Inhibition of Postoxidative Calcium Release in Corn Mitochondria by Inorganic Phosphate

M. J. Earnshaw and J. B. Hanson
Department of Botany, University of Illinois, Urbana, Illinois 61801

Received for publication April 9, 1973

ABSTRACT

Respiration drives the accumulation of a small amount of calcium in corn (Zea mays L.) mitochondria, and this calcium is released when respiration ceases. A postenergized addition of phosphate leads to phosphate uptake and enhanced calcium retention. Oligomycin, KCN, 2,4-dinitrophenol, or mersalyl are without effect on the phosphate-induced calcium retention. Addition of phosphate also inhibits the release of endogenous phosphate which normally accompanies the calcium. It is suggested that passive phosphate uptake retards the release of endogenous phosphate which is complexed with the calcium.

Some years ago Kenefick and Hanson (6) proposed that the contracted state of corn mitochondria produced by respiration in a KCl medium was associated with a conservation of energy which could be used in ion transport even after respiration ceased. Mitochondria contracted in the presence of Ca+ or Pi and centrifuged down through successive wash, reaction, and isotope exchange layers were shown to have accumulated Ca++. Due to the possibility of substrate carry-over, an alternative experiment was devised in which a postenergized addition of Pi to mitochondria contracted with NADH and is oxygenated in the presence of Ca++ led to apparent Ca++ accumulation, thus supporting the centrifugation experiments. We have re-examined this last experiment in some detail, and although the experimental result can be verified the interpretation cannot. It appears that the postenergized addition of phosphate leads to retention of a Ca++ + endogenous Pi complex formed during respiration.

MATERIALS AND METHODS

Mitochondria were isolated from 3-day-old etiolated corn shoots (Zea mays L., WF9 × M14) in 400 mM sucrose, 5 mM EDTA, 50 mM KH,P,O₄, adjusted to pH 7.6 with tris as previously described (6), except that ADP was omitted from the washing medium. The Pi of the isolation medium was labeled with 2 mc of carrier-free ^32P in experiments involving endogenous Pi determination. Protein was determined with the phenol reagent (8) using bovine serum albumin as a standard.

The centrifugation techniques of previous work (6) were not used due to the possibility of substrate carry-over. Mitochondrial reactions were generally carried out in a Pyrex glass cell controlled at 25°C and positioned in the light path of a Beckman Model B spectrophotometer as described previously (3). The medium contained either 200 mM sucrose or 100 mM KCl plus 20 mM Tricine-KOH (pH 7.5), 1 mg/ml bovine serum albumin. Other additives are given with the data. Except for one experiment, mitochondrial respiration and contraction were initiated by the addition of a small amount of NADH. Oxygen uptake was followed using a Clark oxygen electrode (Yellow Springs Instrument Co.) and volume changes as changes in %T at 520 nm. Corn mitochondria oxidize endogenous NADH with ADP:O ratios between 1 and 2 (11), and exhaustion of NADH is clearly signaled by resumption of swelling in a KCl medium (4, 6). As previously reported (4, 6), there is no discernible respiration after the point of reswelling. Additions of Ca++ or Pi or both were made after the initiation of reswelling; samples were withdrawn from the cell, and the mitochondria were analyzed for evidence of postenergized uptake of Ca++ or Pi or both.

Mitochondrial endogenous Pi content was determined using "Ca, and mitochondrial Pi uptake was determined using ^32P. The mitochondria were separated from the reaction medium by either Millipore filtration or centrifugal filtration through a silicone layer as described previously (3). Samples were determined using a Packard liquid scintillation counter with a dual label setting where appropriate.

Mitochondrial endogenous Pi content was determined by Millipore filtration, digestion in 5% trichloroacetic acid, and separation of ^32Pi by the method of Berenblum and Chain (2). Endogenous Pi was estimated by the method of Bartley (1) and ^32P using a Packard liquid scintillation counter as described previously (3).

