Intermediate Reactions of Oxidative Phosphorylation in Mitochondria from Cabbage

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Abstract. Respiratory control ratios between 2.0 and 9.0 were obtained by comparison of the respiratory rates of cabbage mitochondria in the presence and in the absence of individual components of the system used to provide ATP and by comparing the rates before and after exhaustion of added ADP. These results indicate that respiration in cabbage mitochondria is controlled by the availability of ADP, which serves as the phosphate acceptor.

Pentachlorophenol (PCP), 2,4-dinitrophenol (DNP), gramicidin and oleic acid inhibited phosphorylation to a greater extent than respiration in the cabbage mitochondria, but these reagents did not stimulate respiration in the absence of a phosphate acceptor. Respiration was stimulated by DNP only in the presence of added ATP.

2,4-Dinitrophenol, pentachlorophenol, dicumarol and gramicidin did not stimulate ATPase activity either in the presence or absence of added Mg\(^{2+}\). Oleic acid stimulated ATPase activity in the presence of added Mg\(^{2+}\), but did not stimulate respiration even in the presence of added ATP.

The ATP\(^{32}\text{P}\) exchange rate was increased many fold in the presence of added Mg\(^{2+}\). Oleic acid and 2,4-dinitrophenol inhibited the exchange almost completely.

Using digitonin extracts of rat liver mitochondria, Cooper and Lehninger (6) provided evidence that an ATPase activity and the ATP\(^{32}\text{P}\) exchange involve reactions which function in the coupling sequence of oxidative phosphorylation. The ATP-ADP exchange has also been related to this important process (3, 18). Uncoupling agents such as DNP\(^5\), gramicidin, PCP, and dicumarol uncouple oxidative phosphorylation, stimulate ATPase activity, release respiration from its dependence upon a phosphate acceptor and inhibit both of the exchange reactions in freshly prepared mammalian mitochondria (3, 6).

DNP has been shown to uncouple oxidative phosphorylation in many plant mitochondrial preparations (2, 10, 19, 21). Although there have been some reports on ATPase activity in plants (7, 11, 16, 19), only 1 report shows evidence of a small ATPase stimulation by DNP (7). DNP was reported not to stimulate ATPase in cauliflower mitochondria (11, 16, 19); it did not increase respiration in the absence of a phosphate acceptor, but did so in the presence of ATP (11). There have been no reports on the ATP\(^{32}\text{P}\) and ATP-ADP exchange reactions in plant mitochondrial preparations.

In this communication, we wish to report data on respiratory control, 'release' of respiration, ATPase activity, and the ATP\(^{32}\text{P}\) exchange reaction in cabbage mitochondria.

Methods and Materials

Mitochondria, isolated from cabbage, Brassica oleracea var. capitata L. by modifications of the procedure used by Freebairn and Remmert (8), were used for studies of \(\text{O}_2\) uptake. For the ATPase and ATP\(^{32}\text{P}\) exchange studies, the procedure was the same except that the mitochondrial pellet was washed twice with 0.25 M sucrose and then suspended in 0.25 M sucrose.

Warburg constant volume respirometers with air as the gas phase were used for studies of \(\text{O}_2\) consumption at 30\(^\circ\). The ATPase activity was estimated as the amount of Pi formed during the incubation period. A modification of the method of Martin and Doty (13) was used for determinations of Pi. \(^{32}\text{P}\) incorporated into ATP was determined by the method of Nielson and Lehninger (14). The nitrogen contents of mitochondrial
preparations were determined using the micro-
kjeldahl method (1).

The ATP (from muscle), ADP (from muscle),
CoA (from yeast), DPN (from yeast), TPN (from
yeast), and hexokinase (from yeast) were products
of the Sigma Chemical Company. GSH was ob-
tained from Schwarz Laboratories, Inc. and the
TPP was from Nutritional Biochemicals Corpora-
tion. DNP, PCP, isobutyl alcohol, and citric acid
were obtained from Eastman Kodak Company;
dicumarol and gramicidin, from Mann Research
Laboratories. Myristic and stearic acids of the
white label quality were also obtained from East-
man Kodak Company. Oleic acid (U.S.P. grade) was
the product by Matheson, Coleman, and Bell. 32Pi
was obtained as H332PO4 from the Oak Ridge
National Laboratory. Other chemicals used were
of reagent grade.

