Biochimica et Biophysica Acta, 501 (1978) 396—404

BBA 47445

PYRIDINE NUCLEOTIDE INTERACTIONS WITH ISOLATED PLANT MITOCHONDRIA

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(Received July 19th, 1977)

Summary

(1) Intact cauliflower mitochondria reduced added NAD, upon addition of malate, in the presence of antimycin A. The rate of NAD reduction was initially rapid but was greatly reduced within one or two minutes; over the same time period, disrupted mitochondria reduced NAD at a linear rate. Low levels of NADH in the reaction medium dramatically reduced the rate and extent of NAD reduction by intact mitochondria. Exogenous NADP was not reduced.

(2) External NAD reduction in the presence of malate was dependent on added P_i and glutamate but was not affected by effectors of malic enzyme, again showing a need for malate to enter the mitochondria.

(3) Addition of isocitrate to intact mitochondria did not induce NAD reduction, although external NAD relieved rotenone inhibition of citrate oxidation. Disrupted mitochondria reduced NAD in the presence of isocitrate.

(4) Isolated beetroot mitochondria did not oxidize added NADH, but reduced external NAD in the presence of antimycin A and malate. External NAD relieved rotenone inhibition of FeCN reduction with malate as substrate.

(5) The results show that malate oxidation occurs within the mitochondrial matrix but that the reducing power appears as external NADH and are interpreted to provide support for the proposal of a transmembrane hydrogen transfer across the inner membrane of plant mitochondria.

Introduction

Mitochondria isolated from most plant tissues readily oxidize exogenous NADH via a respiratory-linked dehydrogenase located on the outer face of the

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Abbreviations: TES, *N*-tris-(hydroxymethyl)methyl-2-aminoethane-sulfonic acid; FeCN, ferricyanide.

inner membrane [1—3]. These plant tissues do not depend on the operation of complex substrate-shuttle systems, like those found in mammalian mitochondria [4], to transfer reducing power from the cytoplasm to the mitochondria. An exception to this is red-beet tissue, the mitochondria of which cannot oxidize added NADH directly, but can do so by means of a malate-oxaloacetate shuttle [5].

It has been suggested that plant mitochondria can also transfer reducing equivalents out of the matrix directly [2,6]. Exogenous NAD recovered the oxidation of NAD-linked substrates in rotenone- (or piericidin A-) inhibited mitochondria. These mitochondria appear to be impermeable to the added NAD [1,2,6,7]. Two interpretations of this phenomenon have been offered; Coleman and Palmer [1] have suggested that malic enzyme located in the intermembrane space reduced external NAD which was reoxidized by the external NADH dehydrogenase. This suggestion was eliminated for cauliflower mitochondria by Day and Wiskich [2,6] who showed that malate oxidation, both in the presence and absence of added NAD and rotenone, was equally sensitive to *n*-butylmalonate (an inhibitor of malate transport; [4,8]) and was equally dependent on added P_i; i.e., malate had to penetrate the inner membrane to be oxidized. In addition it was shown that added NAD affected citrate and oxoglutarate oxidation in a similar manner [6]. On the basis of this data, another interpretation was proposed [2,6], namely that a transmembrane transhydrogenase was responsible for the reduction of external NAD. Subsequently, malic enzyme has been shown to reside in the matrix of plant mitochondria [9, 10], but interpretation of data has been complicated by the apparent operation of two or more different internal NADH dehydrogenases [7,11]. There is also the possibility that tissue differences with respect to malic enzyme location and activity may exist.

The present study sought to examine pyridine nucleotide interactions with isolated plant mitochondria in more detail, by investigating the reduction of added NAD in the presence of malate and isocitrate. The results presented support our previous suggestion of a transmembrane hydrogen transfer.

