Exogenous NAD$^+$ Effects on Plant Mitochondria

A REINVESTIGATION OF THE TRANSHYDROGENASE HYPOTHESIS

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ABSTRACT

Addition of NAD$^+$ to purified potato (Solanum tuberosum L.) mitochondria respiring α-ketoglutarate and malate in the presence of the electron transport inhibitor rotenone, stimulated O$_2$ uptake. This stimulation was prevented by incubating mitochondria with N-4-azido-2-nitrophenyl-aminobutyryl-NAD$^+$ (NAP$_4$-NAD$^+$), an inhibitor of NAD$^+$ uptake, but not by 1 mM EGTA, an inhibitor of external NADH oxidation. NAD$^+$-stimulated malate-cytochrome c reductase activity, and reduction of added NAD$^+$ by intact mitochondria, could be duplicated by rupturing the mitochondria and adding a small quantity to the cuvette. The extent of external NAD$^+$ reduction was correlated with the amount of extra mitochondrial malate dehydrogenase present. Malate oxidation by potato mitochondria depleted of endogenous NAD$^+$ by storing on ice for 72 hours, was completely dependent on added NAD$^+$, and the effect of NAD$^+$ on these mitochondria was prevented by incubating them with NAP$_4$-NAD$^+$. External NAD$^+$ reduction by these mitochondria was not affected by NAP$_4$-NAD$^+$. We conclude that all effects of exogenous NAD$^+$ on plant mitochondrial respiration can be attributed to net uptake of the NAD$^+$ into the matrix space.

The effects of exogenous NAD$^+$ on O$_2$ consumption and metabolite oxidation by isolated plant mitochondria has been extensively investigated and vigorously debated for the past decade. Coleman and Palmer (2) were the first to note that external NAD$^+$ stimulated malate oxidation and reduced its sensitivity to the electron transport inhibitor rotenone, and also decreased associated ADP/O ratios by one-third. It was consequently proposed that some NAD-linked malic enzyme was localized in the intermembrane space (i.e. between the inner and outer mitochondrial membranes) where it could reduce added NAD$^+$; subsequent re-oxidation of the NADH thus formed, via the external respiratory-linked NADH dehydrogenase (9), would be rotenone-insensitive and coupled to only two phosphorylations. Day and Wiskich (3, 4) confirmed these results but found that malate transport was a prerequisite for the NAD$^+$ effects, and it was subsequently demonstrated that all of the mitochondrial malic enzyme was localized on the matrix side of the inner membrane (7). Stimulation of citrate and α-ketoglutarate oxidation by external NAD$^+$ was also observed (4, 6), and Day and Wiskich postulated the operation of a transmembrane transhydrogenase capable of transferring reducing equivalents from matrix NADH to external NAD$^+$.

Both of these hypotheses were formulated on the assumptions that the inner membrane of the mitochondria was impermeable to exogenous pyridine nucleotides and that the observed rotenone-insensitive O$_2$ uptake was catalyzed by the external NADH dehydrogenase. However, it is now known that plant mitochondria possess an internal pathway for rotenone-insensitive NADH oxidation (1, 11, 12, 23), and it is also firmly established that NAD$^+$ can be accumulated by mitochondria from the external medium (13, 14, 20). Although this net NAD$^+$ uptake is small in extent, it can lead to very substantial increases in the matrix concentration of NAD$^+$, and this in turn can stimulate internal NAD-linked enzyme activity (20) and increase dramatically the rate of electron transport via the internal rotenone-insensitive bypass (14, 18), thus accounting for many of the early observations.

However, it is feasible that more than one mechanism can lead to the NAD$^+$ effects noted above, and operation of a transmembrane transhydrogenase remains a possibility in view of the demonstration that added NAD$^+$ can be reduced externally by intact mitochondria under certain conditions (4, 6; but see Ref. 17). In an attempt to obtain a general consensus on the mechanism underlying effects of exogenous NAD$^+$, we decided to reinvestigate the transhydrogenase hypothesis, using mitochondria purified on Percoll gradients. We conclude that all NAD$^+$ effects on O$_2$ uptake can be accounted for by the uptake of NAD$^+$ into the mitochondria.

MATERIALS AND METHODS

Potatoes (Solanum tuberosum) and cauliflowers (Brassica oleracea botrytis) were purchased from local markets. Mitochondria were isolated and purified as described by Neuburger et al. (15), using self-generating Percoll gradients. NAP$_4$-NAD$^+$ was synthesized as in Neuburger and Douce (14).

