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THE EXTERNAL NADH DEHYDROGENASES OF INTACT PLANT MITOCHONDRIA

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SUMMARY

Evidence is given for the presence of two NADH dehydrogenase systems accessible to exogenous NADH in mitochondria from mung bean hypocotyls. One system, which is located on the outer membrane, contains a flavoprotein and cytochrome b_{555}, is insensitive to antimycin A and is specific for the 4α hydrogen atom of NADH. The second NADH dehydrogenase, which is located on the external face of the inner membrane, is linked to the respiratory chain and is specific for the 4β hydrogen atom of NADH. The intact mitochondria have a large antimycin A-insensitive NADH:ferricyanide oxidoreductase activity which is primarily due to the outer membrane system. The inner membrane dehydrogenase reacts with ferricyanide only after the mitochondria have been osmotically swollen. Such swelling seems to cause detachment of this flavoprotein from the inner membrane.

INTRODUCTION

Although NADH cannot penetrate the inner membrane, mitochondria of plants and fungi can respire with NADH as substrate[1,2]. Von Jagow and Klingenberg[3] recently presented evidence for an NADH dehydrogenase located on the outer surface of the inner membrane of yeast mitochondria, but in the course of their work they neglected the NADH dehydrogenase activity associated with the outer membrane. The results of the present investigation clearly demonstrate the presence of such a dehydrogenase on the outer surface of the inner membrane of plant mitochondria and distinguish it from the outer membrane system.

MATERIAL AND METHODS

The plant material used in these experiments were etiolated mung bean hypocotyls (*Phaseolus aureus*), cut from bean seedlings grown for 5 days in the dark at 28 °C and 60% relative humidity. Mitochondria were prepared and purified by

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methods that have been previously described[4]. Typical preparations start from 1 kg of tissue that is disrupted for 15 s with a Moulinex Mixer 66 (Alençon, France). The yield of purified mitochondria is generally around 70 mg mitochondrial protein. The techniques used for mitochondrial outer membrane isolation are described in the following section.

Oxygen uptake was measured at 25 °C in a 3-ml stirred cell using a Clark oxygen electrode (Yellow Springs Instrument Company) as described by Estabrook[5]. The reaction medium (Medium A) contained: 0.3 M mannitol (or sucrose), 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate buffer (pH 7.2) and 0.3–2.0 mg of mitochondrial protein. The oxygen concentration in air-saturated medium was taken as 250 μM.

Low-temperature difference spectra were performed with a sensitive scanning split-beam spectrophotometer described by Chance[6].

The media and the conditions used in each assay for the determination of enzyme activities were as follows:

(a) Succinate:cytochrome *c* oxidoreductase: Medium A, 50 μM cytochrome *c*, 1 mM KCN, 200 μM ATP and 0.1–1.0 mg of mitochondrial protein. The reaction was initiated with 10 mM succinate.

(b) NAD(P)H:cytochrome *c* oxidoreductase: Medium A, 50 μM cytochrome *c*, 1 mM KCN, and 0.1–1.0 mg of mitochondrial protein. The reaction was initiated with 1 mM NAD(P)H. The final concentration of antimycin A, when used, was 0.5 μg/ml.

(c) NADH:ferricyanide oxidoreductase: Medium A, 1 mM $K_3Fe(CN)_6$, 1 mM KCN and 0.5–1.0 mg of mitochondrial protein. The reaction was initiated with 1 mM NADH. The contribution of the non-enzymatic reduction of $K_3Fe(CN)_6$ was subtracted from the reaction in each assay.

(d) Cytochrome *c*:oxygen oxidoreductase: Medium A, 50 μM reduced cytochrome *c*. The reaction was initiated with 0.1–1.0 mg of mitochondrial protein.

(e) Malate dehydrogenase: Medium A, 1 mM KCN, 0.2 mM NADH. The reaction was initiated with 1 mM oxalacetate.

Spectrophotometric assays were made in a Zeiss spectrophotometer using cuvettes of 1 cm path length. In each assay the final volume was 3 ml. The millimolar extinction coefficients used were: cytochrome *c*, $\varepsilon_{550}=21.0\ mM^{-1}\cdot cm^{-1}$; NADH, $\varepsilon_{340}=6.22\ mM^{-1}\cdot cm^{-1}$; $K_3Fe(CN)_6$, $\varepsilon_{420}=1.03\ mM^{-1}\cdot cm^{-1}$.

