Reduction of Pyridine Nucleotides Induced by Adenosine Diphosphate in Kidney Mitochondria

THE INFLUENCE OF SODIUM, MAGNESIUM, AND INHIBITORS OF OXIDATIVE PHOSPHORYLATION*

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SUMMARY

Rat kidney cortex mitochondria incubated in an isotonic solution containing sodium chloride, EDTA, and Pi exhibit a hitherto unreported phenomenon. The addition of substrate induces a rapid respiratory rate, accompanied by a marked oxidation of pyridine nucleotides measured by dual wave length spectroscopy. In contrast to the typical response, subsequent addition of ADP induces a slowing of respiration associated with reduction of pyridine nucleotides (reverse acceptor control). The phenomenon will not occur if Mg++, rutamycin, or uncoupling agents are present. However, if an uncoupling agent is added after substrate, the ADP effects on respiration and pyridine nucleotides are mimicked. The marked susceptibility of kidney mitochondria to uncoupling in sodium-containing solutions appears to be closely related to the mechanism of this phenomenon.

METHODS

Mitochondria were prepared from the kidney cortex of male Sprague-Dawley rats (150 to 200 g). The animals were killed by decapitation, and both kidneys were removed and immediately placed in iced 0.25 M sucrose. After perirenal fat and capsule were discarded, a longitudinal midsagittal division of each kidney was made. Tissue from the cortex was obtained by dissection with a scalpel on a Petri dish which was cooled on ice and covered with filter paper moistened with 0.25 M sucrose. Tissue from the red (or outer) medulla and white (or inner) medulla was discarded. Mitochondria were prepared from 10 g of kidney cortex by the method of Johnson and Lardy (3). Tissue was homogenized in a motor-driven Potter-Elvehjem homogenizer in 250 mM mannitol, 70 mM sucrose, and 1 mM EDTA. Two subsequent washes were performed in the same medium without EDTA. The final pellet was suspended in mannitol-sucrose, and the protein concentration was adjusted to 10 mg per ml. Liver mitochondria were prepared in an identical fashion.

Changes in the oxidation-reduction state of intramitochondrial pyridine nucleotides were monitored with an Aminco-Chance dual wave length spectrophotometer. Since the optical density changes reflect both types of pyridine nucleotides, this sum will hereafter be referred to as NAD(P):NAD(P)H. Oxygen uptake was measured simultaneously by means of a vibrating platinum electrode inserted in the same cuvette. ATPase activity was assayed by the method of Lardy and Wellman (4). Light-scattering measurements were made in a Gilford recording spectrophotometer at 520 nm. The magnesium content of mitochondria was determined by extracting 1 mg of mitochondrial protein with 5 ml of 0.1 N HCl. The 105,000 g X 30 min supernatant of this extract was analyzed for Mg++ with a Perkin-Elmer atomic absorption spectrophotometer. All studies were performed at 25°, with the exception of the ATPase experiments which were performed at 30°. Protein was determined by the method of Lowry (5).

RESULTS

Kidney mitochondria prepared in mannitol-sucrose media were “well coupled” when studied under suitable conditions. A representative study of respiration and oxidation-reduction state of pyridine nucleotides is shown in Fig. 1 (top). With this incubation medium (0.15 M KCl, 5 mM P_i, 0.25 mM EDTA, 3 mM MgCl,

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† Veterans Administration Research Associate.
ADP induces oxidation of pyridine nucleotides in the K+-containing mitochondria in either K+-containing or Na+-containing media have clearly indicated that the pyridine nucleotides are consistently more oxidized than NAD(P)H in a medium containing 0.15 M NaCl, 5 mM triethanolamine Cl, 2.5 mM MgCl₂, pH 7.4. Complete phosphorylation of ADP results in a more reduced steady state.