Results
and Discussion

Respiratory Control in Cabbage Mitochondria.
It has been shown that the rate of respiration in
mammalian mitochondria is dependent upon the
concentration or availability of a phosphate accep-
tor such as ADP (4, 5). An 'acceptor ratio,' defined
as the ratio of respiration in the presence and in
the absence of a phosphate acceptor, between 4 and
10 and associated with a high P:O ratio has been
considered to be presumptive evidence that respira-
tion is 'tightly coupled' with phosphorylation
(5, 17).

Experiments, designed to determine whether or
not respiration is tightly coupled with phosphoryla-
tion in cabbage mitochondria, are summarized in
Table 1. In these experiments ATP and the hexo-
kinase system were used to provide ADP; in certain
other flasks, components of the hexokinase system
were omitted to obtain respiratory rates in the
absence (or with a lower concentration) of a
phosphate acceptor. When only ATP was omitted
from the system, appreciable respiration occurred,
and comparison to a complete system gave a low
acceptor ratio (about 2.0). However, phosphory-
lization equal to about 20% of that in the complete
system was found to occur, suggesting that endog-
ous adenylic acid was not removed completely even
by 2 washings of the mitochondrial preparations.
Under these circumstances a low acceptor ratio
would be expected when rates with and without
added ATP are compared.

Plant mitochondria have been shown to have
hexokinase firmly bound to them (2, 21) and to
possess invertase activity; when suspended in
sucrose, they may make some glucose available for
the hexokinase reaction (2). In the present studies,
low acceptor ratios (2-4) were obtained when either
glucose and hexokinase or glucose, hexo-
kinase, and ATP were omitted and the respiratory
rates were compared to those of a complete system.

Since Mg2+ is a cofactor for the hexokinase
system, it was used as a variant with glucose, hexo-
kinase and ATP. Acceptor ratios as high as 9.0
were obtained when glucose, hexokinase, ATP, and
Mg2+ were omitted and the respiratory rate was
compared to that of a complete system. Similar
t ratios were obtained by omitting glucose, hexo-
kinase, and Mg2+ but including ATP in the system
(2, 17), suggesting that respiration in these
preparations was inhibited even in the presence of
ATP unless a system for generating ATP was also
included in the medium.

It was pertinent to determine whether or not

<table>
<thead>
<tr>
<th>No of</th>
<th>QO2 (N) with</th>
<th>QO2 (N) with</th>
</tr>
</thead>
<tbody>
<tr>
<td>expts</td>
<td>acceptor system</td>
<td>omission indicated</td>
</tr>
<tr>
<td>1</td>
<td>771</td>
<td>392</td>
</tr>
<tr>
<td>8</td>
<td>893</td>
<td>324</td>
</tr>
<tr>
<td>(720–1160)</td>
<td>(262–382)</td>
<td>(2.38–3.23)</td>
</tr>
<tr>
<td>7</td>
<td>998</td>
<td>314</td>
</tr>
<tr>
<td>(771–1174)</td>
<td>(248–390)</td>
<td>(2.54–3.95)</td>
</tr>
<tr>
<td>4</td>
<td>1029</td>
<td>47</td>
</tr>
<tr>
<td>(773–1174)</td>
<td>(84–128)</td>
<td>(6.60–9.20)</td>
</tr>
<tr>
<td>3</td>
<td>774</td>
<td>111</td>
</tr>
<tr>
<td>(720–830)</td>
<td>(90–122)</td>
<td>(5.90–9.22)</td>
</tr>
</tbody>
</table>