Materials and Methods

Mitochondria were isolated from cauliflower buds and beetroots as described previously [2,12] and subjected to integrity assays. Mitochondria from these tissues displayed the following properties: (1) Low succinate-cytochrome *c* reductase activity (less than 10 nmol $\cdot$ min^{-1} $\cdot$ mg $protein^{-1}$) indicating intact outer membranes [13]. (2) High ADP/O (2.7—2.9 with malate as substrate) and respiratory control (5—10 with malate) ratios, indicating tight coupling. (3) Oxidation of malate and citrate required the presence of P_i, even when uncoupler was added, showing that entry of these substrates occurred only via their transporters on the inner membrane [8]. (4) Exogenous NADH oxidation was unaffected by 15 μM rotenone, indicating impermeability of the inner membrane to this substrate.

Oxygen consumption and cytochrome *c* reduction were measured by published techniques [2]. Protein was estimated by the method of Lowry et al. [14] with bovine serum albumin as standard.

Reduction of exogenous NAD was measured by following the increase in absorbance at 340 nm in a Beckman Acta CIII spectrophotometer at room temperature, using cuvettes of 1 cm light path. The reaction mixture contained 0.1 ml mitochondrial suspension, 0.3 -0.5 mM NAD and 5 μM antimycin A in 3 ml of standard reaction medium which consisted of 0.25 M sucrose/10 mM TES buffer (pH 7.2)/5 mM $MgCl_2$/10 mM KH_2PO_4. NAD reduction was initiated by adding 10 mM malate.

FeCN reduction was followed at 410 nm in 3 ml of standard reaction medium which also contained 1 mM KCN, 0.9 mM $K_3Fe(CN)_6$ and 0.05 ml of mitochondrial suspension.

Results

A. Cauliflower mitochondria

(1) NAD reduction. Exogenous NAD was reduced rapidly and at a steady rate by detergent-disrupted cauliflower mitochondria in the presence of malate and antimycin A (Fig. 1A). Under these conditions NAD is reduced directly by malate dehydrogenase (or malic enzyme) released from the matrix. Since the reaction occurred in a relatively large volume (3 ml), product inhibition by NADH and oxaloacetate was not observed over the period of measurement.

The addition of malate to intact mitochondria resulted in a brief, but rapid, reduction of exogenous NAD, this reduction decreased to a very slow rate with-

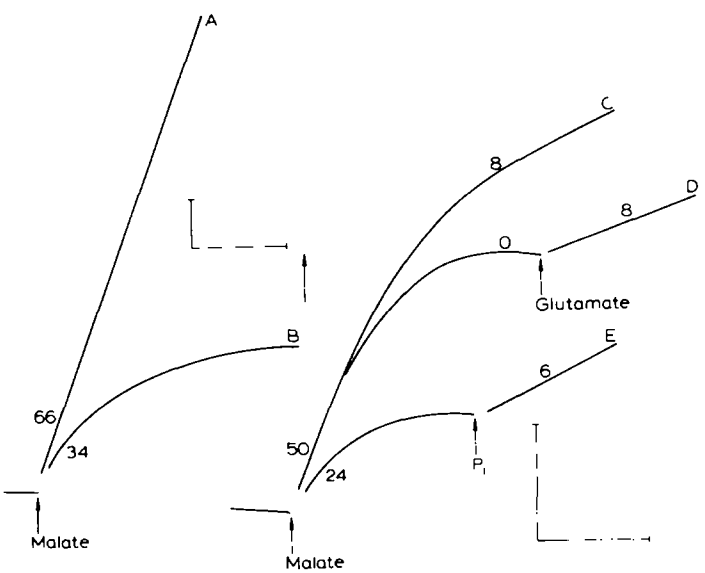

Fig. 1. Exogenous NAD^+ reduction by cauliflower mitochondria. NAD^+ reduction was measured spectrophotometrically as described in Materials and Methods. Malate (10 mM) was added to a reaction medium containing 10 mM glutamate, 5 μM antimycin A, 0.5 mM NAD^+, 10 mM P_i and 1.1 mg mitochondrial protein, except that in (A) 10 μl of "decon 90" detergent concentrate was included; (D), glutamate was omitted; (E), phosphate was omitted. Additions were glutamate (10 mM), P_i (10 mM). Vertical bars represent 0.02 absorbance units; horizontal bars, 1 min. Numbers along the traces represent nmol NADH reduced · min^{-1} · mg $protein^{-1}$.