[^{3}H]NAD$^+$ (specific activity 50.0 Ci/mole) was obtained from Amersham/Searle Corporation. [4β-^{3}H]NADH was prepared by reduction of [^{3}H]NAD$^+$ with ethanol in the presence of α-specific yeast alcohol dehydrogenase, and [4α-^{3}H]NADH was prepared by reduction with UDPG in the presence of β-specific UDPG dehydrogenase[7,8]. In both cases, the NADH-generating systems were inactivated by heat denaturation following the completion of the reaction.

The inability of NADH to penetrate the inner membrane and the absence of proton transfer between exogenous NADH and endogenous NAD$^+$ have been investigated according to von Jagow and Klingenberg[3].

Mitochondrial protein content was determined by a modified Lowry method[9] or the biuret method[10] with crystallized bovine serum albumin (Miles Laboratory, Inc.) as the standard.

RESULTS

Osmotic rupturing of mitochondrial membranes

The properties of mung bean mitochondria purified by the above procedures have been described in detail in a previous paper[4]. Electron micrographs show them to have intact outer and inner membranes and to be uncontaminated by other subcellular material. These mitochondria are well coupled and typically display State 3 oxidation rates of 500 nmoles O_2/min per mg protein for NADH.

It is well known that mitochondria respond morphologically to changes in the osmolarity of suspending media[11,12]. Fig. 1 demonstrates the effect of osmotic

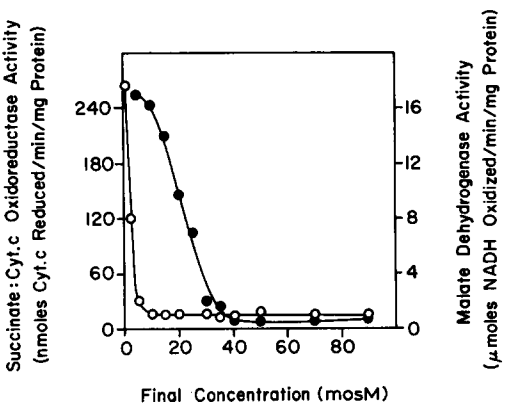

Fig. 1. Effect of final osmolarity of suspending media on mitochondrial enzyme activities. Aliquots of purified mitochondria (20 μl) were added to 5 ml of appropriate sucrose solutions at room temperature. After 6 min the succinate:cytochrome *c* oxidoreductase activity (●—●) of the suspensions were monitored. The mitochondria were then pelleted (8000 × *g*, 10 min) and the supernatants were assayed for malate dehydrogenase activity (○—○).

swelling on two mitochondrial activities. The succinate:cytochrome *c* oxidoreductase activity is a reflection of the accessibility of the exogenous cytochrome *c* to the cytochrome *c* bound to the external face of the inner membrane[13,14]. The malate dehydrogenase activity likewise reflects the accessibility of NADH to the dehydrogenase located in the matrix space (perhaps loosely bound to the inner face of the inner membrane). These two activities can be used to monitor the rupturing of the outer membranes and the inner membranes respectively. As can be seen in Fig. 1, both activities undergo dramatic increases as the mitochondria are exposed to successively more dilute media. The exact concentration at which the two membranes burst varies only slightly between preparations, but if proper care is not taken in the course of mitochondrial preparation, the outer membranes are found to be initially ruptured to a greater extent. However, when the mitochondria are intact initially, the outer membranes invariably rupture at considerably higher osmolarities than the inner membrane.

