Pyruvate and Malate Transport and Oxidation in Corn Mitochondria

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ABSTRACT

Pyruvate oxidation and swelling in pyruvate solutions by corn (Zea mays) mitochondria were inhibited by α-cyano-4-hydroxy-cinnamic acid, an inhibitor of pyruvate transport in animal mitochondria; however, there was no inhibition of pyruvate dehydrogenase activity, and malate and NADH oxidation were not affected. These results suggest the presence of a pyruvate-‘-OH exchange transporter which supplies the mitochondrion with oxidizable substrate. Lactate appears to be transported also, but not dicarboxylic anions or inorganic phosphate. The rate of pyruvate transport was much slower than that of malate, however, and valinomycin was required to elicit appreciable swelling in potassium pyruvate.

Malate oxidation contributed significantly to respiration supported by pyruvate plus malate, and malate did not act solely as a “sparkler” for pyruvate oxidation. NAD’-malic enzyme activity was found in sonicated preparations, and comparison of O2 consumption with CO2 released from 1-14C-pyruvate indicated that transported malate was being converted to pyruvate, particularly as the malate to pyruvate ratio increased. The results suggest that pyruvate transport becomes limiting under conditions of high energy demand, but that rapid malate transport makes up the difference, supplying pyruvate via malic enzyme and replenishing losses of tricarboxylic acid cycle intermediates.

It has been recently demonstrated that rat liver mitochondria possess a relatively specific pyruvate transporter in the inner membrane (8, 10, 19). While it seems definite that this carrier catalyzes pyruvate-hydroxyl exchange and is specifically inhibited by α-cyano-cinnamic acid and its derivatives (8), there is some confusion about its ability to exchange dicarboxylate ions for pyruvate (8, 19, 20, 22). The kinetics of pyruvate uptake suggests that the carrier may be important in the regulation of pyruvate metabolism, particularly gluconeogenesis (8).

Little is known about pyruvate entry into plant mitochondria although swelling studies have suggested that it differs from that of other carboxylic acids (13). Pyruvate oxidation by isolated mitochondria apparently is limited by availability of acetyl-CoA acceptor (23). On the other hand, pyruvate can be generated intramitochondrially from malate, via malic enzyme (2, 16, 18).

The present study sought to determine the means by which pyruvate is taken up by corn mitochondria and to what extent its oxidation, and that of malate, is governed by its transport. It is shown that pyruvate transport occurs via a mechanism similar to that in liver mitochondria, and that malate can act as the primary carbon source of the TCA3 cycle. It is suggested that the relative contributions of malate and pyruvate, when both are supplied, to TCA cycle activity in the plant cell may be determined in part by the respective transport systems.

MATERIALS AND METHODS

Mitochondria were isolated from 3-day-old etiolated corn (Zea mays) shoots essentially as described by Hanson (11) except that TES buffer (pH 7.5) was used instead of KH2PO4 and 0.1% BSA was included in the grinding medium. Oxygen consumption was measured using a Clark O2 electrode in a volume of 4.5 ml at 25 C as described previously (11). The standard reaction medium consisted of 0.25 m sucrose, 10 mM TES buffer, 1 mM MgSO4, and 0.1% BSA, adjusted to pH 7.2 with NaOH. Mitochondrial protein was approximately 1.5 mg/vasell.

Swelling was measured by following changes in absorbance at 520 nm in a Hitachi model 100-10 spectrophotometer at room temperature using cuvettes with a 1-cm light path.

Mitochondrial protein was estimated by the method of Lowry et al. (15) using BSA (fraction V) as the standard.

Pyruvate dehydrogenase activity in detergent-disrupted mitochondria was measured by following NADH production at 340 nm in a Hitachi spectrophotometer. The method was essentially that of Crompton and Laties (5) and the following assay medium was used: 20 mM KH2PO4 (pH 7.3), 0.5 mM EDTA, 1 mM MgCl2, 1 mM NaHSO4, 0.1 mM TPP, 0.15 mM CoA, 0.3 mM NAD+, 1 mM KCN, 1 mg mitochondrial protein, and 0.03% Triton X-100. Pyruvate was added to start the reaction.