1 QO2 (N) values obtained with ATP omitted.
2 QO2 (N) values obtained with glucose and hexokinase omitted.
3 QO2 (N) values obtained with glucose, hexokinase, and ATP omitted.
4 QO2 (N) values obtained with glucose, hexokinase, ATP, and Mg2+ omitted.
5 QO2 (N) values obtained with glucose, hexokinase, and Mg2+ omitted.
our cabbage preparations would show respiratory control when measured as suggested by Chance and Baltscheffsky (4). Since an oxygen electrode was not available for these studies, a single addition of 20 μmoles of ADP was used in manometric measurements of respiratory rates before and after phosphorylation of the added ADP. The results of a typical experiment are shown in figure 1. The high respiratory rate before tipping in the ADP might be due to the uncontrolled oxidation of endogenous substrates, which were used up by the time all of the ADP was converted to ATP. It will be noted that the respiratory rate changed from 2.47 μl O₂/minute in the presence of ADP to 0.27 μl O₂/minute after 7.5 μmoles O₂ had been consumed subsequent to the addition of ADP. The 7.5 μmoles O₂ corresponds to a P:O ratio of 2.66 assuming that all of the ADP was converted to ATP. It was noted in numerous other experiments that, in the presence of ADP and a system for regenerating ADP (hexokinase, glucose, and Mg²⁺), the respiratory rates of these preparations did not diminish significantly or at all during 90-minute incubation periods. Thus, these experiments gave respiratory control ratios as high as 9. In summary, the results of 2 experimental procedures suggest that respiration is tightly coupled with phosphorylation in cabbage mitochondria. Several other investigators (20, 21) have also obtained such high acceptor ratios by studying respiration in plant mitochondria following ADP addition and depletions.

In studies not tabulated here, it was found that DNP, PCP, and gramicidin had a greater inhibitory effect on phosphorylation than on respiration in the cabbage preparations. However, none of these uncoupling agents released respiration from the requirement of a potential phosphate acceptor. The presence of ATP was found to be necessary for stimulation of respiration by DNP (table II).

**Table II. The ATP Requirement for Stimulation of Respiration by DNP**

<table>
<thead>
<tr>
<th>Conc of ATP μmoles</th>
<th>Presence of 0.5 mM DNP</th>
<th>O₂ uptake μg atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>...</td>
<td>21.5</td>
</tr>
<tr>
<td>0.1</td>
<td>+</td>
<td>17.6</td>
</tr>
<tr>
<td>1.0</td>
<td>...</td>
<td>21.5</td>
</tr>
<tr>
<td>1.0</td>
<td>+</td>
<td>22.5</td>
</tr>
<tr>
<td>10</td>
<td>...</td>
<td>21.2</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>44.1</td>
</tr>
</tbody>
</table>

Small quantities of ATP did not permit stimulation, but in the presence of 10 μmoles ATP, DNP at 0.5 mM gave a large increase in respiration in a system without added glucose and hexokinase. The rate of respiration induced by DNP was, however, only 60% that found with the complete system for phosphorylation. The requirement for ATP, for stimulation of respiration by DNP, was also observed in cauliflower mitochondria (11) and in insect mitochondria (9). The large amount of ATP (10 μmoles), required for DNP to stimulate respiration in cabbage mitochondria, might suggest that the increase in respiration is due to stimulation of ATPase activity. This would, of course, provide ADP to serve as a phosphate acceptor, and the 'release' would then be only an apparent one. However, it has not been possible to demonstrate, by direct tests, that DNP stimulates ATPase activity in the cabbage preparations. It is possible, then, that the presence of ATP is essential to maintain the structure of the cabbage mitochondria (11), and that under these conditions DNP can release respiration from its coupling with the phosphorylation of ADP.

Mg²⁺ also was required for stimulation of respiration by DNP (table III). In addition, the concentration of DNP was very critical (table III). In similar experiments, DNP (0.1 mM) could induce respiration when either citrate or α-ketoglu-
Conc of DNP Presence of 5 mm Mg$^{2+}$ O$_2$ uptake

<table>
<thead>
<tr>
<th>M</th>
<th>Presence of 5 mm Mg$^{2+}$</th>
<th>O$_2$ uptake</th>
<th>$\mu$g atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>...</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^{-5}$</td>
<td>...</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^{-4}$</td>
<td>...</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^{-3}$</td>
<td>...</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>19.2</td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^{-3}$</td>
<td>+</td>
<td>21.8</td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^{-4}$</td>
<td>+</td>
<td>30.6</td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^{-5}$</td>
<td>+</td>
<td>9.9</td>
<td></td>
</tr>
</tbody>
</table>

Oleic acid was used as the substrate. However, respiration with succinate as the substrate was twice as much with citrate in the absence of DNP, but addition of DNP (0.1 mm) to this system could not induce any respiration.