Evidence for two external dehydrogenases in plant mitochondria

Similar experiments were conducted in which the NADH:cytochrome *c* oxidoreductase activities were followed as a function of final osmolarity. Fig. 2 shows

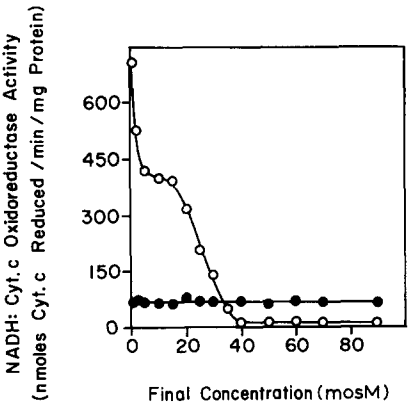

Fig. 2. Effect of final osmolarity of suspending media on NADH:cytochrome *c* oxidoreductase activities. Procedure as in Fig. 1. (●—●), antimycin A-insensitive activity; (○—○), antimycin A-sensitive activity.

the results of a typical experiment. The antimycin A-insensitive activity does not vary while the antimycin A-sensitive activity exhibits three distinct phases. When the outer mitochondrial membranes are intact, the added cytochrome *c* is denied access to the inner membranes[4,14], and the antimycin A-sensitive activity is very low. As the outer membranes rupture, there is a sharp increase in this activity which follows the succinate: cytochrome *c* oxidoreductase activity (see Fig. 1). At very low osmolarities there is a further increase which follows the bursting of the inner membrane as monitored by the malate dehydrogenase activity (see Fig. 1). Since NADH is unable to penetrate the inner membrane, these results demonstrate the presence of three NADH dehydrogenases in plant mitochondria. One is located on the outer membrane, and its electron transfer to exogenous cytochrome *c* is insensitive to antimycin A and to final osmolarity. This implies that the sites of reduction of exogenous cytochrome *c* are located on the outer face of the outer membrane. A second dehydrogenase is located on the outer face of the inner membrane, and a third, common to all mitochondria, on the inner surface of the inner membrane.

Preparation of the outer membrane

To further characterize the outer and inner membrane dehydrogenases, procedures were developed to isolate and purify the outer membranes of mung bean mitochondria.

Basically three techniques were tested for yield and purity of outer membrane fractions. These were: (1) hypotonic treatment[15], followed by contraction in 0.3 M sucrose; (2) swelling–contraction–sonication treatment[16]; and (3) swelling–contraction–Yeda press treatment. The last technique involves very slowly forcing mitochondria, which have been swollen and contracted, through a very small aperture under high N_2 pressure. The apparatus used was the Yeda Press (Rehovot, Israel). For all three techniques, initial hypotonic treatment was done in sucrose solutions with final osmolarities of 10 mosM. Fig. 1 shows that this osmolarity totally ruptures the outer membrane without affecting the inner membrane.

After the various disruptive procedures, the suspensions were layered on top

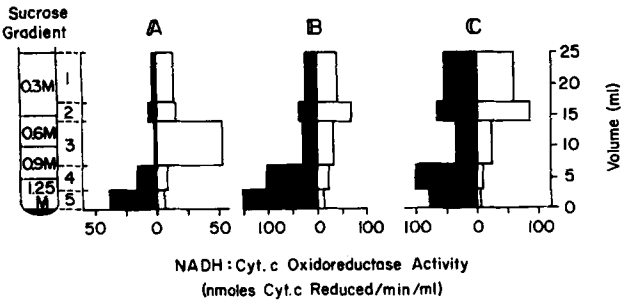

Fig. 3. Profiles of the distribution of NADH:cytochrome *c* oxidoreductase activities after disruption of mitochondria by various techniques. Black areas, antimycin A-sensitive activity; blank areas, antimycin A-insensitive activity. (A) Hypotonic treatment (10 mosM, 15 min) followed by contraction in 0.3 M sucrose. (B) Swelling–contraction treatment as in A, followed by "Yeda Press" treatment. Flow rate, 1 ml/5 min. (C) Swelling–contraction treatment as in A followed by sonication in 3-ml aliquots for 15 s using a Branson "sonifier-cell disruptor", Model W 185, special microtip, output control setting 3. Numbers to the right of gradient tube refer to the various fractions. Note the use of different scales along the abscissa.