Pyruvate accumulation was measured by incubating 1 to 2 mg mitochondrial protein in 3 ml of standard reaction medium containing 0.4 μmol NADH, 20 μM rotenone, 2 mM arsenite, and 2 mM pyruvate. Other additions were made as given in Table V. The reaction was terminated by adding 0.1 mM CHCA and the mixture centrifuged at 30,000g for 2 min in a Sorvall RC-2 centrifuge at 2 C. The supernatant layer was decanted and the pellet rinsed with water and resuspended in 6% (v/v) cold perchloric acid. Precipitated protein was removed by centrifugation and the supernatant layer neutralized with KOH prior to assaying. Controls were run with 10 μM antimycin A in the medium to allow for passive penetration and intermembrane pyruvate. Pyruvate was determined as described by Bucher et al. (3) using lactate dehydrogenase.

Pyruvate oxidation was determined by measuring 14CO2 evolution from 1-14C-pyruvate. Mitochondria were incubated for 10 min at 25 C in 25-ml flasks containing 5 ml standard reaction medium (pH 6.8) to which had been added 1 mM ADP, 50 μM CoA, 50 μM TPP, 10 mM KH2PO4, 20 units hexokinase, 20 mM

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3 Abbreviations: CHCA: α-cyano-4-hydroxy-cinnamic acid; val: valinomycin; BM: n-butyl malonate; TCA: tricarboxylic acid; PEP: phosphoenolpyruvate; TPP: thiamin pyrophosphate.
glucose, and 1 mM 1-14C-sodium pyruvate (0.01 µCi/mmol). Varying amounts of unlabeled malate were also added. The flasks were fitted with serum stoppers and a small plastic well, containing 0.1 ml 20% (w/v) KOH and a filter paper wick, was suspended from the stopper by copper wire. The flasks were gently shaken in a water bath and the reaction was terminated by injecting perchloric acid (final concentration = 7%) into the mixture and the flasks were shaken for an additional 30 min. The wells were then removed, placed in Aquasol scintillation fluid (10 ml), and counted in a Beckman LS-230 scintillation counter. Radioactivity was determined using the channels-ratio method. In parallel experiments, O2 uptake was measured polarographically under the same conditions.

Malic enzyme was assayed as described by Hatch and Kagawa (12). Sonicated mitochondria (about 1 mg) were added to 3 ml of a medium containing 10 mM TES buffer (pH 7), 0.2 mM EDTA, 5 mM dithiothreitol, 5 mM malate, 2 mM NAD+, and 75 µM CoA. The reaction was started by adding 5 mM MgCl2 and NADH production was measured spectrophotometrically at 340 mm; 10 µM antimycin A was added to prevent reoxidation of NADH.

1-14C-Pyruvate (Na salt) was purchased from Amersham/Searle Corp. (Chicago, Ill.). Aquasol scintillation cocktail was a gift from New England Nuclear (Boston, Mass.). α-Cyano-4-hydroxy-cinnamic acid was purchased from Aldrich Chemical Co. (Milwaukee, Wis.), and dissolved in absolute ethanol. Other chemicals and enzymes were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS AND DISCUSSION

Effect of α-Cyano-4-Hydroxy-Cinnamic Acid on Pyruvate Oxidation. When malate alone was added to isolated corn mitochondria, a slow rate of O2 uptake was observed which could be markedly stimulated by adding pyruvate providing cofactors were also present (Fig. 1). However, when low concentrations of CHCA were added prior to pyruvate, the stimulation was prevented. CHCA did not prevent enhancement of respiration by glutamate (Fig. 1), and had little effect on NADH oxidation or pyruvate dehydrogenase (Table 1). These results suggest that CHCA inhibition of malate plus pyruvate oxidation lies at the level of pyruvate entry to the matrix and suggest the existence of a specific pyruvate transporter, such as that shown to operate in liver mitochondria (8, 10, 19, 22).

The data shown in Figures 2 and 3 support these ideas. Figure 2 depicts the effect of varying concentrations of CHCA on the pyruvate stimulation of malate oxidation; 50% inhibition was achieved with 1.8 µM CHCA. The inhibition of malate and pyruvate oxidation was noncompetitive with respect to pyruvate (Fig. 3); and the Km for pyruvate was 0.53 mM. On the other hand, the apparent Km of pyruvate dehydrogenase was found to be 0.21 mM (see also ref. 5). These results suggest that the kinetics of pyruvate oxidation reflects that of the transporter rather than the dehydrogenase, and that the transporter may play a role in the regulation of pyruvate oxidation.