O$_2$ uptake was measured at 25°C with a Clark-type O$_2$ electrode system from Hansatech Ltd. (Hardwick Industrial Estate, Norfolk, U.K.). The reaction medium contained 0.3 mM mannitol, 6 mM MgCl$_2$, 10 mM KCl, 10 mM phosphate buffer (pH 7.2), 0.1% (w/v) defatted BSA, and known quantities of mitochondrial protein in a volume of 1 ml. The O$_2$ concentration in air-saturated medium was taken as 240μM. Mitochondrial integrity was estimated using succinate Cyt c reduction (8) or Cyt c-dependent O$_2$ uptake (15).

Mitochondrial protein was estimated by the method of Lowry et al. (10).

Cyt c reduction was measured spectrophotometrically at 550 nm in 3 ml of O$_2$ reaction medium (see above) containing 0.5 mM KCN and 50 μM Cyt c. The reaction was initiated by adding 10 μM substrate.

Reduction of exogenous NAD$^+$ was measured spectrophoto-

1Abbreviations: NAP$_4$-NAD$^+$, N-4-azido-2-nitrophenyl-aminobutyryl NAD$^+$; MDH, malate dehydrogenase; OAA, oxaloacetate.
metrically at 340 nm in 3 ml of O₂ reaction medium containing 5 μM antimycin A and 0.5–1 mM NAD⁺. The reaction was initiated by adding 10 mM substrate.

Malate dehydrogenase was measured by following NADH oxidation in the presence of OAA (16).

Mitochondrial NAD⁺ content was measured as described by Tobin et al. (20).

RESULTS AND DISCUSSION

Figure 1 illustrates the well known effects of rotenone and NAD⁺ on NAD-linked substrate oxidation, in this case with purified potato mitochondria. Identical effects of these compounds were seen with malate and a-ketoglutarate. The degree of inhibition by rotenone in the absence of added NAD⁺ is correlated with the level of endogenous NAD⁺ (14, 20), and this varies between species and between different preparations from the same tissue. The NAD⁺ content of the mitochondria depicted in Figure 1 was 4.4 nmol mg⁻¹ protein, and rotenone inhibited a-ketoglutarate oxidation by 56% (Fig. 1A). When malate is substrate, rotenone resistance also depends on the relative activities of malate dehydrogenase (MDH) and malic enzyme, because OAA levels influence the rate of O₂ uptake. Thus, when MDH activity is greater than that of malic enzyme, OAA accumulates; subsequent addition of rotenone causes the level of NADH in the matrix to rise also, and since MDH equilibrium favors malate formation, the increase in both OAA and NADH will severely restrict O₂ uptake. If malic enzyme is active, the accumulated OAA is removed by MDH at the expense of NADH formed during the conversion of malate to pyruvate, and O₂ uptake recovers (18, 21, 22). At pH 6.5, the condition used in the experiment shown in Figure 1C, malic enzyme alone contributes to O₂ uptake (20), OAA does not accumulate, and rotenone inhibition is the same for both malate and other NAD-linked substrates. The same result is obtained when care is taken to remove OAA (e.g. by transamination with glutamate) during operation of MDH (21, 22). Hence, there is no need to postulate a specific link between malic enzyme and the rotenone-insensitive pathway of electron transport (19).

In the presence of external NAD⁺, rotenone had little, if any, effect on O₂ uptake by potato tuber mitochondria oxidizing α-ketoglutarate or malate (Fig. 1). Recently (14), a specific inhibitor (NAP⁺-NAD⁺) of NAD⁺ uptake has been synthesized. In the dark, this compound competes with NAD⁺ for the innermembrane transport system, but in the light it binds covalently to the transporter and irreversibly inhibits NAD⁺ uptake (14). When potato mitochondria were preincubated in the light with NAP⁺-NAD⁺, NAD⁺ had little effect on O₂ uptake (Fig. 1B). In other respects, O₂ uptake was not affected by the inhibitor, which also had no effect on the oxidation of external NADH (Table I). EGTA, on the other hand, does inhibit external NADH oxidation (2), but prior addition of this did not prevent NAD⁺ stimulation of O₂ uptake (Fig. 1A), in agreement with Palmer et al. (18).