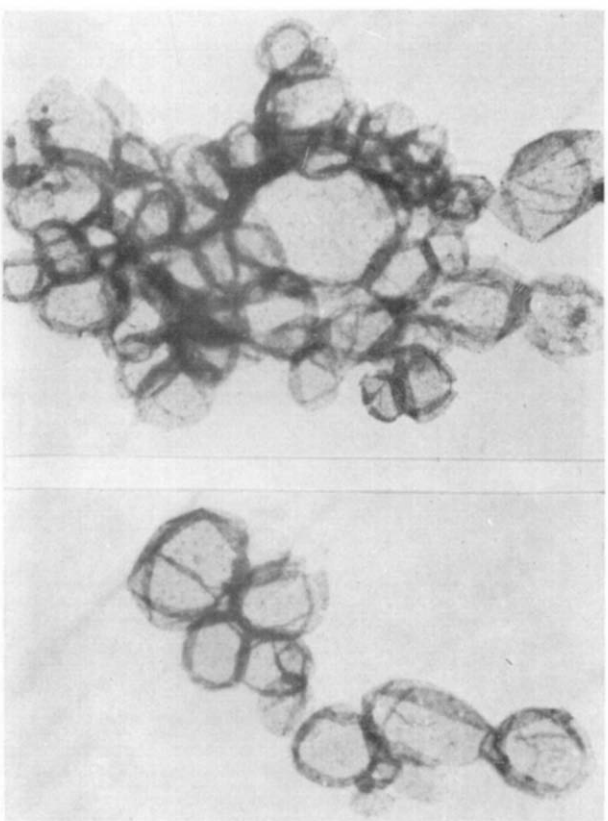

Fig. 4. Negatively stained preparation of purified mung bean hypocotyl mitochondria outer membranes (Fraction 3, Fig. 3A). The outer membranes were suspended in 10 mM phosphate buffer and fixed for 1 min at room temperature with 1% uranyl acetate. The sample was directly examined with a Philips 200 electron microscope operating at 60 kV. Magnification ×20000.

of discontinuous sucrose gradients (0.6, 0.9 and 1.25 M) and spun at $40000 \times g$ for 60 min.

Fig. 3 illustrates the effectiveness of the three techniques used to isolate outer membranes. In each case, the total antimycin A-insensitive NADH:cytochrome *c* oxidoreductase activity of the starting material is recovered, taking into account the activity remaining in the pellet. Swelling–contraction treatment alone results in a recovery of 30% of this outer membrane activity in Fraction 3, along with a barely detectable antimycin A-sensitive activity. Subsequent disruption, whether by sonication or Yeda press treatment furthers the separation of outer membranes from the mitochondria but dramatically increases inner membrane contamination, as shown by the high antimycin A-sensitive activity in all fractions. In addition, each fraction was also assayed for cytochrome oxidase and succinate: cytochrome *c* oxidoreductase activities. The profiles of these activities were found to closely follow those of the antimycin A-sensitive NADH:cytochrome *c* oxidoreductase activity and confirm the purity of the outer membrane fraction (Fraction 3, Fig. 3A).

NADPH:cytochrome *c* oxidoreductase activity was also assayed and found to be negligible in the outer membrane fraction. Hence this fraction is essentially free of microsomal contamination.

The purity of this outer membrane fraction was confirmed by two other methods. Electron micrographs of this fraction after uranyl acetate fixation (Fig. 4) clearly show the typical folded-bag appearance of outer mitochondrial membranes[15] with no other structures visible. In addition, low temperature difference spectra of this fraction (Fig. 5) indicate the presence of the flavoprotein and *b*-type cyto-

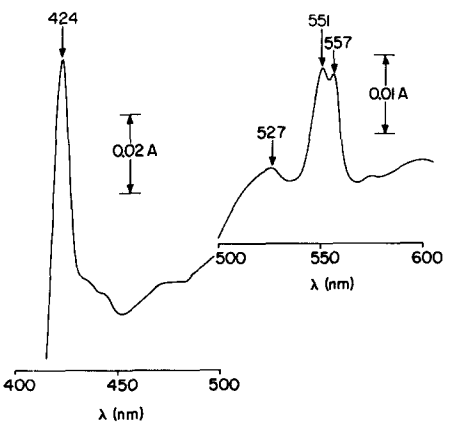

Fig. 5. Difference spectrum of outer membrane fraction (Fraction 3, Fig. 3A). Dithionite-reduced minus aerobic. Path length 2 mm, liquid-nitrogen temperature, 1.4 mg protein per ml.

chrome associated with the outer membrane NADH:cytochrome *c* oxidoreductase[17]. The room temperature difference spectrum of this cytochrome shows an α absorption band centered at 555 nm. Therefore, by accepted rules of nomenclature, we have called this pigment cytochrome b_{555}. Again, there is no evidence of inner membrane cytochrome contamination.