Other components of the electron transfer chain were also studied by dual wave length spectroscopy: flavoproteins (465/510), cytochrome b (564/575), cytochrome c (550/541), cytochrome a (605/630), and cytochrome a₃ (445/460). In contrast to the pyridine nucleotides, these components exhibited no differences between the NaCl-EDTA and incubation mixtures in the NaCl-EDTA medium (where ADP induces reduction of pyridine nucleotides) the NAD(P):NAD(P)H ratio is initially relatively more oxidized.

Light-scattering measurements of mitochondria studied under identical conditions have indicated rapid swelling upon placement into the sodium medium, further swelling after addition of substrate, followed by contraction induced by ADP (see below). To eliminate the possibility that apparent variations in the oxidation-reduction state of pyridine nucleotides were due to changes in turbidity, similar experiments were performed with the dual wave length spectrophotometer using 340/310 nm and 340/300 nm as the wave length pairs. In these cases, artifacts due to light scattering would be recorded in a direction opposite to that when 374 nm is used as the reference wave length. Changes in pyridine nucleotides: oxidation-reduction state were virtually identical, regardless of the wave length pair utilized, indicating that these observations reflect true alterations in NAD(P):NAD(P)H.

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Conditions Favorable for ADP-induced Reduction of Pyridine Nucleotides—Reduction of pyridine nucleotides induced by ADP can consistently be demonstrated with rat kidney cortex mitochondria. Rat liver mitochondria prepared in a similar manner do not demonstrate this effect when incubated in NaCl-EDTA medium. When exposed to a KCl-EDTA medium (0.15 M KCl, 2.5 mM EDTA, 5 mM P₅, 10 mM triethanolamine chloride, pH 7.4) rat liver mitochondria have occasionally but not consistently exhibited reverse acceptor control. Kidney mitochondria always show ADP-induced reduction of pyridine nucleotides when sodium or lithium chloride are the major osmotic components of the incubation medium. Rubidium and cesium salts will also permit this phenomenon to occur, although the effects are less striking.

Fig. 2 illustrates the marked influence of sodium on changes

Fig. 1. Effects of ADP on respiration and pyridine nucleotides. Experiments were performed with 1.0 mg of mitochondrial protein, total volume 3.0 ml, 25°, pH 7.4. Wave length settings of dual wave length spectrophotometer were 340/374. Full scale (set at 100%) equivalent to a ΔNAD(P)H of 10.5 nmol. Slt = 3.3 nm. Bracketed numbers refer to respiratory rate (microgram atoms of oxygen min⁻¹ X mg⁻¹ of protein). Top, incubation medium optimum for oxidative phosphorylation: 0.15 M KCl, 10 mM triethanolamine chloride, 5 mM triethanolamine P₅, 0.25 mM EDTA, 3 mM MgCl₂, pH 7.4. Bottom, NaCl-EDTA medium optimum for reverse acceptor control: 0.15 NaCl, 10 mM triethanolamine chloride, 5 mM triethanolamine P₅, 2.5 mM EDTA, pH 7.4. Glut-Mal, glutamate-malate.

10 mM triethanolamine chloride, pH 7.4) the addition of oxidizable substrate, in this case 3 mM glutamate and 3 mM malate, causes a reduction of NAD(P)H to NAD(P)H and an increase in the respiratory rate (State 4). ADP addition markedly stimulates respiration (State 5) associated with a rapid oxidation of NAD(P)H (4.1 nM). Complete phosphorylation of ADP results in a return of respiration to State 4 and a return of NAD(P) to a more reduced steady state.

A strikingly different response to substrate and ADP is observed when mitochondria from the same preparation are studied in a medium containing 0.15 M NaCl, 2.5 mM EDTA, 10 mM triethanolamine chloride, 5 mM P₅, pH 7.4 (NaCl-EDTA) (Fig. 1, bottom). Before the addition of substrate, a slow drift in the oxidized direction of pyridine nucleotides is seen. Substrate addition initially causes a marked reduction of pyridine nucleotides, similar to that seen under the previous well coupled conditions. In marked contrast, this brief period of NAD(P) reduction is followed by a steady and progressive shift in the oxidized direction. This is associated with a State 4 respiratory rate (0.060 µg atom of oxygen per min per mg of protein) significantly greater than under well coupled conditions. The subsequent addition of ADP results in a pronounced and rapid reduction of pyridine nucleotides (NAD(P)H increases by 3.6 nmol) associated with a slowing of respiration (0.006 µg atoms of oxygen per min per mg of protein). Within a few minutes, the NAD(P)H slowly reoxidizes, and the cycle may be repeated with additional pulses of ADP. This phenomenon will henceforth be referred to as reverse acceptor control.