The above results suggest very strongly that NAD⁺ acts within the matrix to relieve inhibition by rotenone, rather than being reduced externally by a transmembrane transhydrogenase. However, added NAD⁺ was observed to promote both malate and α-ketoglutarate-Cyt c reductase activity in mitochondria which were more than 97% intact (Figs. 2, A and B). Cyt c cannot penetrate the outer membrane of mitochondria and NAD⁺ stimulation of Cyt c reduction was previously considered as evidence for the direct transfer of reducing equivalents from internal NADH to external NAD⁺ via a transmembrane transhydrogenase. The NADH thus formed extramitochondrially could be oxidized via the outer membrane antimycin-insensitive NADH-Cyt c reductase (3, 4). (This result cannot be explained solely on the basis of some external MDH or malic enzyme since it is seen also with α-ketoglutarate as substrate; Fig. 2B.) Although the NAD⁺ stimulation of Cyt c reduction can appear quite substantial (Fig. 2, A and B), it is misleading since the stimulated rate of Cyt c reduction with malate and α-ketoglutarate was no faster than that observed with succinate as substrate (Fig. 2C), and this can be accounted for by the small percentage of mitochondria with ruptured outer membranes present in the preparations (note that the inner membrane of these mitochondria may also have been damaged). Furthermore, all of the α-ketoglutarate-Cyt c reductase activity was inhibited by antimycin A showing that it was mediated by the inner membrane. We suggest that the little Cyt c reduction observed with this substrate was also due to a small proportion of mitochondria with ruptured outer membranes, allowing access of Cyt c to the inner membrane. If these damaged mitochondria were depleted of NAD⁺, as might be

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Table 1. Effect of NAP⁺-NAD⁺ on the Oxidation of α-Ketoglutarate and Exogenous NADH by Purified Potato Mitochondria

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Oxygen Consumption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 3</td>
</tr>
<tr>
<td></td>
<td>nmol min⁻¹ mg⁻¹ protein</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>95</td>
</tr>
<tr>
<td>α-Ketoglutarate + NAP⁺-NAD⁺</td>
<td>102</td>
</tr>
<tr>
<td>NADH</td>
<td>133</td>
</tr>
<tr>
<td>NADH + NAP⁺-NAD⁺</td>
<td>136</td>
</tr>
</tbody>
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expected if the inner membrane integrity had been affected, then adding NAD\(^+\) would stimulate \(\alpha\)-ketoglutarate dehydrogenase (14) and thus Cyt c reduction.

The malate Cyt c reductase, on the other hand, was largely insensitive to antimycin A (Fig. 2A), suggesting that Cyt c was reduced by the outermembrane system. This activity in the intact mitochondrial preparation could be duplicated by deliberately rupturing the mitochondria and adding a small quantity (5%) of the protein to the reaction cuvette (Fig. 2A, broken line). It is thus feasible that the malate Cyt c reductase activity observed in the presence of antimycin was due to some contaminating external MDH. If so, upon presentation of malate and NAD\(^+\), NADH will be produced extramitochondrially and be available to the outer membrane reductase. Although it has been shown previously that small quantities of MDH are bound (presumably during preparation) to the outer mitochondrial membrane (5), it is also possible that the activity found in our preparations could have originated from the few ruptured mitochondria present.

Fig. 2. Cyt c reduction by intact, purified potato mitochondria. Where indicated, 10 mM malate, 5 mM \(\alpha\)-ketoglutarate (KG), 10 mM succinate, 0.2 mM NAD\(^+\), and 5 \(\mu\)M antimycin A were added. Numbers on traces refer to nmol min\(^{-1}\) mg\(^{-1}\) protein; 0.13 mg protein was used in 3 ml of reaction medium. The broken line in A shows the rate of Cyt c reduction by 6.5 \(\mu\)g (equivalent to 5% of the quantity of intact mitochondria) of ruptured mitochondria. The rate of succinate-Cyt c reduction in ruptured mitochondria was 826 nmol min\(^{-1}\) mg\(^{-1}\) protein. Mitochondria were ruptured by swelling in water for 1 min.

FIG. 3. External NAD\(^+\) reduction by purified potato mitochondria. A, Intact mitochondria 1 mM NAD\(^+\); B, intact mitochondria 0.5 mM NAD\(^+\); C, 5% ruptured mitochondria 1 mM NAD\(^+\). Additions as indicated were; 10 mM malate, and 10 mM \(\alpha\)-ketoglutarate (KG). For other details, see “Materials and Methods” and Figure 2. The mitochondria were judged to be 97.5% intact.