in 1 min (Fig. 1) and eventually stopped. The rate and extent of reduction could be varied, depending on the presence of P_i and glutamate; when glutamate was omitted from the reaction medium less NAD was reduced and the reaction quickly slowed to zero (Fig. 1D). A subsequent addition of glutamate restored the final steady rate. In the absence of P_i, NAD reduction was limited more severely (Fig. 1E). These results suggest that the reduction of added NAD by intact mitochondria is governed by the rate of malate oxidation within the matrix, which can be limited by the rate of malate entry or the rate of malate dehydrogenase activity. The observed decrease in the rate of NAD reduction was probably due to the accumulation of internal NADH equilibrating the malate dehydrogenase reaction. Since antimycin A was present, NADH in the matrix could not be oxidized via the respiratory chain and therefore would accumulate; although reducing equivalents can leave the matrix (either as NADH or [H]), only a small quantity would need to be retained to cause inhibition of malate oxidation (since the malate dehydrogenase equilibrium does not favour this reaction). When P_i was omitted, malate entry was restricted [8] and NAD reduction was restricted; when glutamate was omitted, oxaloacetate accumulation restricted malate oxidation [2] and, hence, NAD reduction. Glutamate did not contribute directly to NAD reduction since no reduction was observed until malate was added (Fig. 1).

Addition of relatively small amounts (10% of the NAD concentration) of NADH to the external medium prevented the initial rapid phase of NAD reduction and susequent reduction was slow (Fig. 2B). This implies that a large concentration gradient of NADH (high in the matrix, low in the medium) is needed for rapid efflux of reducing equivalents. Exogenous NADP was not reduced by these mitochondria (Fig. 2C).

The results shown in Fig. 3 suggest that very little NAD reduction was attributable to malic enzyme activity. In the absence of glutamate, CoA, an activator

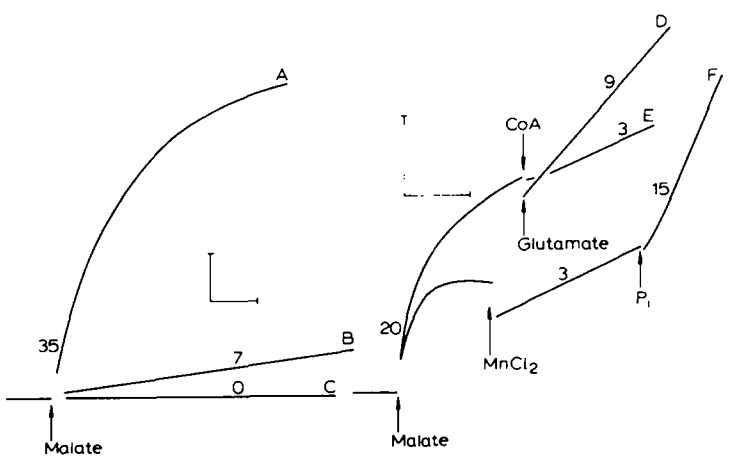

Fig. 2. Exogenous NAD(P) reduction by cauliflower mitochondria. Assayed as described in Fig. 1, with 2.4 mg mitochondrial protein, except that in (B) 0.5 mM NADH was included; (C), 0.5 mM $NADP^+$ replaced NAD^+; (D) and (E), glutamate was omitted; (F), P_i was omitted. The final concentrations of additions were CoA (0.1 mM), glutamate (10 mM), P_i (10 mM) and $MnCl_2$ (6 mM). Vertical bars represent 0.02 absorbance units; horizontal bars, 1 min. Numbers along the traces are rates expressed as nmol NADH · min^{-1} · mg $protein^{-1}$.

