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Isolation and Characterization of the Tricarboxylate Transporter from Pea Mitochondria

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ABSTRACT

The tricarboxylate transporter was solubilized from pea (Pisum sativum) mitochondria with Triton X-114, partially purified over a hydroxylapatite column, and reconstituted in phospholipid vesicles. The proteoliposomes exchanged external [14C]citrate for internal citrate or malate but not for preloaded D,L-isocitrate. Similarly, although external malate, succinate, and citrate competed with [14C]citrate in the exchange reaction, D,L-isocitrate and phosphoenolpyruvate did not. This tricarboxylate transporter differed from the equivalent activity in animal tissues in that it did not transport isocitrate and phosphoenolpyruvate. In addition, tricarboxylate transport in isolated plant mitochondria, as well as that measured with the partially purified and reconstituted transporter, was less active than the transporter isolated from animal tissues.

The inner membrane of mitochondria must serve two very divergent functions. It must maintain the ion gradients that are essential intermediates between electron transport and ATP synthesis, and it must at the same time allow for rapid equilibrium of substrates between the cytosol and the mitochondrial matrix. This problem is solved by a family of transporters that allow the rapid exchange of adenylates, phosphate, and organic and amino acids across the membrane. These include an adenylate exchanger, a phosphate/OH\(^-\) antiporter, a pyruvate/OH\(^-\) antiporter, the dicarboxylate transporter that exchanges dicarboxylates and phosphate, and a tricarboxylate transporter that will exchange dicarboxylates and tricarboxylates. These transporters or carriers have been biochemically distinguished by their substrate and inhibitor specificities (7, 9, 17, 30).

Although mitochondrial transporters are found in both plants and animals, there are some differences in the types of transporters present. Plant leaf mitochondria, for example, rapidly exchange glycine and L-serine to support the respiratory conversion of glycine to serine (19, 27). Plant mitochondria also contain a unique oxaloacetate transporter that interacts with NAD\(^+\)-dependent malate dehydrogenase in the cytosol and matrix to shuttle reducing equivalents across the inner mitochondrial membrane (20).

The adenylate and phosphate transporters have been isolated, purified, and sequenced (1). A gene for the adenylate transporter has been isolated from corn (28) and the clone for the phosphate transporter has been obtained from Arabidopsis (our unpublished data). Several organic acid transporters have been solubilized from mitoplasts, partially purified, and incorporated into liposomes. The monocarboxylate, dicarboxylate (24), glutamate/aspartate (25), and α-ketoglutarate (10) transporters have been isolated from plant mitochondria and incorporated into proteoliposomes for detailed study. However, sequence information for the organic acid transporters is not yet available from either plants or animals.

The tricarboxylate transporter from animal mitochondria has been studied in considerable detail (6, 16, 21–23). This transporter from rat (4) and bovine (6) mitochondria has been isolated and purified 1000- and 400-fold, respectively. The preparation from rat liver showed a protein with an apparent molecular mass of 30 kD, whereas the preparation from bovine liver had a predominant 37- to 38-kD protein on SDS-PAGE gels. No conclusive proof is available that either band is the mammalian tricarboxylate transporter. Both transporters exchanged citrate, isocitrate, malate, malonate, and phosphoenolpyruvate. In this paper, we report the solubilization, partial purification, reconstitution, and analysis of the tricarboxylate transporter from pea (Pisum sativum L.) mitochondria.

MATERIALS AND METHODS

Plants and Chemicals

[U-14C]Citrate and malonate were from New England Nuclear. Dowex, azolectin, cardiolipin, Percoll, and other standard reagents were from Sigma. Hydroxylapatite was from Bio-Rad.

Peas (Pisum sativum L. var Alaska or Scout) were allowed to imbibe in a pipet washer overnight and grown in vermiculite in the dark. Etiolated seedlings were harvested after 10 d. Mitochondria were isolated by grinding and differential centrifugation and further purified on a self-generating Percoll gradient (26). The mitochondria were washed to remove Percoll, resuspended in 20 mm Mops, pH 7.2, and subjected to two freeze-thaw cycles (−20°C) to break the membranes and release the matrix proteins.

Isolation and Enrichment of the Tricarboxylate Transporter Activity

The mitochondrial membranes were solubilized and the transporter-enriched fraction was prepared by a modification of the methods of Kaplan et al. (13, 14) and Vivekananda et
al. (24). All procedures were carried out at 4°C unless stated otherwise. The frozen lysed mitochondria were thawed at room temperature and centrifuged for 20 min at 27,000 g to pellet the membranes. The membranes were suspended in Kaplan and Pedersen (13) buffer B (10 mM KPi, 20 mM KC1, 1 mM EDTA, pH 7.2) and protein content was determined. The membranes were centrifuged as above and resuspended in ice-cold buffer B to a final concentration of 30 mg of protein mL\(^{-1}\). An equal volume of 6% (v/v) Triton X-114 in buffer B containing 6 mg of cardiolipin mL\(^{-1}\) was added and the solution was incubated for 30 min. Solubilized membranes were then centrifuged for 35 min at 37,000g and the supernatant was removed. This crude Triton X-114 extract was then chromatographed on cold dry hydroxylapatite (0.5 g per column, 0.5 mL of crude extract per column). Elution was done with buffer B and the first 1.0 mL off each column was collected and pooled.