Dual wave length spectroscopy indicates changes in oxidation-reduction state but does not record the NAD(P):NAD(P)H in absolute terms. However, repeated comparisons of mitochondria in either K⁺-containing or Na⁺-containing media have clearly indicated that the pyridine nucleotides are consistently more oxidized prior to addition of ADP in the latter. When ADP induces oxidation of pyridine nucleotides in the K⁺-containing medium, the NAD(P):NAD(P)H ratio is initially relatively reduced; whereas in the NaCl-EDTA medium (where ADP induces reduction of pyridine nucleotides) the NAD(P):NAD(P)H ratio is initially relatively more oxidized.

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Fig. 2 illustrates the marked influence of sodium on changes
influence on this phenomenon.

The order of addition of the components other than ADP has no must be added after the substrate, mitochondria, and phosphate. prior to phosphate, no reverse acceptor control occurs; the ADP organic phosphate and ADP are added to the cuvette is critical to the demonstration of reverse acceptor control. If ADP is added for inorganic phosphate : acetate, nitrate, borate, thiocyanate, NADH. A wide variety of anions was found not to substitute concentrations from 0 to 0.09 mM, ADP induced a slight oxidation of acceptor control to occur. When phosphate was present in con- in their light-scattering properties. Fig. 4 illustrates the most powerful influence on NAD(P) :NAD(P)H following substrate ADP by the adenylate kinase reaction. Nevertheless, SMP fails although usually this did not occur. ADP is the only nucleotide tested that induces this effect. ATP (in final concentrations up to 6 mM) has no effect on pyri- dine nucleotides or respiration. AMP will induce State 3 respira- tion and oxidation of pyridine nucleotides in kidney mitochondria when studied under conditions optimum for oxidative phosphory- lation. This occurs presumably because of the formation of ADP by the adenylate kinase reaction. Nevertheless, AMP fails to affect the oxidation-reduction status of pyridine nucleotides when mitochondria are incubated in NaCl-EDTA medium. Presumably the high concentration of EDTA and absence of Mg++ inhibit adenylyl kinase.

Reduction of pyridine nucleotides induced by ADP is strikingly pH-dependent (Fig. 8). Within the range of probable mito- chondrial viability, more acid conditions favor reverse acceptor control. This is in contrast to the broad pH optimum for oxidative phosphorylation under well coupled conditions. pH has a powerful influence on NAD(P) :NAD(P)H following substrate additions, prior to ADP. More acid conditions induce more oxidized pyridine nucleotides, apparently permitting increased NAD reduction following ADP.

As mentioned previously, incubation of kidney mitochondria in sodium-containing solutions is associated with marked alteration in their light-scattering properties. Fig. 4 illustrates the most significant results of these studies. Addition of oxidizable sub-

Table I indicates that reduction of pyridine nucleotides induced by ADP is strongly influenced by ADP concentration.

![FIG. 3. Effects of pH upon reduction of NAD induced by ADP. Conditions were identical with Fig. 1 (bottom).](http://www.jbc.org/)

![FIG. 4. Light-scattering changes of kidney and liver mitochondria. The change in optical density at 520 nm in a Gilford spectrophotometer is recorded. Conditions were identical with Fig. 1 (bottom) (for kidney and liver Na+-EDTA) and (top) (for kidney K+-EDTA). Glut-Mal, glutamate-malate.](http://www.jbc.org/)
Table II

Respiration and phosphorylation by kidney mitochondria

KCl and NaCl-EDTA media are the same as Fig. 1, 3 mm glutamate-3 mM malate as substrate. Incubations were performed at 24°C. Respiration was determined polarographically. Phosphorylation was determined by measuring the ΔP_i following ADP addition.