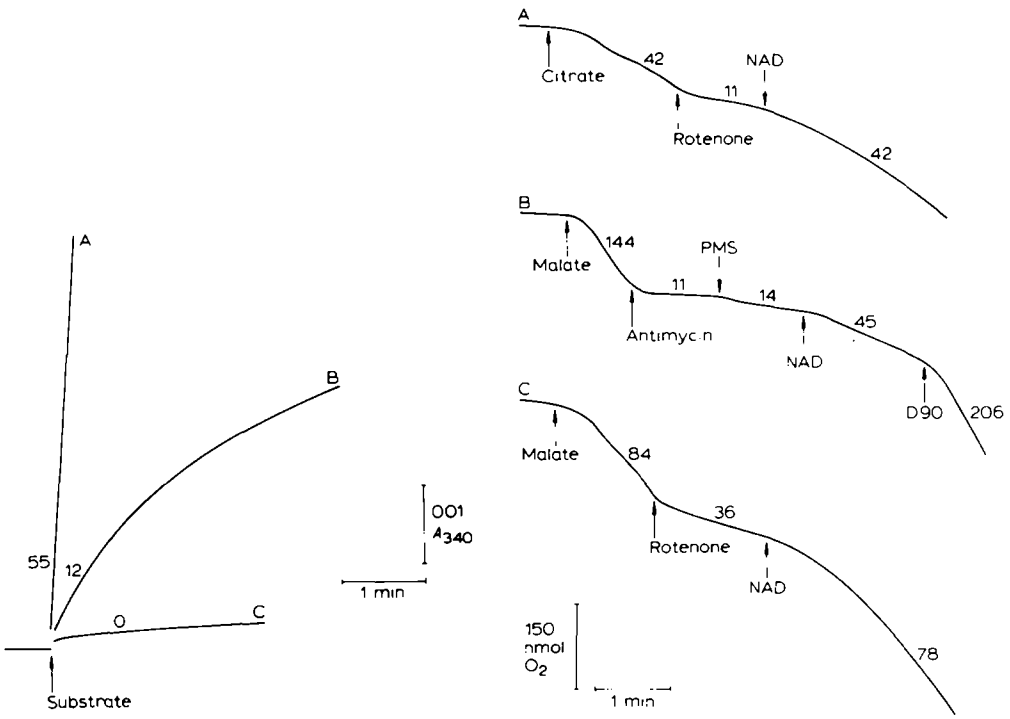

Fig. 3. Exogenous NAD^+ reduction by cauliflower mitochondria. Assay conditions are described in Fig. 1 except that in (A) and (C) isocitrate (10 mM) replaced malate as substrate. In (A) 10 μl "decon 90" detergent was included in the medium. In (A) and (C) 1.4 mg mitochondrial protein was used, while in (B) 0.7 mg was used. Rates are expressed as nmol NADH · min^{-1} · mg $protein^{-1}$.

Fig. 4. Oxygen consumption by cauliflower mitochondria. Oxygen uptake was measured as described in Materials and Methods. To 3 ml of standard reaction medium, 3.0 mg mitochondrial protein, 1 mM ADP, 10 mM glutamate (B and C) and either 10 mM citrate (A) or 10 mM malate (B and C) were added. After a steady rate was obtained the following additions were made, as shown: 15 μM rotenone, 5 μM antimycin A, 10 μM phenazine methosulphate (PMS), 0.5 mM NAD^+ and 10 μl "decon 90" detergent (D90). Rates are expressed as nmol O_2 · min^{-1} · mg $protein^{-1}$.

of malic enzyme [10,15] did not affect NAD reduction (Fig. 2E), although glutamate stimulated it (Fig. 2D). When P_i was absent, $MnCl_2$, also a cofactor of malic enzyme [10,15], stimulated NAD reduction only slightly; P_i had a more marked affect (Fig. 2F). Thus, under these conditions, malate dehydrogenase plays a more important role than malic enzyme in reducing NAD, and removal of oxaloacetate is essential for the maintenance of this activity.

