Oxidation of Reduced Nicotinamide Adenine Dinucleotide Phosphate by Isolated Corn Mitochondria

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ABSTRACT
Isolated corn (Zea mays L.) mitochondria were found to oxidize reduced nicotinamide adenine dinucleotide phosphate in a KCl reaction medium. This oxidation was dependent on the presence of calcium or phosphate or both. Strontium and manganese substituted for calcium, but magnesium or barium did not. The oxidation of NADPH produced contraction of mitochondria swollen in KCl. Further evidence that the oxidation of NADPH was coupled was observed in respiratory control and adenosine diphosphate-oxygen ratios that were comparable to those reported for reduced nicotinamide adenine dinucleotide. The pathways of electron flow from NADH and NADPH were compared through the addition of electron transport inhibitors. The only difference between the two dinucleotides was that amytal was found to inhibit almost totally the state 3 oxidation of NADPH, but had little effect on the state 3 oxidation of NADH. The hypothetical pathways for electron flow from NADPH are discussed, as are the possible sites of calcium and phosphate stimulation.

Isolated plant mitochondria have been reported by numerous workers to possess the ability to oxidize exogenous NADH (1, 3, 7, 9, 11, 14, 21, 22), a characteristic not found in isolated animal mitochondria. This oxidation of exogenous NADH typically yields P/O, or ADP/O, values indicating the involvement of only two coupling sites (1, 3, 9, 11, 21). The question of why plant mitochondria, but not animal mitochondria, have evolved a means of oxidizing exogenous NADH has not been satisfactorily answered. There has been no evidence indicating in vivo mitochondrial oxidation of exogenous NADH even though the back flow of reducing equivalents out of the mitochondrion has been suggested (16). Kennedy and Lehninger (15) were the first to report that fatty acid oxidation in animals occurs exclusively in the mitochondria with the NADH produced being oxidized via the normal mitochondrial NADH dehydrogenase linked pathways. In recent plant studies, however, the complete \( \beta \)-oxidation of fatty acids by mitochondria has been questioned. Cooper and Beevers (6) reported that in the endosperm of germinating castor bean the enzymes of \( \beta \)-oxidation are localized primarily in the glyoxysomes, and not in the mitochondria, but that the NADH produced by such oxidation is not utilized in the glyoxysomes. Thus the transfer of the NADH to the mitochondria of this tissue and its oxidation is possible.

Like NADH, NADPH is known to be cytoplasmically generated and utilized in several reactions, being notably produced in the dehydrogenation of glucose-6-P. To our knowledge, the possible oxidation of NADPH by isolated mitochondria has been only minimally studied, even though there exist, in certain tissues, cytoplasmic concentrations of NADPH that are probably comparable to those of NADH. Stern and Johnston (20) studied NADPH oxidation in wheat embryos and ascribed much of the NADH and NADPH oxidation to particulate structures other than mitochondria. The oxidation of cytoplasmic NADPH has been reported (10), but again this oxidation has not been linked to the mitochondria.

This paper reports that isolated corn mitochondria do oxidize NADPH via a coupled pathway under specific reaction media conditions.

MATERIALS AND METHODS
Corn seedlings (Zea mays L., Wf9 \times M14) were grown in the dark on paper toweling saturated with 0.1 mM CaCl\(_2\), at 29 ± 0.5 C. Mitochondria were isolated by the procedure of Miller et al. (18).

Experiments were performed in a glass reaction vessel equipped with a Clark oxygen electrode (Yellow Springs Instrument Co.) and fitted in the light path of a Bausch and Lomb Spectronic 70 spectrophotometer. Polarographically measured oxygen concentration and percentage of transmittance changes were simultaneously recorded on a dual channel recorder. The reaction media (4 ml) were magnetically stirred and temperature controlled (27 ± 0.2 C). Amounts of mitochondria added to the reaction media were 0.6 to 0.7 mg of mitochondrial protein as determined by the method of Lowry et al. (17).

The assay for NAD\(^{+}\) was carried out using the procedures of Estabrook and Maitra (8). Other reaction conditions are given in the figure and table legends.