Soluble NADH dehydrogenase

According to Sottocasa *et al.*[16], the outer membrane cytochrome *b* of rat liver mitochondria tends to become detached from the membranes during the course of their isolation. Experiments were done to determine whether our procedure for outer membrane isolation resulted in the loss of cytochrome b_{555} from these membranes. Outer membranes were isolated by hypotonic treatment without contracting the mitochondria before layering on the sucrose gradient. This did not significantly decrease the yield of outer membranes in Fraction 3 and insured that the supernatant (Fraction 1) would be free of membrane-bound systems.

The low temperature difference spectra of this supernatant (Fig. 6) are those of a flavoprotein and not of cytochrome b_{555} as might have been expected. The NADH-reduced *minus* aerobic spectrum indicates that this flavoprotein is an NADH dehydrogenase. To determine whether this NADH dehydrogenase belongs to the outer or inner membrane systems, the stereospecificity of both dehydrogenases was determined. Fig. 7 shows that the NADH dehydrogenase found in the super-

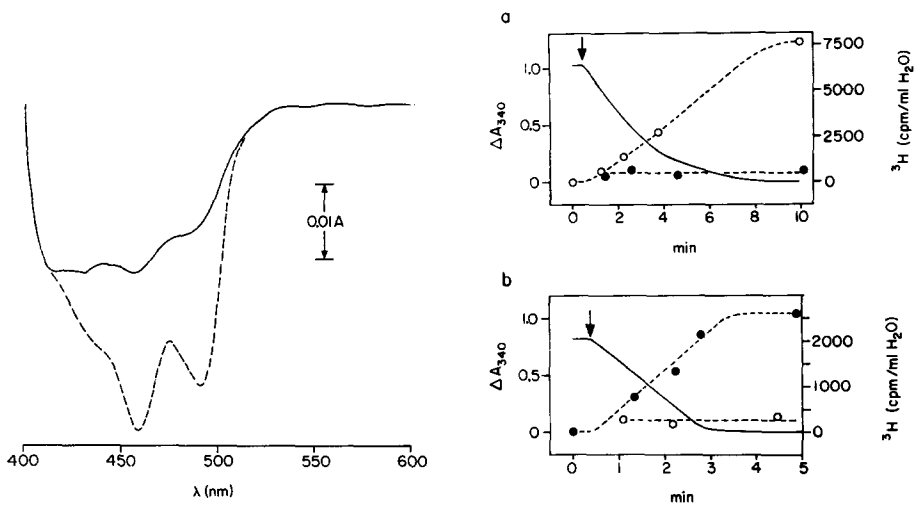

Fig. 6. Difference spectra of supernatant: NADH-reduced *minus* aerobic (———), dithionite-reduced *minus* aerobic (— — —). Path length 2 mm, liquid-nitrogen temperature. This supernatant, obtained by hypotonic treatment of 40 mg mitochondrial protein, was concentrated at 30 °C using a Büchi evaporator. NADH concentration 1 mM.

Fig. 7. Stereospecificity of the NADH dehydrogenases of the supernatant and the outer membrane from hypotonically treated mitochondria. NADH oxidation (———), detritiation of [4α-^{3}H] NADH (●---●) and of [4β-^{3}H]NADH (○---○) by supernatant (a) and outer membrane (b) fractions with ferricyanide as acceptor. Conditions as in Material and Methods for NADH: ferricyanide reductase assay except for initial NADH concentrations (0.16 mM in a, 0.13 mM in b). Reactions were initiated (arrows) by addition of 5 μl of concentrated supernatant (see legend of Fig. 6) and 50 μl of outer membrane suspension (1 mg/ml). Data points indicate the times at which 0.25 ml of the reaction mixture were assayed for incorporation of ^{3}H into water according to the procedure of Lee *et al.*[18]. The first sample in each figure ($t = 0$) was actually taken 2 min after the addition of ferricyanide. The extent of ^{3}H incorporation up to that point due to non-enzymatic detritiation was subtracted from the total incorporation of subsequent samples.

natant is specific for the 4β hydrogen atom of NADH, while the outer membrane dehydrogenase involves the 4α hydrogen atom.