Unlike malate, iso-citrate did not induce external NAD reduction in the presence of antimycin A in intact mitochondria (Fig. 3), although NAD was rapidly reduced when the mitochondria were disrupted. Von Jagow and Klingenberg [16] also failed to see external NAD reduction with citrate as substrate, while Palmer and Arron [7] found citrate to be a poorer substrate in this respect than malate. Nonetheless, as shown in Fig. 4 the addition of NAD relieved rotenone-inhibition of citrate oxidation.

(2) Oxygen electrode studies. Interaction of exogenous NAD with cauliflower mitochondria was also investigated using the oxygen electrode. In Fig. 4 phenazine methosulphate was used to link NAD reduction to oxygen consumption; addition of phenazine methosulphate alone to antimycin A-inhibited mitochondria had little effect suggesting that phenazine methosulphate did not penetrate the inner membrane and react with internal NADH. Addition of NAD caused a slow, but steady, rate of oxygen uptake which was dramatically stimulated by detergent (Fig. 4). The stimulation of O_2 uptake by NAD, in the presence of phenazine methosulphate and antimycin A approximated the initial rate of NAD reduction upon addition of malate (Fig. 1), and the NAD-stimulation of rotenone-inhibited malate oxidation. The linearity of the rates shown in Fig. 4, where externally formed NADH was reoxidized (either by phenazine methosulphate or the respiratory chain), is in contrast to the rapid decline seen in the NAD reduction studies (Fig. 1) and supports the suggestion that accumulation of external NADH inhibits external NAD reduction.

B. Beet mitochondria

In contrast to other plant mitochondria, those from fresh beetroot do not

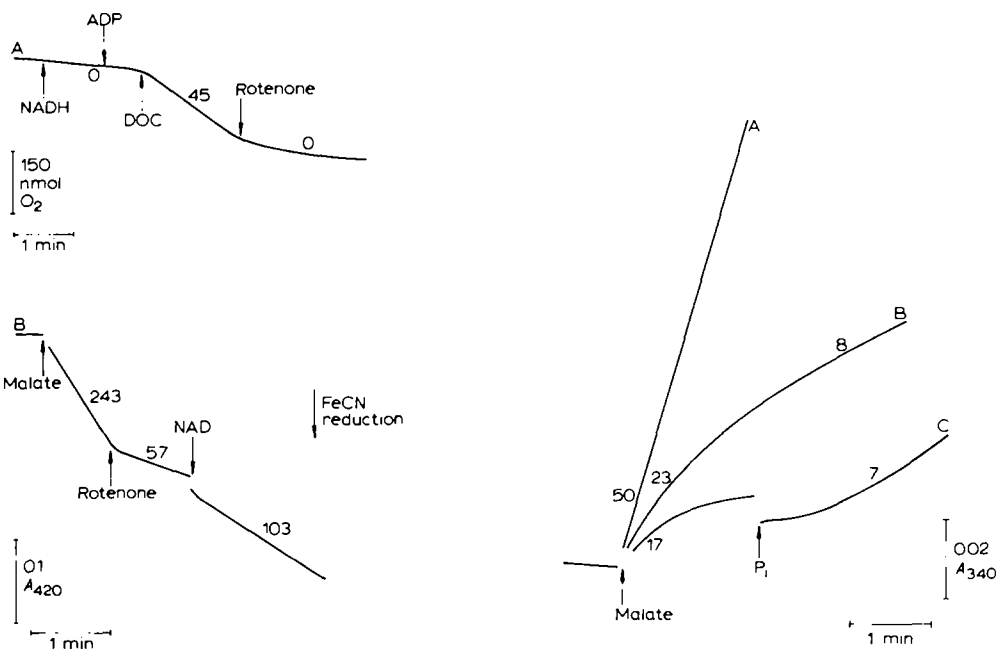

Fig. 5. (A) Oxygen consumption by beetroot mitochondria. Beetroot mitochondria were added to 3 ml of standard reaction medium; subsequent additions were, as shown, 1 mM NADH, 0.25 mM ADP, 0.05% deoxycholate (DOC) and 10 μM rotenone. (B) FeCN reduction by beetroot mitochondria. FeCN reduction was measured at 410 nm as described in Materials and Methods; 10 mM glutamate and the mitochondria were added to a cuvette containing 3 ml of reaction medium. Subsequent additions, as indicated, were 10 mM malate, 10 μM rotenone and 0.5 mM NAD. Rates are expressed as nmol FeCN · min^{-1} · mg $protein^{-1}$.