Swelling Studies and Pyruvate Uptake. Swelling was measured in a variety of K+ salts of monocarboxylic acids (Fig. 4). Very little passive swelling in 10 mM K+ salt was observed (Fig. 4A). Respiration-driven swelling in pyruvate and lactate solutions was slight unless valinomycin was present (Fig. 4, B and C). On the other hand swelling in acetate and propionate, which cross the inner membrane as undissociated acids (4), was much more pronounced (Fig. 4, D and E), suggesting that pyruvate and lactate enter the mitochondria in a different fashion than the other monocarboxylic acids.

The NADH-driven swelling in pyruvate and valinomycin was severely inhibited by CHCA, and was also inhibited by mersaryl (Fig. 5A), indicating that the pyruvate carrier is —SH-sensitive.

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** Effect of α-cyano-4-hydroxy-cinnamic acid on pyruvate oxidation. Mitochondria (1.2 mg protein) were added to 4.5 ml of standard reaction medium to which had been added: 5 mM KH2PO4, 50 µM CoA, 0.1 mM TPP, and 4.5 µmol ADP. Where indicated, 2 mM malate, 20 mM pyruvate, 20 µM CHCA or 6 mM glutamate were added. Oxygen uptake is expressed as nmol O2/min·mg protein−1.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Expt</th>
<th>Control</th>
<th>+10 µM CHCA</th>
<th>+50 µM CHCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>1</td>
<td>212</td>
<td>212</td>
<td>217</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>183</td>
<td>183</td>
<td>185</td>
</tr>
<tr>
<td>5 mM malate</td>
<td>1</td>
<td>150</td>
<td>145</td>
<td>140</td>
</tr>
<tr>
<td>5 mM glutamate</td>
<td>2</td>
<td>127</td>
<td>127</td>
<td>123</td>
</tr>
<tr>
<td>2 mM malate</td>
<td>1</td>
<td>125</td>
<td>22</td>
<td>17</td>
</tr>
<tr>
<td>2 mM pyruvate</td>
<td>2</td>
<td>98</td>
<td>17</td>
<td>11.5</td>
</tr>
<tr>
<td>pyruvate</td>
<td>1</td>
<td>75</td>
<td>73.5</td>
<td>--</td>
</tr>
<tr>
<td>dehydrogenase</td>
<td>2</td>
<td>42.5</td>
<td>42.5</td>
<td>42.5</td>
</tr>
</tbody>
</table>

Table 1. Effect of α-cyano-4-hydroxy-cinnamic acid on corn mitochondria

Oxygen uptake rates shown are state 3 rates; CHCA was added during state 3. When pyruvate was used 50 µM CoA and 0.1 mM TPP were included in the reaction medium. Pyruvate dehydrogenase was assayed as described in Materials and Methods; CHCA, when present, was added to the medium.

![Figure 2](https://example.com/fig2.png)