**Preparation of Proteoliposomes**

Dried azolectin was resuspended in buffer C (120 mM Hepes, 50 mM KC1, 1 mM EDTA, pH 7.5) under N₂ gas at 120 mg/mL and bath sonicated (Laboratory Supplies Co.) at room temperature until the solution was translucent. Six microliters of a 1-m solution of the organic to be loaded into the proteoliposome (citrate, malate, etc.) was added to 540 µL of liposomes and then 460 µL (6.5 µg of protein) of the hydroxylapatite eluate (or 200 µL of the crude Triton X-114 extract containing about 4 mg of protein and 260 µL of buffer B) was added. The sample was vortexed and frozen at −80°C.

Immediately prior to the assay, the proteoliposomes were thawed for 15 min in a room-temperature water bath, cooled on ice for 5 min, and then sonicated with a probe sonicator. Proteoliposomes were allowed to stand at room temperature for 5 min and chromatographed on a 5-cm Dowex 1X8 Pasteur pipet column and eluted with buffer C. The opalescent fraction (1.2 mL) was collected and assayed for transport activity.

**Assay of Tricarboxylate Transporter Activity**

A 300-µL sample of proteoliposomes was incubated for 5 min at room temperature before the addition of 20 µL of either buffer C (control) or the transport inhibitor (n-butylmalonate or 1,2,3-benzenetricarboxylate as indicated) in buffer C. After 2 min, the transport reaction was started by the addition of 20 µL of 20 mM citrate (0.5 µCi). After the desired time, 160 µL of the reaction was removed and added to 340 µL of ice-cold 20 mM inhibitor and the reaction chromatographed on a 0.5 × 5.0 cm Dowex 1X8 column equilibrated with buffer C to remove unincorporated isotope (4). The sample was eluted with 1 mL of buffer C and the eluate was collected and counted. All assays were done in duplicate or triplicate. Proteoliposomes prepared without internal organic acids carried very little \[^{14}C\]citrate through the Dowex column, showing that the reaction measured was an exchange for internal organic acids and not binding to the proteoliposome surface.

**Other Methods**

Protein was estimated with the Bio-Rad Bradford protein reagent dye with BSA as the standard. Samples containing Triton X-114 were precipitated by 10% TCA (final concentration); pellets were washed with ice-cold acetone, air dried, and redissovled in 0.1 N NaOH before assay for protein content.

**RESULTS AND DISCUSSION**

Mitochondria were isolated from etiolated peas and purified on a Percoll gradient. After freezing and thawing to release the matrix, the membranes were solubilized in Triton X-114 containing cardiolipin. The transporter fraction was passed over a hydroxylapatite column. Unlike the case with transporters from some sources, passage of the crude Triton extract over a Celite column either after or simultaneous with the hydroxylapatite column did not increase the purification (4, 10). We have shown that this transporter fraction contains the dicarboxylate, α-ketoglutarate, monocarboxylate, and glutamate/aspartate transporters.

When this fraction was incorporated into azolectin vesicles and the vesicles were preloaded with citrate, they rapidly exchanged internal citrate for external \[^{14}C\]citrate. Compared with the crude Triton X-114 extract, the flow-through from the hydroxylapatite column apparently contained all of the transporter activity but only 0.19% of the initial protein (Table I). This 500-fold purified fraction was used for characterizing the tricarboxylate transporter.

Figure 1 shows the purification of the transporter–enriched fraction from Percoll-purified pea mitochondria. Some purification occurred during the isolation of the membrane fraction and the solubilization with Triton X-114. The significant purification, however, resulted when the crude Triton extract was passed over the hydroxylapatite column. The flow-through from this column contained the tricarboxylate transporter activity. On SDS-PAGE, this fraction revealed a minimum of 10 bands. The two major bands at 33.5 and 35 kD are traditionally identified as transporters based largely on the size of the adenylate and phosphate transporters. After acetone precipitation, there are three additional bands at 13, 13.5, and 14 kD (the last is the H-protein of glycine decarboxylase).

One of the difficulties encountered when working with tricarboxylate transport in plant mitochondria is that 1,2,3-

### Table I. Purification of the Crude Triton X-114 Fraction on Hydroxylapatite

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Yield</th>
<th>Specific Activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton</td>
<td>125</td>
<td>866</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>0.28</td>
<td>1012</td>
<td>116</td>
<td>3614</td>
<td>516</td>
</tr>
</tbody>
</table>
benzenetricarboxylate, a highly specific inhibitor of this carrier in animal mitochondria (21), is not as potent an inhibitor with plant mitochondria (7). To inhibit the oxidation of 2 mM citrate by pea mitochondria over 90%, it was essential to use 10 to 20 mM 1,2,3-benzenetricarboxylate (data not presented). Citrate-dependent O2 uptake was more sensitive to n-butylmalonate, with 5 mM inhibiting the rate about 90%.