Fig. 6. Exogenous NAD^+ reduction by beetroot mitochondria. NAD^+ reduction was measured as described in Fig. 1. In (A) 10 μl "decon 90" detergent was included in the medium; in (C) P_i was omitted from the medium and added as shown (to a concentration of 10 mM). Rates are expressed as nmol NADH · min^{-1} · mg $protein^{-1}$.

oxidize exogenous NADH [5] unless the inner membrane is broken and then NADH oxidation is inhibited by rotenone (Fig. 5). This implies that isolated beetroot mitochondria are not permeable to pyridine nucleotides; yet external NAD reduction by beetroot mitochondria was very similar to that by cauliflower mitochondria (Fig. 6 vs. Fig. 1), and addition of NAD to the medium relieved rotenone inhibition of malate oxidation (Fig. 5). In the latter experiment malate oxidation was followed by measuring reduction of FeCN, a non-penetrant which accepts electrons from externally-facing cytochrome *c* on the inner membrane [17,18] and from the outer-membrane electron transport chain [19]. Added NAD was reduced via the inner membrane hydrogen-transferring system and oxidized by the outer membrane NADH dehydrogenase, leading to FeCN reduction. Hence, Fig. 4C and 5B are analagous although different external NADH dehydrogenases are involved.

These considerations also explain the results of Palmer and Arron (Table 6 in ref. 7) and eliminate the need to postulate activation of unknown pathways. That is, the addition of NAD would stimulate FeCN reduction via the NADH dehydrogenase on the outside of the inner membrane (some of this, being linked through cytochrome *c*, would be antimycin-sensitive [13,5]) and via the NADH dehydrogenase on the outer membrane (antimycin-insensitive). With beetroot mitochondria, external NAD will recover the rotenone inhibition of malate oxidation, but re-oxidation of the NADH has to be coupled to the outer-membrane NADH dehydrogenase system which is antimycin-insensitive [5].

Discussion

The results reported here show that reducing power can be transferred from the matrix of intact plant mitochondria, upon oxidation of malate, to externally supplied NAD. External NAD reduction was not due to the activity of external (or internal) malic enzyme, as suggested for Jerusalem artichoke mitochondria [7]. In addition to the data of Fig. 2B, the kinetics of NAD reduction are not compatible with an external enzyme being the primary reductant of exogenous NAD. An external enzyme system should yield a sustained linear rate of reduction since the products would be diluted in a relatively large volume. This pattern was only realised when the mitochondria were disrupted (Fig. 1A).

It appears that in intact cauliflower mitochondria, external NAD is in some balance with internal NADH during malate oxidation. The rate of NAD reduction is governed by (1) the rate of malate penetration of the inner membrane (i.e., malate concentration in the matrix), (2) removal of oxaloacetate and (3) the NAD/NADH ratio in the external medium. Observation (3) may simply reflect a restriction of NAD(H) diffusion across the inner membrane. On the other hand, if [H] transfer involves a transmembrane transhydrogenase [2,6], the inhibition of NAD reduction by external NADH may involve competition between the reduced and oxidized nucleotide for binding sites on the outer surface of the inner membrane.