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>KCl media</th>
<th>Sodium-EDTA media</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 4 respiration</td>
<td>0.08</td>
<td>0.13</td>
</tr>
<tr>
<td>State 3 respiration</td>
<td>0.25</td>
<td>0.10</td>
</tr>
<tr>
<td>Phosphorylation rate</td>
<td>0.76</td>
<td>0.10</td>
</tr>
<tr>
<td>Respiratory control</td>
<td>3.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table III

ATPase activities of kidney and liver mitochondria

All incubating solutions contained 6 mM ATP, 10 mM triethanolamine chloride, pH 7.4, 30°C, final volume = 1.0 ml. Incubation was started by adding mitochondria (1 mg of protein). After 10 min, the reaction was terminated with 1 ml of 10% trichloroacetic acid.

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Kidney*</th>
<th>Liver*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15 M KCl and 3 mM MgCl</td>
<td>0.06</td>
<td>0.0</td>
</tr>
<tr>
<td>0.15 M NaCl</td>
<td>0.24</td>
<td>0.09</td>
</tr>
<tr>
<td>0.15 M NaCl and 2.5 mM EDTA</td>
<td>0.75</td>
<td>0.20</td>
</tr>
</tbody>
</table>

* ATPase activity is expressed as micromoles of P_i mg⁻¹ of protein in 10 min.

ADP-induced slowing of respiration, in contrast to the usual State 3 stimulation, has been observed by several previous investigators (Table V). Apparently, this phenomenon was first briefly described by Chance and Hagihara using a preparation of aged pigeon mitochondria (6). Lehninger and Gregg
and was noted by Chance and Hagihara (6) and Elliott (8). These workers found that rat liver mitochondria incubated in 0.1 M NaCl and 1.2 mM EDTA without magnesium had a rapid State 4 respiration, which was slowed by the addition of dinitrophenol (Figs. 1 and 4 (16)) and in some cases ADP (Table I and Fig. 4 of Reference 16). No spectroscopic studies of electron transport chain components were performed, but the authors suggested that sodium uncouples at the rotenone-sensitive site, and that ADP may have protected against the detrimental action of sodium on this system.

The oxidation reduction state of the pyridine nucleotides (NAD(P):NAD(P)H) is controlled by several factors including substrate availability, activity of the substrate dehydrogenase, as well as the activity of the flavoprotein NADH dehydrogenase which is governed by the rate of electron transport through the respiratory chain. The coincident slowing of respiration, associated with the NAD reduction described herein, strongly implies an inhibition of the electron transport process. This is because NAD reduction, secondary to an increase in substrate availability or dehydrogenase activity, would tend to accelerate rather than to diminish oxygen consumption. Furthermore, two separate observations suggest that this inhibition of electron transport occurs at a rather specific site, i.e. between the pyridine nucleotides and flavoproteins. First is the absence of alterations in the oxidation-reduction state of flavoproteins and cytochromes. These findings are underscored by the studies using succinate as substrate; marked reverse acceptor control occurs with succinate, but the addition of rotenone restores the usual pattern of ADP-stimulated State 3 respiration. The rotenone effect is particularly puzzling, for it suggests that ADP may induce a slowing of succinate-supported respiration at a site which is not directly in the path of electrons from succinate dehydrogenase to oxygen! This is similar to the observations of Azzone and Ernster (17) that succinate oxidation by liver mitochondria was inhibited by treatment with arsenate and dicumarol, and was restored by ATP addition. The presence of amytal prevented this inhibitory phenomenon.