**ATPase Activity.** Earlier studies on oxidative phosphorylation and ‘release’ of respiration in cabbage mitochondria indicated that these preparations had a low content of endogenous Mg$^{2+}$. ATPase activity was stimulated by increasing the concentration of Mg$^{2+}$ (1 μM to 10 μM). As mentioned previously, DNP (3 μM to 100 μM) did not stimulate ATPase activity either in the presence or absence of added Mg$^{2+}$ (0.2 mm to 10 mm).

Other uncoupling agents such as PCP, dicumarol and gramicidin were also found not to stimulate cabbage mitochondrial ATPase, even in the presence of added Mg$^{2+}$. Failure of the uncoupling agents mentioned to stimulate ATPase, or to release respiration, in the cabbage mitochondria suggests that they may inhibit phosphorylation by different mechanisms in cabbage and mammalian mitochondria.

Oleic acid uncouples oxidative phosphorylation, stimulates ATPase activity, inhibits the ATP-$^{32}$Pi exchange reaction and releases respiration in rat liver mitochondria (12). On the basis of these results, it may be speculated that DNP and oleic acid have the same point of action on oxidative phosphorylation in mammalian mitochondria. It was considered pertinent, therefore, to determine whether or not oleic acid would uncouple oxidative phosphorylation and stimulate ATPase activity in cabbage mitochondria.

In experiments not tabulated here, oleic acid at 0.3 mm was found to inhibit phosphorylation and O$_2$ uptake in cabbage mitochondria by 100% and 33%, respectively. Oleic acid did not stimulate respiration in a system which contained ATP but no added glucose or hexokinase. In this respect, oleic acid differed from DNP which, in the presence of ATP, did stimulate respiration (table II).

Oleic acid stimulated cabbage mitochondrial ATPase activity only in the presence of added Mg$^{2+}$ (table IV). The stimulation was about 3-fold with 1 mm oleic acid and about 4-fold with 3 mm. The ATPase of cabbage mitochondria could also be stimulated by saturated fatty acids such as myristic acid and stearic acid. Myristic acid gave a larger stimulation than did stearic or oleic acid at the same concentration. Similar results were obtained with ATPase activity in rat liver mitochondria (15).

**Table IV. Effects of Fatty Acids on the ATPase of Cabbage Mitochondria**

Each flask contained tris-maleate buffer, pH 7.4, 0.1 mm ATP (pH 7.4), 10 mm mitochondria, 0.05 to 0.1 mg N/mi; sucrose, 0.083 m; where indicated MgCl$_2$, 1 mm; additions as indicated and glass distilled water to a final volume of 3 ml. Incubated 30 minutes at 30°C. Each value is the average from duplicate flasks.

<table>
<thead>
<tr>
<th>Expt no</th>
<th>Fatty acid</th>
<th>Conc</th>
<th>$\mu$moles Pi released</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oleic</td>
<td>1</td>
<td>0.67</td>
</tr>
<tr>
<td>2</td>
<td>Oleic</td>
<td>3</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>Myristic</td>
<td>3</td>
<td>1.83</td>
</tr>
<tr>
<td></td>
<td>Searic</td>
<td>3</td>
<td>3.13</td>
</tr>
</tbody>
</table>

**ATP-$^{32}$Pi Exchange.** Mg$^{2+}$ was found to be required for the ATP-$^{32}$Pi exchange reaction in the cabbage preparations, since the rate was increased many fold by addition of Mg$^{2+}$ at 1 mm to 10 mm (table V). It should be noted that the apparent

**Table V. Dependence of the ATP-$^{32}$Pi Exchange and ATPase Activity on Magnesium Concentration**

The flask contents were as described in table IV except that $^{32}$Pi equivalent to about 10$^6$ counts per minute was added to each flask. Incubated 30 minutes at 30°C. Each value is the average from duplicate flasks.