Fig. 4. External NAD\(^+\) reduction by cauliflower mitochondria. For details, see Figure 3. The concentration of NAD\(^+\) was 1 mM. A, Washed mitochondria (Mw), 0.24 mg protein; B, purified mitochondria (Mp), 0.46 mg protein. Mw were judged to be 90% intact and Mp 96.5%. External MDH activity (OAA reduction) was 754 and 280 nmol min\(^{-1}\) mg\(^{-1}\) protein in Mw and Mp, respectively. Numbers on traces refer to nmol NAD\(^+\) reduced min\(^{-1}\) mg\(^{-1}\) protein.

Fig. 5. O\(_2\) consumption (A and B) and external NAD\(^+\) reduction (C and D) by 12-h aged mitochondria. Potato mitochondria were purified on Percoll gradients and stored at 0\(\degree\)C for 72 h prior to experiments. The NAD\(^+\) content of the mitochondria was 0.3 nmol mg\(^{-1}\) protein after aging. A and C, Control mitochondria; B and D, mitochondria treated with NAP\(_e\)-NAD\(^+\) (see Figure 1 and the text for details). In A and B, 0.22 mg mitochondrial protein was used; in C and D, 0.13 mg protein. The mitochondria were judged to be 96% intact.

Purified potato mitochondria reduced added NAD\(^+\) in the presence of antimycin A (to prevent re-oxidation of NADH by the respiratory chain) and malate, as observed previously with mitochondria from other tissues (6, 17). The extent of this reduction depended on the level of added NAD\(^+\), but was not seen with \(\alpha\)-ketoglutarate as substrate (Fig. 3). However, using a
few per cent of ruptured mitochondria gave a pattern of NAD+ reduction similar to that by intact mitochondria (Fig. 3C).

Figure 4 shows a comparison of external NAD+ reduction by washed and by purified cauliflower mitochondria. This comparison was made because the original experiments were done with washed cauliflower mitochondria (3, 4, 6), and it is possible that crude preparations contain larger quantities of contaminating external enzymes. Indeed, NAD+ was reduced substantially more quickly and to a larger extent by the washed preparation (Fig. 4), and this was correlated with a larger proportion of external MDH. In fact, the ratio of external MDH activity in the washed preparation to that in the purified preparation was 2.65, which is virtually identical to the ratio of initial NAD+ reduction rates (2.55).

The above results are consistent with, but do not prove, the idea that external NAD+ reduction by plant mitochondria is catalyzed by extramitochondrial MDH rather than a transmembrane transhydrogenase. We sought to provide more direct evidence by using mitochondria depleted of cofactors. Plant mitochondria, notably these from potato tubers, display remarkable longevity when carefully purified on Percoll gradients and stored on ice (14). Even as much as 5 d after isolation, the mitochondria show rapid rates of O2 uptake, good respiratory control, and largely intact (greater than 90%) outer membranes (14; see also Fig. 5A). The main effect of aging the mitochondria is to deplete them of endogenous co-factors, particularly NAD+ (see legends to Figs. 1 and 5). Hence, O2 uptake with malate as substrate is completely dependent on added NAD+, and stimulation by NAD+ is severely depressed by pre-treating the mitochondria with the inhibitor of NAD+-transport, NAPe-NAD+ (14; see also Fig. 5B). Similar effects were seen with α-ketoglutarate substrate (not shown). Since operation of a transmembrane transhydrogenase would also require NAD+ to be taken up into the matrix of these NAD-depleted mitochondria, treating the aged mitochondria with NAPe-NAD+ prior to addition of NAD+ should inhibit external NAD+ reduction, by at least the same extent as it inhibits O2 uptake. Yet the rates and extent of external NAD+ reduction by aged, intact potato mitochondria were the same whether treated with NAD+-transporter inhibitor or not (Fig. 5, C and D). Clearly, then, the observed reduction of external NAD+ by mitochondria in the presence of malate is due to the presence of a small quantity of external MDH, as proposed by Palmer (17). However, this external MDH does not in any way contribute to the relief of rotenone-inhibited O2 uptake by added NAD+ (Fig. 1).

**CONCLUDING REMARKS**

The present results, together with those in Neuburger and Douce (14), Palmer et al. (18), and Tobin et al. (20), demonstrate conclusively that the stimulation of respiration of intact plant mitochondria by added NAD+ is due to uptake of NAD+ into the matrix space. The consequent increase in the concentration of endogenous NAD+ stimulates the activity of all NAD-linked enzymes (14, 20), thus providing more reducing power to the respiratory chain and stimulating the rotenone-insensitive pathway. Since the rotenone-insensitive pathway has a lower affinity for NADH than the rotenone-sensitive pathway (12), it shows a greater dependence on added NAD+ than does the rotenone-sensitive pathway.

**LITERATURE CITED**