The mechanism by which ADP induces NAD reduction and respiratory inhibition appears to be closely linked to the process of oxidative phosphorylation. Despite a diminished oxygen consumption, some phosphorylation of ADP does occur, albeit at a rate which is suboptimal (Table II). The absolute requirements for ADP and Pi tend to support further this concept. This is in contrast to the observations of Augustyn et al. (13), who found inhibition of respiration induced by AMP, UDP, CDP, and GDP in mitochondria treated with snake venom. Finally, inhibition of reverse acceptor control by uncouplers, as well as by rutamycin and aurovertin which are specific inhibitors of the ATP-synhetase enzyme, provides further support for involvement of the processes of oxidative phosphorylation in the mechanism of reverse acceptor control. The restoration of ADP-induced oxidation of pyridine nucleotides by rutamycin remains unexplained. This may be reminiscent of the recoupling of oxidative phosphorylation of submitochondrial particles by oligomycin described by Lee and Ernster (18). The observation that uncouplers added after substrate (if added before substrate the usual effects of uncouplers are seen and no reverse acceptor control occurs after ADP) produce a reduction of pyridine nucleotides and slowing of respiration is strikingly similar to the response to ADP. Respiratory inhibition by uncoupling agents has been noted by many workers (19-20) and, in most instances, is secondary to diminished substrate entry into the mitochondrion (21). Inhibition of substrate uptake could not account for the present observations. Although no complete explanation is apparent for the inhibition by uncoupler of electron transport reported here, the similarity between the response to ADP and uncouplers is noteworthy, since both accelerate electron transport in well coupled mitochondria. A further property

| TABLE IV |
| Magnesium content of mitochondria |

Three minutes after ADP addition the mitochondria were centrifuged at 30,000 x g for 30 min. Pellet was then extracted with 0.1 N HCl as described under "Methods."  
<p>| Mg²⁺ (μmol/mg protein) |</p>
<table>
<thead>
<tr>
<th>Kidney</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final mitochondria pellet after preparation...</td>
<td>31.8</td>
</tr>
<tr>
<td>Mitochondria 3 min after ADP addition in KCl medium...</td>
<td>32.1</td>
</tr>
<tr>
<td>Mitochondria 3 min after ADP addition in NaCl-EDTA medium...</td>
<td>31.5</td>
</tr>
</tbody>
</table>

| a Conditions identical with Fig. 1 (top). |
| b Conditions identical with Fig. 1 (bottom). |

| TABLE V |
| Previous reports of reverse acceptor control |

| Chance and Hagihara (7) | Aged pigeon mitochondria |
| Lehninger and Gregg (2) | Submitochondrial particles |
| Elliot et al. (7-13) | Liver mitochondria treated with snake venom, phospholipase, fatty acids |
| Krall and Dougherty (15) | Kidney mitochondria from rats fed lead |
| Scott et al. (14) | Heart mitochondria treated with lead incubation mixture |
| Gomez-Puyou et al. (16) | Liver mitochondria in NaCl |

(2) observed ADP-induced respiratory inhibition in rat liver submitochondrial particles and coined the term "reverse acceptor control." Sonic fragments demonstrated this phenomenon when NADH, β-hydroxybutyrate, succinate, or choline was utilized as substrate. Submitochondrial particles prepared with digitonin showed reverse acceptor control when supplemented with at least 10⁻³ M exogenous NAD, but only with NAD-linked substrates. Elliott and co-workers have also observed reverse acceptor control in rat liver mitochondria treated with snake venoms (7-13). Brierley and co-workers have noted that heart mitochondria treated with lead salts demonstrate inhibition of respiration when dinitrophenol is added (14). The only previous observation of reverse acceptor control in kidney mitochondria has been with rats fed lead for several months (15). Therefore, prior observations of reverse acceptor control have been noted in a variety of different mitochondria preparations. However, a common feature of all of these preparations appears to be a diminished integrity of the mitochondrial membrane secondary to aging (6), detergents (2), snake venom (7-13), or lead (14, 15). These conditions might be associated with a diminished magnesium content and enhanced permeability to cations. All of these preparations appeared to be uncoupled; i.e. respiration in the absence of acceptor was rapid. An initial oxidized state of pyridine nucleotides may therefore be presumed and was noted by Chance and Hagihara (6) and Elliott (8).