RESULTS
The oxidation of exogenous NADH by isolated corn mitochondria occurs in KCl reaction media in the absence of either Ca\(^{2+}\) or P\(_i\), (Fig. 1) (18). This oxidation was accelerated by the addition of Ca\(^{2+}\) or P\(_i\), and was further stimulated when both Ca\(^{2+}\) and P\(_i\), were present in the reaction media (Fig. 1). Exogenous NADPH, on the other hand, was not oxidized in KCl reaction media without Ca\(^{2+}\) or P\(_i\), but was oxidized after the addition of either Ca\(^{2+}\) or P\(_i\), (Fig. 1), or when Ca\(^{2+}\) or P\(_i\), were present initially in the reaction medium (Fig. 2). As with NADH, the oxidation of exogenous NADPH was further stimulated by the addition of Ca\(^{2+}\) or P\(_i\), whichever was not.

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were: NADPH, and deflection.

The reaction media contained 200 mM KCl, 20 mM tris-HCl, pH 7.4, and 1 mg/ml bovine serum albumin. Percent transmittance changes are represented by the solid line (a downward deflection represents swelling), and oxygen uptake by the dashed line (rates per mg mitochondrial protein are given). Amounts of compounds added were: NADPH, 1 μmole; CaCl₂, 20 μmoles; and P_i, 16 μmoles. The reaction media volumes were 4 ml.

Fig. 1. Swelling-contraction and oxygen uptake of isolated corn mitochondria oxidizing exogenous NADH or NADPH. The reaction media contained 200 mM KCl, 20 mM tris-HCl, pH 7.4, and 1 mg/ml bovine serum albumin. Percent transmittance changes are represented by the solid line (a downward deflection represents swelling), and oxygen uptake by the dashed line (rates per mg mitochondrial protein are given). Amounts of compounds added were: NADPH, 1 μmole; CaCl₂, 20 μmoles; and P_i, 16 μmoles. The reaction media volumes were 4 ml.

Fig. 2. Effect of calcium or phosphate in the reaction media on the swelling-contraction and oxygen uptake of isolated corn mitochondria oxidizing exogenous NADH or NADPH. Reaction media were as given in Figure 1, but with the addition of 4 mM P_i in A and B and 5 mM CaCl₂ in C and D. Additions of NADH and NADPH were 1 μmole as indicated. Percent transmittance is represented by the solid lines (a downward deflection represents swelling), and oxygen uptake by the dashed lines (rates per mg mitochondrial protein are given).

Fig. 3. Effect of NADH and NADPH concentration on the rates of their oxidation by isolated corn mitochondria. Reaction media were as in Figure 1. Addition of substrate was 3 min after addition of mitochondria to the reaction media. Apparent Km values were 60 μM for NADPH and 33 μM for NADH.

Since Ca²⁺ by itself was found to facilitate the oxidation of exogenous NADPH, other divalent cations were also tested (Table I). In these experiments Sr²⁺ or Mn²⁺ were found to substitute for Ca²⁺, but Mg²⁺ or Ba²⁺ were not. Concentrations of these divalent cations were similar to those used in a study of divalent cation stimulation of the oxidation of exogenous NADH (18).

Figures 1 and 2 show the similarity of the percentage of transmittance (% T) traces when isolated corn mitochondria were oxidizing either exogenous NADH or NADPH. These changes included a strong decrease in % T in the presence of both Ca²⁺ and P_i, due probably to calcium phosphate deposit-
Table I. Effect of Several Divalent Cations Other than Calcium on the Rate of Oxidation of NADPH

<table>
<thead>
<tr>
<th>Compound Added</th>
<th>Rate before Addition</th>
<th>Rate after Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles O_2/min-mg protein</td>
<td></td>
</tr>
<tr>
<td>MgCl_2, 5 mm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SrCl_2, 5 mm</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>BaCl_2, 5 mm or 10 mm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MnCl_2, 5 mm</td>
<td>0</td>
<td>32</td>
</tr>
</tbody>
</table>

Addition of exogenous NADPH or NADH was made to passively swelling mitochondria in a KCl reaction medium containing Ca^{2+} (Fig. 2). Contraction (% T decrease) is more difficult to compare since P_i generally tends to reduce a % T decrease after substrate addition (Fig. 2). In the presence of Ca^{2+}, however, both exogenous dinucleotide substrates did exhibit contraction (% T decrease) after Ca^{2+} addition (Fig. 1).