RESULTS

The experiment described in Figure 1 is a more detailed analysis of the previously reported effect of a postenergized Pi addition on mitochondrial Ca++ content (6). When placed in KCl containing 1 mM "CaCl₂, corn mitochondria swell passively and bind a small amount of Ca++. Addition of NADH results in contraction and rapid accumulation of Ca++. Reswelling occurs upon depletion of NADH and is associated with release of Ca++. To a level approaching the prior nonenergized binding of Ca++ (3, 4). The postenergized addition of Pi reduces the rate of reswelling as previously described (6). However, the action of Pi is to retard Ca++ loss rather than promote the postenergized uptake of Ca++. As shown by ^32P tracer, the ion which undergoes postenergized uptake is actually phosphate, and this uptake presumably retards Ca++ loss and re-
Fig. 1. The effect of a postenergized Pi addition on Ca\(^{2+}\) release and associated light scattering changes in KCl. Cell contained 100 mM KCl, 20 mM Tricine-KOH (pH 7.5), 1 mg/ml bovine serum albumin, 1 mM \(^{40}\)CaCl\(_2\), 200 \(\mu\)g/ml mitochondrial protein with 0.78 \(\mu\)mole NADH/mg protein added at 5 min, and 1 mM KH\(_2\)PO\(_4\)-KOH (pH 7.5), labeled with \(^{32}\)Pi) added upon NADH depletion. The Pi data are adjusted for a nonenergized control. Samples collected by Millipore filtration. Solid trace: (→) Pi; dotted trace: (+) Pi.

Table I. Postenergized Pi Uptake and Ca\(^{2+}\) Retention in KCl

Experimental conditions are as described under "Materials and Methods" and Figure 1. Mitochondrial protein was 910 \(\mu\)g/ml. NADH (0.37 \(\mu\)mole/mg protein) was added at 5 min. Termination of NADH oxidation was detected with the \(O_2\) electrode at 6 min 29 sec, and \(^{32}\)Pi was added at 6 min 45 sec. Duplicate samples were taken at 10 min. Pi values were adjusted for a nonenergized control.

<table>
<thead>
<tr>
<th>Sampling Method</th>
<th>Pi Addition</th>
<th>Ca(^{2+})</th>
<th>Net Ca(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Millipore filtration</td>
<td>+</td>
<td>27</td>
<td>98</td>
</tr>
<tr>
<td>Centrifugal filtration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>−</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>29</td>
<td>64</td>
</tr>
</tbody>
</table>

The reason for the loss of accumulated mitochondrial Pi some 2 min following the postenergized Pi addition (Fig. 1) is not known but is possibly linked with the concomitant decline in Ca\(^{2+}\) retained as a result of the Pi addition.

The validity of the Pi uptake data was checked by comparing the Millipore filtration with centrifugal filtration (Table I). Postenergized Pi uptake and Ca\(^{2+}\) retention occur with similar values for both sampling methods.

If our interpretation that a postenergized Pi addition merely retards Ca\(^{2+}\) loss rather than promotes Ca\(^{2+}\) accumulation is correct, then postenergized Ca\(^{2+}\) uptake would not be expected to occur following a postoxidative Ca\(^{2+}\) addition. Figure 2A is a summary of the experiment described in Figure 1. The greater level of Ca\(^{2+}\) retained is in part a result of a longer Ca\(^{2+}\) accumulation period than in the experiments described in Figure 1 and Table I. Indeed, the postenergized addition of Ca\(^{2+}\) to mitochondria contracted in the presence of Pi (Fig. 2B) or the postenergized addition of Ca\(^{2+}\) plus Pi (Fig. 2C) do not result in significant Ca\(^{2+}\) uptake.

Previous work indicates that Ca\(^{2+}\) accumulation in the ab-
sence of exogenous Pi (Fig. 1) involves the mersalyl-sensitive formation of a complex with endogenous Pi which is dissipated upon NADH depletion (3). It seemed possible that the postenergized uptake of Pi (Fig. 1, Table I) might represent a passive exchange of added Pi for endogenous Pi. To assess this the endogenous Pi was labeled with 32P during isolation of the mitochondria and the postrespiration addition was made with unlabeled Pi (Fig. 3). The postenergized release of Ca++ is associated with a reduction in endogenous 32Pi, and retention of Ca++ as a result of the exogenous Pi addition is associated with retention of endogenous 32Pi. Little exchange between endogenous 32Pi and added exogenous Pi could have occurred, since otherwise the endogenous 32Pi content should have declined sharply.