An association between sodium and reverse acceptor control has been suggested by Gomez-Puyou et al. (16). These workers found that rat liver mitochondria incubated in 0.1 M NaCl and 1.2 mM EDTA without magnesium had a rapid State 4 respiration, which was slowed by the addition of dinitrophenol (Figs. 1 and 4 (16)) and in some cases ADP (Table I and Fig. 4 of Reference 16).
in common between ADP and uncouplers is the induction of matrix condensation. A number of previous reports suggest that reduction in matrix volume inhibits respiration (22) by an undefined mechanism. Perhaps the swollen state of kidney mitochondria in sodium-containing solutions enhances the susceptibility to respiratory inhibition by matrix condensation.

The importance of magnesium to this phenomenon cannot be overemphasized. Under the conditions studied, EDTA is an absolute requirement, while EGTA will not support reverse acceptor control. The affinity of EDTA for Mg++ is 1000 times that of EGTA (23). The inhibition of reverse acceptor control produced by addition of Mg++ in excess of EDTA adds additional emphasis to the importance of this divalent cation. Although no changes in Mg++ content were measured during reverse acceptor control, the Mg++ content of kidney mitochondria was significantly lower than that of liver. This may explain the greater susceptibility of kidney mitochondria to the induction of reverse acceptor control. Gear and Lehninger (24) showed that rat liver mitochondria suspended in a solution of NaCl tended to lose K+ and Mg++ in proportion to the concentration of Na++. However, these experiments were performed without substrates, phosphate, or ADP, conditions quite different from the present studies. Azzi et al. (25) have demonstrated that Mg++ chelating agents, including EDTA, induced an increased permeability of rat liver mitochondria to Na+ and other alkali metal cations, permitting enhanced swelling. This resulted in an uncoupling of oxidative phosphorylation, i.e. stimulation of respiration and enhanced ATPase activity. The addition of Mg++ inhibited swelling and restored the "tightly coupled state" confirmed in the present observations.

The way in which Na+, in conjunction with EDTA, sets the stage for reverse acceptor control in kidney mitochondria is not completely clear. However, the rapid State 4 respiratory rate, oxidized pyridine nucleotides, and high ATPase activity all indicate that the mitochondria are "uncoupled." This uncoupling might be secondary to a rapid energy-dependent flux of sodium. An alternative explanation might be that the swollen mitochondria have a diminished integrity, allowing enhanced penetration by protons. According to chemiosmotic concepts, this would diminish the proton motive force faced by the respiratory chain and permit more rapid flux of electrons from the pyridine nucleotide dehydrogenase to oxygen.

We have concluded that NAD(P)H reduction and respiratory inhibition induced by ADP or uncouplers results from inhibition of electron transport at or close to site I. One possible explanation is that the matrix condensation secondary to ADP (26) and uncouplers results in a diminished permeability to protons, causing a recoupling. Our observation that ADP-induced contraction resembles in some aspects an enhanced coupling of mitochondria may indicate that the physiological role of ADP is more than solely as an acceptor of Pi in the phosphorylation reaction. ADP may also serve to condense the inner membrane to a configuration that permits more efficient production of ATP (27). This property of ADP is a less specific phenomenon and may be related to a binding to the adenine nucleotide translocase (28). Other mechanisms involving chemiosmotic phenomena (29), "high-energy intermediate" formation (30), and conformational changes (31) might be speculated upon. Nevertheless, ADP-induced inhibition of electron transport is a phenomenon to be accounted for by any generally accepted theory of oxidative phosphorylation.

Acknowledgments—The many helpful discussions with Dr. Shelagh Ferguson are gratefully appreciated, as well as the expert technical assistance of Ms. Margaret Lu and Doris Ostoff.

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