Comparative studies of respiratory control (RCR) and ADP/O ratios of mitochondria oxidizing exogenous NADH, exogenous NADPH, malate-pyruvate, or succinate are reported in Figure 4. From these studies two factors emerge: (a) the oxidation of exogenous NADPH by isolated corn mitochondria is coupled, and (b) electrons passed from exogenous NADPH apparently pass through two coupling sites similar to those from exogenous NADH.

To determine whether exogenous NADPH or NADH were both being oxidized via similar electron transport pathways, the effects of several specific electron transport inhibitors were measured. These studies were carried out in KCl reaction media with added Ca^{2+} and/or P_i. For comparative purposes, the inhibitions of both NADH and NADPH are reported. Antimycin A, KCN, and rotenone induced almost identical inhibitions of O_2 uptake when mitochondria were oxidizing either NADH or NADPH (Table II). Amytal, however, which had a small inhibitory effect on state 3 respiration and a slight stimulatory effect on state 4 respiration of exogenous NADH, produced almost complete inhibition in the rate of exogenous NADPH oxidation.

The results of inhibitor addition on the rate of oxidation of exogenous NADH reported in this paper are somewhat different from those previously reported (19). It is likely that these differences result from different reaction media (KCl or sucrose) and the respiratory states the mitochondria were in at the time of inhibitor addition. Previous reports (19, 22) in-
Table II. Effects of Various Inhibitors on Mitochondria Oxidizing NADH or NADPH

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition state 3</th>
<th>Inhibition state 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antimycin A, 0.3 μM</td>
<td>89</td>
<td>78</td>
</tr>
<tr>
<td>Rotenone, 30 μM</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>KCN, 1 mM</td>
<td>98</td>
<td>86</td>
</tr>
<tr>
<td>Amytal, 5 mM</td>
<td>24</td>
<td>+25 (stimulation)</td>
</tr>
<tr>
<td>NADPH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antimycin A, 0.3 μM</td>
<td>78</td>
<td>69</td>
</tr>
<tr>
<td>Rotenone, 30 μM</td>
<td>38</td>
<td>7</td>
</tr>
<tr>
<td>KCN, 1 mM</td>
<td>100</td>
<td>96</td>
</tr>
<tr>
<td>Amytal, 5 mM</td>
<td>89</td>
<td>90</td>
</tr>
</tbody>
</table>

dicate that the effect of inhibitors on the rate of oxidation of exogenous NADH become greater as the mitochondria are coupled (i.e., greater inhibitions have been observed in the presence of ADP + Pi, than in the presence of only Pi). Other than the selective inhibition of the oxidation of NADPH by amytal, considerable similarity exists between the oxidation patterns of NADH and NADPH. This relative similarity raised the questions: (a) is it possible that NADPH is converted to NADH and that NADH is then oxidized, or (b) is there a transhydrogenase reaction involved transferring the electrons first to NAD⁺ which in turn would feed them into the electron transport system? To answer the first question the presence of NAD⁺ was determined after the complete oxidation of NADH using the procedure of Estabrook and Maitra (8). No NAD⁺ was found. If a transhydrogenase were functioning the addition of NAD⁺ should probably affect the oxidation of NADPH in the absence of Pi and Ca²⁺. No NADPH oxidation was observed after NAD⁺ addition (Fig. 5). However, NAD⁺ stimulated the oxidation of NADPH in the presence of Pi or Ca²⁺ (Fig. 5), an observation for which we have no ready explanation.

**DISCUSSION**

The capacity of isolated corn mitochondria to transfer electrons from exogenous NADPH to oxygen in the presence of Pi and/or Ca²⁺ presents some interesting questions. The first is whether the pathway of electron flow in the oxidation of exogenous NADPH is identical to that of exogenous NADH and secondly, if these pathways are not identical, where do electrons from NADPH enter the electron transport chain?