From these experiments several conclusions can be drawn. The outer membrane NADH dehydrogenase exhibits the same stereospecificity as that reported for the outer membrane dehydrogenase of rat liver mitochondria[16]. In addition, neither this flavoprotein nor the cytochrome b_{555} becomes detached from the outer membrane in the course of its preparation to any detectable extent. On the other hand, the NADH dehydrogenase found in the supernatant displays the same stereospecificity as that of energy-linked NADH dehydrogenases[19]. It is unlikely that this is the dehydrogenase bound to the inner face of the inner membrane. Although such dehydrogenases have been isolated from animal mitochondrial particles, the techniques involved (snake venom[20,21] or phospholipase treatment[22], acid–ethanol extractions[23]) were much more drastic than the hypotonic treatment used here. For example, swelling at 10 mosM final osmolarity results in no detectable soluble succinate dehydrogenase activity, and the bulk of the malate dehydrogenase activity is not released from the mitochondria (Fig. 1). Hence, it is most likely that the flavoprotein released by hypotonic treatment is the NADH dehydrogenase bound to the outer face of the inner mitochondrial membrane.

Experiments with ferricyanide as acceptor

With the aim of further demonstrating the localization of an NADH dehydrogenase on the external face of the inner membrane, experiments were done using ferricyanide as the acceptor of electrons from NADH oxidation. It is well known that ferricyanide is able to react directly with flavoprotein dehydrogenases[24,25] and that it is unable to penetrate the inner mitochondrial membrane[26].

The results of such experiments are shown in Fig. 8. As can be seen, antimycin A inhibits about 50% of the total NADH: ferricyanide oxidoreductase activity in

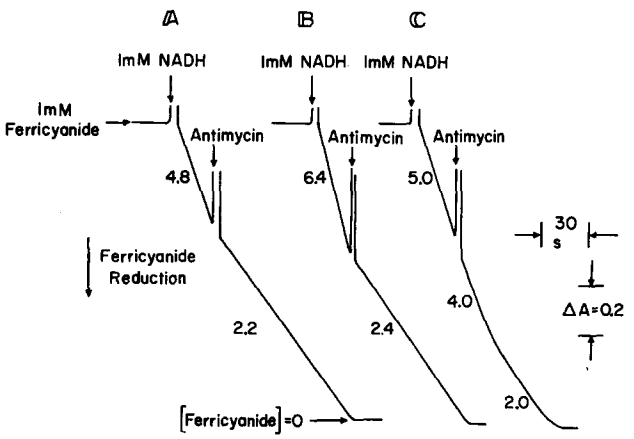

Fig. 8. NADH:ferricyanide oxidoreductase activity of purified mung bean mitochondria. (A) Intact mitochondria, (B) After addition of 170 μM ATP and 30 μM uncoupler (1799). (C) After hypotonic treatment (final osmolarity, 20 mosM). The numbers along the traces indicate μmoles ferricyanide reduced/min per mg protein. In each case the amount of mitochondrial protein was 0.5 mg. Antimycin A (0.5 μg/ml) was added when indicated.

intact coupled mitochondria (Fig. 8A). Also the addition of uncoupler induces a noticable acceleration of the initial rate of reduction but does not change the antimycin A-insensitive rate (Fig. 8B). These results differ from those reported by von Jagow and Klingenberg[3] for yeast mitochondria. They found no such inhibition by antimycin A and concluded that the ferricyanide was reacting directly with the flavoprotein. In addition, they neglected to consider the contribution of the outer membrane NADH dehydrogenase system to the rate of ferricyanide reduction*. In fact (Fig. 8C), when mung bean mitochondria are swollen in a solution of low osmolarity (20 mosM), the results are similar to those reported by von Jagow and Klingenberg[3]; namely, there is little or no inhibition by antimycin A. Note that at 20 mosM the inner mitochondrial membranes are still intact (Fig. 1).