Several observations, including the integrity assays described in Materials and Methods, suggest that NAD did not cross the inner membrane. No NAD reduction occurred when isocitrate was substrate instead of malate (Fig. 3), despite

the fact that external NAD relieved rotenone inhibition of isocitrate oxidation (Fig. 4, ref. 6 and 7). This discrepancy is difficult to explain but probably involves the susceptibility of isocitrate dehydrogenase to inhibition by NADH [20] and slow isocitrate transport. In the presence of antimycin A inhibition of electron transport is virtually complete and no potential is generated across the membrane to drive citrate transport. Rotenone, on the other hand is an incomplete inhibitor of plant respiration [2,9,21] thus allowing some oxidation of internal NADH (and hence, generation of a proton motive force) without interfering with external NADH oxidation at all [2,3,21]. Alternatively, accumulation of internal NADH in the presence of antimycin A may restrict isocitrate dehydrogenase activity; it is conceivable that the internal NADH level may be high enough to inhibit isocitrate oxidation but not high enough to drive transmembrane hydrogen transfer.

Although beetroot mitochondria do not oxidize external NADH (apparently lacking the dehydrogenase on the outside of the inner membrane [5]), they do reduce external NAD. Obviously, NADH did not enter these mitochondria or it would have been oxidized by the rotenone-sensitive internal NADH dehydrogenase (as it is when detergent is added, Fig. 5). This in turn, implies that NAD did not cross the inner membrane.

In our view, the best explanation of these results is the operation of a transhydrogenase across the inner membrane, capable of transferring reducing equivalents from within the matrix to exogenous NAD in the intermembrane space, upon oxidation of NAD-linked substrates. The alternative explanations of Palmer and colleagues [1,7,9] are not necessary. For example, the results of Palmer and Arron [7] can be explained simply on the basis of oxaloacetate inhibition without invoking separate matrix compartments and other piericidin A-insensitive pathways for NADH oxidation.

The postulation of a transmembrane transhydrogenase is not novel; Moyle and Mitchell [22] suggested that the energy-linked transhydrogenase of rat liver mitochondria was positioned across the membrane and Kohler and Saz [23] have demonstrated the functioning of an NADH-NAD transhydrogenase in mitochondria from *Ascaris* muscle. Interestingly enough, this latter enzyme appears to transfer [H] from external NADH to intramitochondrial NAD upon oxidation of extramitochondrial malate. Although transhydrogenase activity itself was not assayed for in cauliflower mitochondria, Hackett [24] reported NAD-NADH transhydrogenase activity in mitochondria isolated from a number of plant tissues. The rates of NAD reduction measured by Hackett [24] were of the same order of magnitude as those reported here.

In contrast to the results presented here, Davis et al. [25] found that exogenous NAD was not reduced by addition of malate to beef heart mitochondria unless they were disrupted. In fact it seems that most animal mitochondria are impermeable to pyridine nucleotides and NAD(H) does not communicate across the membrane [26,27], except, perhaps, in the presence of Ca^{2+} [28, 29].

Since plant mitochondria oxidize exogenous NADH readily via their respiratory chain [1—3], are permeable to oxaloacetate [5,30] and are capable of exporting reducing equivalent from the matrix (this report), it seems unlikely that the difference in redox potential between mitochondrial and cytoplasmic

NAD couples, observed with mammalian tissues [4,31] can be maintained in the plant cell, unless the postulated transmembrane hydrogen transfer is regulated. The oxidation of cytoplasmic NADH should present little difficulty for most plant tissues. The real question is, do they maintain their intramitochondrial pyridine nucleotide pool more reduced? If the two pools are not in redox equilibrium in vivo there must be some restriction on the operation of the transmembrane transfer, since, as shown here, such equilibrium can occur in isolated mitochondria. There is no supporting evidence to suggest that the transfer is energy linked [6]. However, it could require a threshold gradient (or level of internal NAD reduction) to be functional. Thus, malate dehydrogenase (with glutamate to remove oxaloacetate) or malic enzyme can achieve this gradient whilst isocitrate dehydrogenase becomes inhibited before it reaches this gradient; hence no reduction of external NAD.

The interaction of cytoplasmic and mitochondrial NAD couples, and the maintenance of their redox states, represent a key metabolic difference between animal and plant cells.

Acknowledgements

This research was supported by a grant from the Australian Research Grants Committee; D.A. Day was the recipient of an Australian Commonwealth Postgraduate Award. We wish to thank Ms. C. Wilkins for typing the manuscript.

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