Table II. Effect of Ion Uptake Inhibitors on Ca++ Retention and Reswelling in KCl

Experimental conditions are as described under “Materials and Methods” and Figure 1. Mitochondrial protein was 160 μg/ml. NADH (0.96 μmole/mg protein) added at 5 min. Reswelling commenced at 8 min 13 sec, and Pi ± inhibitor was added at 15 to 30 sec later. Samples were taken (Millipore filtration) 3 min after Pi addition.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Pi Addition</th>
<th>Ca++ Release</th>
<th>Re-Swelling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/3 min-mg protein</td>
<td>% T/2 min</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>–</td>
<td>118</td>
<td>8.3</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>13</td>
<td>2.1</td>
</tr>
<tr>
<td>KCN, 1 mM</td>
<td>+</td>
<td>7</td>
<td>2.1</td>
</tr>
<tr>
<td>Oligomycin, 1 μg/ml</td>
<td>+</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>2,4-Dinitrophenol, 0.1 mM</td>
<td>+</td>
<td>0</td>
<td>3.4</td>
</tr>
<tr>
<td>Mersalyl, 63 nmoles/mg</td>
<td>+</td>
<td>0</td>
<td>2.9</td>
</tr>
</tbody>
</table>

The crucial question then arises as to whether the postenergized uptake of added Pi is an energy-linked or a passive event. Several inhibitors were checked for an effect on postenergized Ca++ retention (Table II), but none was found to be effective. Cyanide would not be expected to have an effect since there was no detectable respiration. However, if a conserved energy potential was responsible for the Ca++ retention the uncoupler 2,4-dinitrophenol should have dissipated it, and it did not, although there was a small increase in the rate of reswelling. Oligomycin was without effect, as was mersalyl, an inhibitor of the Pi-OH antiporter (10). Hence, the postenergized Pi uptake and Ca++ retention have none of the characteristics of normal respiration-linked transport (3, 5).

Respiration-linked contraction in KCl is now known to be due to an efflux pumping of the KCl that enters during passive swelling (7). Is this physical shrinkage responsible for the Pi uptake and Ca++ retention, or could the same result be obtained with nonpenetrating sucrose?

Uptake of Ca++ occurs during respiration in a sucrose medium (Fig. 4), and this is accompanied by a Ca++-activated swelling, possibly due to succinate transport in this experiment. The basis for the Ca++-activated swelling in sucrose medium is not clear yet, but quantitatively it depends on the anions present, acetate being more effective than chloride (research in progress). A rapid release of Ca++ occurs on exhaustion of NADH in sucrose medium (Fig. 5). Addition of Pi in the postenergized state sharply reduces the rate of Ca++ release just as in the KCl medium. Thus, contraction and reswelling per se are not obligatory for the Pi uptake and Ca++ retention.

**DISCUSSION**

In the absence of exogenous Pi a small amount of Ca++ is taken up during respiration and is rapidly released after respiration ceases. Previous work shows that the Ca++ uptake is dependent on endogenous Pi and is mersalyl-sensitive (3), indicating that Pi transport from the intermembrane space to the matrix space is involved. The passive release of Ca++ is strongly retarded by the addition of Pi as is the reswelling (Fig. 1). Re-
swelling is due to re-entry of KCl (7), so addition of Pi must retard both the efflux of Ca\(^{2+}\) and endogenous Pi and the influx of K\(^+\) and Cl\(^-\). However, the retarding action of added Pi on Ca\(^{2+}\) loss is also seen with a sucrose medium (Fig. 5), and thus contraction and reswelling are not obligatory.

Some of the added Pi is taken up by the mitochondria (Fig. 1, Table I), and this undoubtedly has significance in retarding the loss of Ca\(^{2+}\). However, none of the usual inhibitors of Pi uptake (5, 6) affect the action of added Pi (Table II). Presumably, the entry of Pi must be by diffusion. Sites for diffusive entry of ions do exist as evidenced by the rapid passive swelling in KCl. Passive influx of Pi probably offsets passive efflux to a large degree, slowing net Pi loss and thereby the loss of Ca\(^{2+}\).

Since there is no evidence that the postenergized uptake of Pi is energy-linked, the hypothesis of Kenefick and Hanson (6) that a conserved energy potential for transport exists in the contracted state cannot be supported. This does not mean that no potential exists, since there is obviously a diffusion potential for the entry of salt which produces the reswelling, but it does mean that this potential is not translatable into energized calcium + phosphate uptake under the conditions of these experiments. It should be noted that in those cases where K\(^+\) flux yields a potential for Ca\(^{2+}\) transport it is an efflux, not an influx (9).

Acknowledgment—We are grateful to D. M. Madden for expert technical assistance.

LITERATURE CITED