Another indication that ferricyanide is acting after the flavoprotein in intact mitochondria was given by experiments similar to those done by Sottocasa and Sandri[28] on rat liver mitochondria. Fig. 9A is a polarographic trace showing the

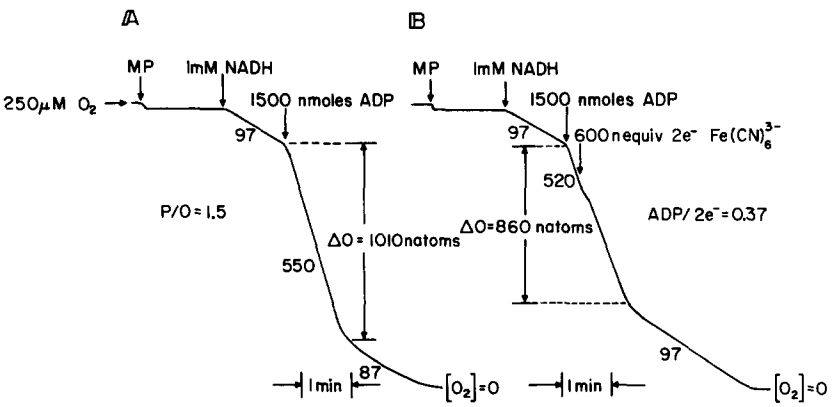

Fig. 9. Polarographic traces showing oxidation of NADH by purified mung bean mitochondria. (A) Normal respiration. (B) After addition of ferricyanide in State 3. The numbers along the traces indicate nmoles O_2 consumed/min per mg of mitochondrial protein. MP = purified mitochondria.

typical State 4–State 3 transition that occurs when limiting amounts of ADP are added to intact purified mitochondria oxidizing exogenous NADH. Fig. 9B shows that addition of ferricyanide in State 3 results in a visible inhibition of respiration. This inhibition is reversed as the added ferricyanide is reduced (followed spectrophotometrically at 420 nm). Moreover, for the same amount of ADP added, the absolute amount of O_2 taken up in State 3 is decreased by this addition of ferricyanide. This result indicates that some electrons used in the reduction of ferricyanide are going through phosphorylation Site II (the initial phosphorylation site for NADH oxidation). In fact, the ADP/$2e^-$ ratio calculated by the method of Sottocasa and Sandri[28] for the electrons accepted by ferricyanide is generally around 0.3. One might expect this ratio to be around 0.75, since the P/O ratio in the absence of

*Ohnishi *et al.*[27] have shown that mitochondria from the strain of yeast (*Saccharomyces carlsbergensis*) used by von Jagow and Klingenberg[3] have considerable outer membrane NADH dehydrogenase activity.

ferricyanide is 1.5. However, the reductase system located on the outer membrane is simultaneously operating, thus lowering the apparent ADP/$2e^-$ ratio.

These results indicate that when intact mitochondria are oxidizing NADH, the ferricyanide is receiving electrons after the site of antimycin A inhibition; that is, from the *c* cytochromes bound to the outer face of the inner membrane[13]. The antimycin A-insensitive activity is at least partly due to the outer membrane reductase system and may also be due to a direct interaction between ferricyanide and the flavoprotein on the external surface of the inner membrane. However, there are several indications that in intact coupled mitochondria the latter contribution to this activity is very small and is appreciable only after the mitochondria have been swollen.

The apparent K_m of the soluble NADH dehydrogenase for ferricyanide has been determined to be around 300 μM while that of the outer membrane is much less, <5 μM. If the ferricyanide were directly reacting to any considerable extent with the flavoprotein on the external face of the inner membrane of intact mitochondria, one would expect the trace of Fig. 8A to curve at low ferricyanide concentrations in the presence of antimycin A (reflecting the large K_m of the dehydrogenase for ferricyanide). In fact, however, this is not seen unless the mitochondria are swollen as in Fig. 8C. In the latter trace, the initial rate of ferricyanide reduction after the addition of antimycin A is faster than that for intact mitochondria, but approaches this rate at low ferricyanide concentrations.