**Fig. 2.** Inhibition of pyruvate stimulation of respiration by α-cyano-4-hydroxy-cinnamic acid. O2 uptake was measured as in Figure 1B. Respiration was started with 2 mM malate; when a steady rate was obtained, variable concentrations of CHCA were added, and then 10 mM pyruvate. The rate of O2 uptake before addition of pyruvate was subtracted from that after pyruvate was added, and this value was plotted against CHCA concentration.
TABLE II. Pyruvate accumulation by corn mitochondria.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pyruvate content (mmol/mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM pyruvate</td>
<td>10.3 (± 3.4)</td>
</tr>
<tr>
<td>2 mM pyruvate + 50 μM CHCA</td>
<td>9.4 (± 0.2)</td>
</tr>
<tr>
<td>2 mM pyruvate + 25 μM mersalyl</td>
<td>4.0 (± 1.4)</td>
</tr>
<tr>
<td>2 mM pyruvate + 2 mM Pi + 2 mM malonate</td>
<td>9.1 (± 1.2)</td>
</tr>
<tr>
<td>2 mM pyruvate + 3 mM lactate</td>
<td>5.3 (± 1.9)</td>
</tr>
</tbody>
</table>
Malate versus Pyruvate Oxidation. Since malate is transported faster than pyruvate, it may contribute much respiratory NADH both in being oxidized to oxaloacetate and to pyruvate. When butyl malonate was added to inhibit malate transport under conditions where “sparker” pools should have been established (Fig. 7A) malate plus pyruvate respiration declined by approximately 32%; subsequent addition of CHCA inhibited further. Butyl malonate inhibited malate plus glutamate oxidation more severely (by approximately 63%) while CHCA had no effect (Fig. 7B). Obviously, sustaining maximum pyruvate plus malate oxidation involves consumption of exogenous malate and requires continuous malate transport; that is, malate does not act solely as a sparker. The more rapid rate of malate plus glutamate oxidation suggests a more rapid rate of oxaloacetate turnover than when pyruvate is supplied. Hence, the rate-limiting step in malate plus pyruvate oxidation is probably pyruvate transport or oxidation to acetyl-CoA.

The rate of malate oxidation can be affected by the pH of the medium also, as noted previously by Macrae (16). Cofactors necessary for the oxidation of pyruvate (derived via malic enzyme) are effective in promoting $O_2$ uptake at pH 6.8 (Fig. 8B), but not at pH 7.2 unless pyruvate is supplied (Fig. 8A). Macrae (16) ascribed this effect of pH to malic enzyme activity which was found to have optimum activity at pH 6.8. However, lowering the pH may also affect substrate penetration since swelling of corn mitochondria in response to substrate (and Pi) anions is greater at lower pH values (11; Bertagnolli and Hanson, unpublished results).

In order to be certain that malic enzyme was present, determinations were made of malic enzyme activity in sonicated corn mitochondria. Rates varying from 120 to 175 nmol NADH/min·mg protein$^{-1}$ were found. This compares favorably with the range of activities reported by Macrae (17).

Table IV shows the effect of increasing malate concentration on the $^{14}CO_2$ released from $^{14}C$-pyruvate. Assuming no signifi-

![Fig. 6. Swelling in potassium malate and pyruvate. Mitochondria (1.4 mg protein) were added to 2.9 ml standard reaction medium containing 30 mm rotenone and swelling measured as described under “Materials and Methods.” Swelling was driven by NADH, as shown in Figure 4, B and C. Phosphate (1 mm), when used, was added immediately after NADH and 15 mm K$^+$ pyruvate or malate added when Pi-induced swelling was completed (see ref. 6). Numbers on the curves are initial swelling rates in $\Delta_{\text{swell}}$ min$^{-1}$. Solid lines: controls; dashed lines: prior addition of Pi.](Image)

Table III. Swelling rates of corn mitochondria in pyruvate and malate solutions.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Expt</th>
<th>Initial rate of swelling control</th>
<th>+ valinomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM malate</td>
<td>1</td>
<td>0.027</td>
<td>0.094</td>
</tr>
<tr>
<td>2</td>
<td>0.028</td>
<td>0.088</td>
<td></td>
</tr>
<tr>
<td>10 mM malate + 1 mM Pi</td>
<td>1</td>
<td>0.056</td>
<td>0.154</td>
</tr>
<tr>
<td>2</td>
<td>0.009</td>
<td>0.066</td>
<td></td>
</tr>
<tr>
<td>10 mM pyruvate</td>
<td>1</td>
<td>0.01</td>
<td>0.048</td>
</tr>
</tbody>
</table>

Fig. 7. Effects of transport inhibitors on malate and pyruvate oxidation. Mitochondria (1.4 mg protein) were added to 4.5 ml standard reaction medium (pH 7.2) which also included 5 mm K$_2$HPO$_4$, 50 mm CoA, 0.1 mm TPP, and either 5 mm pyruvate (A) or 5 mm glutamate (B). Malate (1 mm) was added to initiate $O_2$ uptake and 5 mmol ADP, 10 mm butylmalonate, and 50 mm CHCA were added as indicated. $O_2$ uptake is expressed as nmol/min$^{-1}$. mg protein$^{-1}$.

