedimentation coefficient is proportional to r^2 (for constant particle density) and $1/\omega^2 t$ (e.g. 14) and the mean-radius (I) was obtained by linear interpolation. The NADH-oxidase activity was calculated on the basis of the protein content, i.e., on constant volume (μM NADH/min X mg protein (J)) and on the basis of constant surface area (in arbitrary units, $r^2 = r^3/r_{mean}$ (K)).

reactions, i.e., the reduction of intramitochondrial NAD by succinate and the metabolic changes induced by ADP addition. The NADH oxidase activity was in some preparations temporarily increased by ageing (cf. fig. 2). This aged NADH-oxidase was, however, considerably less sensitive to antimycin A as was also the oxidase obtained after the decline in activity (90 min ageing). In some preparations a further increase of activity with ageing was obtained after the decline. This increase would continue until the final destruction of the mitochondria as evidenced by complete loss of all respiratory activity.

Another possible explanation of the NADH oxidation was that it was catalyzed by particles different from the mitochondria. This possibility seemed, however, very unlikely because of the high activity of the oxidase relative to the other respiratory activities of the preparation. The specific activity of the NADH-oxidase was furthermore not decreased by 7 subsequent washings of the mitochondria (67% protein was lost in the washings (7700 g)).

Further fractionation of the mitochondria was undertaken in order to investigate the homogeneity of the preparation (table 2). The various fractions did not show constant specific activity of NADH-oxidase although the specific activity of succinate (+ glutamate) oxidase and of succinate-endogenous NAD-reductase were virtually constant. It was also attempted to correlate the activity of the NADH-oxidase to the surface area of the particles. In calculating the relative surface areas of the various fractions it was assumed: (1) that the mitochondria were spheres (no substantiation); (2) that the density of the particles in all fractions was identical (in accordance with the constancy of the mitocrit relative to the protein content); (3) that the average radius of the particles in a fraction was equal to the value calculated by linear interpolation; and (4) that the sedimentation laws were valid for the conditions in the angle-rotor (cf. ref. [12]). Taking these limitations in consideration the NADH-oxidase activity per surface unit was surprisingly constant in the 8 fractions (263 ± 16 s.e.m. arbitrary units). The mitochondrial NADH-oxidase activity for added NADH therefore appeared to be a function of the mitochondrial surface rather than the mitochondrial volume (i.e., protein content).

Fig. 1 shows an experiment with fresh mitochondria. NADH was not oxidized in the absence of oxygen. In the presence of oxygen the respiration was completely independent of the redox state of the endogenous NAD. The oxygen uptake due to NADH oxidation was additive to the one caused by succinate and glutamate in the presence or absence of ADP (and phosphate) even though the total respiratory rate thereby significantly exceeded the "maximal" respiratory rate which could otherwise be obtained with any substrate combination tested such as glutamate, succinate, α-oxoglutarate, pyruvate and combinations of these. The oxidized NADH was apparently not available to any mitochondrial enzymes as indicated by the amount of reduction in fig. 1 (point marked ADP = 0). The respiratory rate in the presence of NADH was not influenced by ADP and inorganic phosphate (fig. 1) or by 2,4-dinitrophenol, atractyloside or oligomycin. The respiration of added NADH therefore appeared to be disconnected from the phosphorylating mechanism. Enzymatic analysis of ATP [13] further substantiated this. Neither the endogenous ADP (about 2 μM) nor the added ADP was phosphorylated during the NADH oxidation in contrast to for instance the succinate (+ glutamate) respiration which was accompanied by a very rapid phosphorylation of endogenous ADP (fig. 1).

The enzymatic system which was responsible for the oxidation of added NADH appeared, however, to involve the normal components of the respiratory chain. The NADH oxidation could be inhibited completely by amytal, antimycin A (fig. 2), cyanide or sulfide. About 5×10^{-4} M amytal or about 5×10^{-8} M antimycin A caused 50% inhibition of the NADH respiration. In fresh mitochondria, the NADH oxidation could be inhibited 70–80% by antimycin A without any significant inhibition of the succinate (+ glutamate) respiration (fig. 2). As mentioned above was the sensitivity of the NADH oxidation to antimycin A markedly altered by ageing of the mitochondria in contrast to for instance the succinate (+ glutamate) respiration (fig. 2). The NADH oxidation in fresh mitochondria was considerably more sensitive than the oxidation in the aged mitochondria, which appeared to exhibit a sensitivity pattern for NADH very similar to that shown by other substrates (e.g. α-oxoglutarate (+ malonate) and the succinate (+ glutamate) respiration). In the presence of 5×10^{-8} M antimycin A was the rate of NADH respiration in the 30 min aged preparation more than twice the comparable rate in the fresh preparation.

4. Discussion

The NADH oxidase of pigeon heart mitochondria apparently involves a complete and normally composed respiratory chain as substantiated by the sensitivity to the respiratory chain inhibitors in normal concentrations. This in addition to the high rate of NADH respiration (equal to the succinate + glutamate respiration) is strong evidence in favour of the assumption that the reaction is a mitochondrial reaction. Repeated washings of the mitochondria do furthermore not alter the relative activities of the reactions.

However, the respiratory chain which carries out the NADH oxidation seems for the following reasons to exist in another compartment than the one which carries out the tricarboxylic acid cycle oxidations: the respiration of NADH does not result in any ATP synthesis although flux in the normal respiratory chain of these mitochondria leads to the theoretical phosphorylation at all three sites. The NADH respiration is additive to the tricarboxylic acid cycle respirations and the two types of reactions exhibit very different sensitivity to ageing of the mitochondria. The NAD formed from added NADH can not be reduced by, e.g., succinate, which causes rapid and complete reduction of endogenous NAD.

The present mitochondria appear to be rather impermeable as evidenced by K_m-values in the mM range for most substrates and by inability to respire for instance glutamate when completely fresh. Other mitochondria which appear more permeable for substrates than these are, however, virtually impermeable for NADH, and this makes NADH-permeability of the present mitochondria unlikely. It seems therefore probable that the oxidation of NADH by pigeon heart mitochondria is the result of the presence of a separate normally composed respiratory chain located outside the permeability barrier of the mitochondria. This is further supported by the very low K_m-value for the reaction and by the finding that the activity is closely related to the surface area of the particles.

It is well established that damage of mitochondria increases their permeability for NADH [5] and the ageing pattern of the present mitochondria might be explained as the result of two effects: destruction of the exterior NADH-oxidase and increased permeability resulting in oxidation of NADH by the normal respiratory chain. The relative contributions of these two effects during the ageing may vary considerably among the preparations resulting in some cases in the biphasic ageing pattern described above. The antimycin A sensitivity seems moreover to be in accordance with this assumption, i.e., high sensitivity when the external oxidase dominates and sensitivity similar to the sensitivity of the substrate oxidation when the NADH is oxidized after permeation.

The observation of high activities of a labile NADH oxidase in all preparations of pigeon heart mitochondria and subfractions thereof, but not in liver mitochondria, seems to exclude that the enzyme activity is an artifact. The reaction may under *in vivo* conditions exert a very important function. Because of the low K_m-value and relatively high turnover number a high cytoplasmic NAD/NADH ratio can be maintained and lactate formation from glucose prevented. The pyruvate preserved in this way will by oxidation in the tricarboxylic acid cycle lead to a high energy yield per mole glucose even when the non-phosphorylating oxidation of cytoplasmic NADH is taken in consideration. The non-phosphorylating character of this reaction may even be advantageous because the oxidation of cytoplasmic NADH will be controlled only by the NADH/NAD ratio.

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