The NADH pathway has been elucidated by Storey (21) for mung bean mitochondria, and shows no interaction with coupling site 1 (13), producing P/O ratios in the range of 1.2 to 1.5. Storey (21) speculates that exogenous NADH is dehydrogenated on the outside of the inner membrane with the reducing equivalents being passed directly to the respiratory chain in the ubiquinone, cytochromes b, and the high potential flavoprotein region. As in mung bean mitochondria, electrons from the oxidation of exogenous NADH in isolated corn mitochondria also bypass coupling site 1 (22) and are not sensitive to inhibition by rotenone or amytal (22). Our results, at least superficially, suggest a similar pathway of electron flow in the oxidation of NADPH and NADH. Only two coupling sites were involved in the oxidation of each dinucleotide and the general oxidation of each was stimulated by Ca²⁺. However, there was a substantial rate of oxidation of exogenous NADH in the absence of Ca²⁺ or Pi, while there was none with exogenous NADPH. This argues against complete similarity in the oxidation of these two dinucleotides.

Regardless of the path of oxidation of NADPH or NADH, the swelling-contraction traces show that the energy usage in ion transport contraction are similar if not identical. Since the swelling-contraction process is an index of membrane function that is not always elucidated by respiration, this observation is significant. This similarity is particularly apparent in Figure 2 where, in the presence of Ca²⁺, the initiation of respiration caused an initial swelling followed by contraction. This phenomenon, which has not been explained, has only been observed with NADH (18).

The effects of various electron transport inhibitors on the rate of oxidation of exogenous NADPH were studied with the hope of elucidating the pathways of electron flow. When the state 3 inhibited rates of mitochondria oxidizing exogenous NADH or NADPH are compared, only the effect of amytal was substantially different for these two dinucleotides. If both amytal and rotenone affect the same step in electron transport as is commonly reported (2), then they should both similarly influence the rates of oxidation of exogenous NADH and NADPH. It is possible, however, that amytal and rotenone do not work specifically at site 1 as suggested by Chance et al. (5) and by Chance et al. (4), but that rotenone more specifically inhibits the transfer of electrons from the flavoprotein F₄₅₀ and amytal from the flavoprotein F₄₅₀. If electrons from

![Fig. 5. Effect of NAD⁺ on the rate of oxidation of exogenous NADPH by isolated corn mitochondria. Reaction media were as given in Figure 1. Lines represent recorder traces of oxygen uptake with associated numbers being rates on a mg protein basis. Amounts of compounds added as indicated were: Pi, 16 μmoles; CaCl₂, 20 μmoles; NAD⁺, 1 μ mole; NADPH, 1 μ mole; and mitochondria (M), approximately 0.6 mg of protein.](https://example.com/diagram.png)
NADPH could enter the chain at FAD, this could account for the observed results if electrons from exogenous NADH enter at another flavoprotein which is little affected by either inhibitor.

If we utilize what is known about rotenone and amytal inhibitions from the literature, then we must conclude one of the following: (a) that exogenous NADPH is initially oxidized via a flavoprotein that is neither the flavoprotein involved with endogenous NADH (amyl and rotenone sensitive) oxidation or with exogenous NADH (amyl and rotenone insensitive) oxidation, or (b) that amyl has an inhibitory effect on a possible transhydrogenase reaction involving an NADPH to NAD$^+$ electron transfer. The second of these possibilities seems the least likely for several reasons. First, if subsequent to a transhydrogenase reaction the NADH produced was oxidized via the endogenous NADH pathway, the rate of oxidation should be highly susceptible to the inhibition by rotenone (which it was not) and three coupling sites should have been involved (only two sites were in our experiments). Secondly, if the NADH produced as a result of a transhydrogenase reaction from NADPH were oxidized via the pathway for exogenous NADH, then the addition of exogenous NAD$^+$ should have permitted some electron flow from NADPH which was not the case. On the other hand, it is possible that the hypothetical transhydrogenase involved here is inhibited by amyl, but has an absolute requirement for Ca$^{2+}$ and/or P$. This possibility is not suggested by the literature.

It seems more likely that there is yet another flavoprotein associated with corn mitochondria that is specific for the oxidation of NADH, that requires Ca$^{2+}$ or phosphate, and that is inhibited by amyl. A NADPH-specific diaphorase has been purified from nongreen rice embryo tissue (12). While we have not attempted to purify the flavoprotein involved with the mitochondrial oxidation of exogenous NADPH, it is possible that the NADPH diaphorase purified by Idi and Morita (12) from rice embryo could be similar to that associated with isolated corn mitochondria since they did not differentiate the cellular location of their enzyme.

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LITERATURE CITED