<table>
<thead>
<tr>
<th>Conc of Mg$^{2+}$</th>
<th>Pi released $\mu$mole</th>
<th>Mean Pi concentration $\mu$m</th>
<th>ATP-$^{32}$Pi exchange $\mu$mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^{-3}$</td>
<td>0.85</td>
<td>0.51</td>
<td>1.6</td>
</tr>
<tr>
<td>$1 \times 10^{-4}$</td>
<td>1.20</td>
<td>0.56</td>
<td>2.2</td>
</tr>
<tr>
<td>$1 \times 10^{-5}$</td>
<td>1.18</td>
<td>0.56</td>
<td>2.8</td>
</tr>
<tr>
<td>$1 \times 10^{-6}$</td>
<td>2.43</td>
<td>0.77</td>
<td>57.7</td>
</tr>
<tr>
<td>$1 \times 10^{-7}$</td>
<td>5.26</td>
<td>1.24</td>
<td>185.0</td>
</tr>
</tbody>
</table>

1 The mean Pi concentration, used in calculating radio-active counts per $\mu$mole of Pi, was determined in each case as the initial Pi concentration plus one-half of the increase resulting from ATP hydrolysis (14).
rates of the ATP-$^{32}$Pi exchange reaction (table V) cannot be compared meaningfully with the rates of phosphorylation given in table I for several reasons. First, the initial Pi concentration for studies of oxidative phosphorylation (table I) was 66.6 mM, whereas the mean Pi concentration in the exchange studies was approximately 1 mM (table V). It is reasonable to assume that the actual rate of ATP-$^{32}$Pi exchange would have been considerably higher if the Pi concentration had been 66 mM, although this was not tested. Secondly, addition of Mg$^{2+}$ (1-10 mM) stimulated ATPase activity as well as the ATP-$^{32}$Pi exchange, giving an obvious loss of AT$^{32}$P formed through the exchange reaction. Thirdly, such experiments (table V) measure only the apparent rate of ATP-$^{32}$Pi exchange, since $^{32}$Pi may be recycled (incorporated into AT$^{32}$P followed by hydrolysis by ATPase) several times after entrance into, and before exiting from, the mitochondrion. Finally, the medium used for the exchange studies (table V) was necessarily very different from that used for studies of oxidative phosphorylation (table I); a different medium might affect the rate observed for the exchange, for oxidative phosphorylation, or both. The data in table V are presented as evidence for an ATP-$^{32}$Pi exchange activity in cabbage mitochondria, even though absolute reaction rates were not determined.

The ATP-$^{32}$Pi exchange rate was very sensitive to aging. It was decreased by 81% after aging the mitochondria for 4 hours, whereas phosphate esterification and the P:O ratio had decreased by only 37 and 19%, respectively. However, after 20 hours, both the exchange activity and phosphorylation were lost almost completely. These results neither prove nor disprove that the ATP-$^{32}$Pi exchange represents reactions which are part of the process of oxidative phosphorylation, because the medium used for the exchange studies may have reduced all activities in partially aged mitochondria more than did the different medium used for studying oxidative phosphorylation.

Table VI. Inhibition of the ATP-$^{32}$Pi Exchange by DNP and Oleic Acid

<table>
<thead>
<tr>
<th>Conc of DNP (mM)</th>
<th>Conc of oleic acid (mM)</th>
<th>ATP-$^{32}$Pi exchange (mamoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>...</td>
<td>...</td>
<td>189.4</td>
</tr>
<tr>
<td>0.5</td>
<td>...</td>
<td>3.7</td>
</tr>
<tr>
<td>1</td>
<td>...</td>
<td>3.8</td>
</tr>
<tr>
<td>...</td>
<td>0.5</td>
<td>3.9</td>
</tr>
<tr>
<td>...</td>
<td>1</td>
<td>3.0</td>
</tr>
</tbody>
</table>

The effects of DNP and oleic acid on the ATP-$^{32}$Pi exchange in cabbage mitochondria are shown in table VI. The exchange activity was inhibited almost completely when either reagent was added at 0.5 mM, similar to the results obtained with mammalian mitochondria (3, 6, 12).

The results of this paper indicate that respiration and the formation of ATP are tightly coupled in cabbage mitochondria, as in mammalian mitochondria. However, the effects of DNP, fatty acids, and other uncoupling agents, which were different with cabbage mitochondria from those observed with mammalian mitochondria, should be considered in relation to proposed mechanisms of oxidative phosphorylation.

Literature Cited


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