Fig. 8. Effect of pH and cofactors on malate oxidation. $O_2$ uptake was measured as described in Figure 7 except that no pyruvate or glutamate was present in the medium, and 5 mm malate was added to initiate $O_2$ uptake. Subsequently, 5 mmol ADP, cofactors (CoF; 50 mm CoA + 0.1 mm TPP) and 5 mm pyruvate were added as indicated. The pH of the medium (pH 7.2 in A and pH 6.8 in B) was adjusted prior to assaying. $O_2$ uptake is expressed as nmol/min$^{-1}$. mg protein$^{-1}$.
significant accumulation of acetyl-CoA, the amount of transported pyruvate entering the TCA cycle is proportional to $^{14}\text{C}$O$_2$ released. If malate is serving as a source of "cold" pyruvate via malic enzyme, in addition to serving as a source of oxaloacetic acid, there will be extra O$_2$ consumption not accounted for by $^{14}\text{C}$O$_2$ released from transported pyruvate.

Oxidation of transported pyruvate tends to saturate above 1 mM malate, but O$_2$ consumption does not (Table IV). Since oxidation via the TCA cycle requires acetyl-CoA production, malate must have been oxidized via malic enzyme and pyruvic dehydrogenase. At 4 mM malate, 42% of the pyruvate being consumed is derived from malate (provided there is no loss of TCA cycle intermediates from the matrix). This figure agrees reasonably well with those estimated from blocking malate or pyruvate transport (Fig. 7). Clearly, the relative contributions made by direct pyruvate transport and by malic enzyme will be a function of substrate concentrations, but the data also suggest that pyruvate transport rates can become limiting.

**GENERAL DISCUSSION**

The results obtained demonstrate the operation in corn mitochondria of a relatively specific pyruvate-transporting system, which apparently catalyzes the exchange of pyruvate for hydroxyl ions. Lactate also appears to be transported by this carrier which, apart from this feature, resembles that found in liver mitochondria, particularly with respect to CHCA inhibition. Halestrap (9) has suggested that CHCA attacks sulfhydryl groups on the carrier, and the inhibition by mersaryl observed here supports this. The specificity and potency of CHCA make it a potentially useful inhibitor for in vivo studies.

Plant cells are furnished with sugars which are converted in the cytoplasm to PEP, and from PEP produce two respiratory substrates, pyruvate and malate (Fig. 9). Malate appears to be ubiquitous in plants and is often accumulated in large amounts; the requisite enzymes (PEP carboxylase, PEP carboxykinase, and malic dehydrogenase) are commonly found (7, 21); dark CO$_2$ fixation is proving to have a central role in many aspects of plant metabolism (1, 7). Lips and Beever (14) have reported dark fixation of CO$_2$ into malate and turnover of this malate by the TCA cycle under conditions which stimulate respiration (e.g. uncoupling). The role of intramitochondrial malic enzyme in utilizing vacuolar malate during repression of glycolysis and in intermediary metabolism has been discussed elsewhere (12, 18). Hence, it is important to recognize that two sources of respiratory substrate are furnished to plant mitochondria, not just pyruvate, as is commonly taught.

Isolated corn mitochondria require a continuous supply of both pyruvate and malate for maximum oxidation rates (Fig. 7; Tables I and IV). This was not expected, because respiratory-driven malate uptake is very rapid (6), and sparker levels of TCA cycle intermediates should be abundant after a minute or two. There are two possible explanations. First, the rates of pyruvate transport are not very large (16-33% of malate transport rates, Table III), and appear to be inadequate to sustain maximum respiration; malate transport and oxidation via malic enzyme will supplement here (Table IV). Second, during respiration, the mitochondria may be exporting TCA cycle intermediates which would normally be utilized in intermediary metabolism of the cell. These can be replaced by malate uptake but not by pyruvate. Depending on metabolic demands, it is possible that dark CO$_2$ fixation diverts much PEP from pyruvate to malate formation, with malate entering the mitochondria and filling two roles — replenishing the TCA cycle pool, and furnishing part of the respiratory carbon (see Fig. 9).

**LITERATURE CITED**


PYRUVATE TRANSPORT AND OXIDATION

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