TABLE I

RESULTS OF A TYPICAL EXPERIMENT SHOWING THE SPECIFIC ANTIMYCIN A-INSENSITIVE NADH OXIDOREDUCTASE ACTIVITIES FOR INTACT AND SWOLLEN MITOCHONDRIA AND THE PURIFIED OUTER MEMBRANE

Acceptor	*Antimycin A-insensitive NADH oxidoreductase (μmoles acceptor reduced/min per mg protein)*		
	Intact mitochondria	*Swollen mitochondria*	*Outer membrane*
Ferricyanide	2.0	4.0	14
Cytochrome *c*	0.08	0.08	0.7
Ratio	25	50	20

Table I summarizes the specific NADH dehydrogenase activities for intact and swollen mitochondria and for the outer membrane fraction after addition of antimycin A. When the mitochondria are intact, the ratio between the specific activities using ferricyanide and cytochrome *c* as acceptors is close to that for the outer membrane system alone (25 *versus* 20); however, this ratio increases significantly when the mitochondria are swollen (50), and it is very large (400) for the soluble inner membrane dehydrogenase. (This last ratio is difficult to measure since the apparent K_m of the soluble dehydrogenase for cytochrome *c* is on the order of 1 mM). The similarities between these ratios for the outer membrane and intact mitochondria and the increase seen upon swelling again indicate that ferri-

cyanide does not interact readily with the external dehydrogenase of the inner membrane in intact mitochondria.

DISCUSSION

Fig. 10 illustrates the pathway of electron transfer suggested by the results of these experiments. It is postulated that, in intact mitochondria, ferricyanide reacts with the inner membrane reductase system primarily after the antimycin A block. Upon swelling, the inner membrane dehydrogenase becomes less firmly bound to the membrane and may even detach completely. This would account both for the increase in antimycin A-insensitive NADH:ferricyanide oxidoreductase activity in swollen mitochondria and for the large activity found in the supernatant after hypotonic treatment.

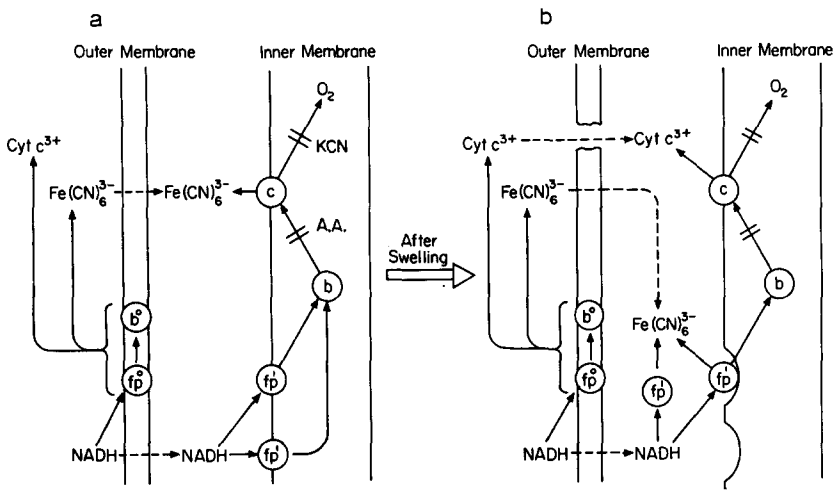

Fig. 10. Schematic representation of principal electron transfer pathways during the oxidation of externally added NADH by different acceptors. (a) Intact mitochondria. (b) After hypotonic treatment. Dotted lines indicate passage of NADH and acceptors into the intermembrane space. Solid lines indicate pathways of electron transfer. b° and fp° indicate cytochrome b_{555} and the flavoprotein NADH dehydrogenase of the outer membrane. fp' is the NADH dehydrogenase located on the outer face of the inner membrane. The NADH dehydrogenase located on the inner face of the inner membrane is not shown.

From the experiments using ferricyanide as the acceptor for the NADH dehydrogenases of plant mitochondria it is clear that the outer membrane system contributes significantly to the total activity. Such experiments were repeated on intact mitochondria from rat liver, rat heart and frog muscle. In each case, although mitochondria were unable to oxidize externally added NADH, they showed strong antimycin A-insensitive NADH:ferricyanide activities (typical activities were in the range of 1 to 3 μmoles ferricyanide reduced/min per mg protein). Hence, the true demonstration of the presence of an NADH dehydrogenase on the outer face of the inner membrane does not lie in the ferricyanide experiment alone. The results of Fig. 2 are, in fact, the clearest indication of this flavoprotein's localization.

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