Figure 2 shows the time course for n-butylmalonate- and 1,2,3-benzenetricarboxylate-sensitive citrate/citrate exchange by proteoliposomes reconstituted with the transporter-enriched fraction. Both inhibitor-stop reagents gave very similar results when present at 20 mM. Because both inhibitors gave the same results and n-butylmalonate was more straightforward to work with, it was used as the inhibitor-stop reagent for the remaining experiments. Citrate/citrate exchange was linear for 6 min.

The n-butylmalonate-sensitive citrate/citrate exchange showed saturation kinetics (Fig. 3) when external [14C]citrate was varied between 0.1 and 5 mM. The exchange reaction exhibited an apparent Ko.5 of 0.66 mM. This value is similar to the Ko.5 of 0.12 mM reported for citrate transport in intact rat liver mitochondria (16) and 0.13 mM for the reconstituted bovine transporter (6). The Vmax for the pea transporter (14 nmol mg−1 min−1), however, is substantially lower than that measured for other isolated tricarboxylate carriers, 376 nmol mg−1 min−1 for bovine (6) and 450 to 1350 nmol mg−1 min−1 for rat (4). Similarly, the rate for the citrate transporter is much lower than that measured in this laboratory for other substrate transporters from pea mitochondria (24, 25) but similar to the rates measured with intact plant mitochondria.

A range of potential inhibitors and substrates were tested for their effect on citrate/citrate exchange in the reconstituted
system (Table II). n-Butylmalonate, the sulfhydryl reagent p-chloromercuribenzoate, 1,2,3-benzenetricarboxylate, and the arginine reagent p-hydroxyglyoxal inhibited [14C]citrate uptake completely. Partial inhibition was noted with high concentrations of pyridoxal 5-phosphate, a reagent that reacts with lysine residues, and α-cyano-4-hydroxycinnamate, a sulfhydryl reagent that usually inhibits the monocarboxylate transporter at low concentrations but apparently shows lower specificity at the high concentrations used in this study. These results are very similar to those reported for mammalian mitochondrial transport (4, 6).

External succinate and malate inhibited citrate/citrate exchange (Table II). Malate and malonate included inside the proteoliposomes were capable of exchanging for external [14C]citrate (Table III). This suggests that citrate will exchange for these dicarboxylic acids on the tricarboxylate transporter.

Pyruvate, α-ketoglutarate, phosphate, and ATP, substrates specific for the monocarboxylate, α-ketoglutarate, dicarboxylate, and adenylate transporters, respectively, had no effect on the rate of citrate/citrate exchange. These observations agree with the information available on the tricarboxylate transporter from animal tissues (17, 22, 23).

Curiously, when malonate was provided outside during citrate/citrate exchange, it doubled the rate of citrate uptake, which might indicate that malonate is imported by a separate transporter in the system and is then exchanged with external citrate. Alternatively, the malonate might be binding to a citrate transporter, thereby activating it. The structural similarity between malonate and malate or succinate makes it difficult to resolve why they have such different effects.

The citrate transport reaction in this reconstituted system does exhibit some striking differences from other (mammalian) tricarboxylate transporters. For example, citrate/citrate exchange by the pea mitochondrial transporter was not inhibited by isocitrate (Table II), and proteoliposomes pre-loaded with isocitrate did not exchange it for external [14C]citrate (Table III). Similarly, phosphoenolpyruvate did not compete with the citrate/citrate exchange reaction.

## CONCLUSIONS

Tricarboxylate transport in plant mitochondria is not well understood. To date, three mechanisms have been postulated for citrate transport into plant mitochondria. These are (a) a proton/citrate symport with maximal activity at pH 4.5 (2, 3); (b) a direct citrate/phosphate antiport in corn and beetroot (15, 29); and (c) the classic tricarboxylate transporter (8, 12). All of these transporters have low rates of citrate transport measured between 5 and 20 nmol mg⁻¹ protein min⁻¹, which is substantially lower than the rates of citrate uptake and metabolism that can exceed 120 nmol mg⁻¹ protein min⁻¹ in potato mitochondria (11) but is not dissimilar from the rates of 20 to 30 nmol mg⁻¹ protein min⁻¹ often reported (31).

We have solubilized the classic tricarboxylate transporter (tricarboxylate/dicarboxylate exchanger) from pea mitochondria and reconstituted it into artificial membranes. The transporter will exchange citrate/citrate or citrate/malate across the membrane. This transporter activity is differentiated from the dicarboxylate carrier by not responding to phosphate (Table II). The transport reaction was inhibited by high concentrations of 1,2,3-benzenetricarboxylate and n-butylmalonate. Interestingly, it was neither inhibited by nor able to transport isocitrate and phosphoenolpyruvate. The inability of isocitrate to interact with this transporter was confirmed by its inability to interfere with citrate/citrate exchange (Table II) as well as the absence of any exchange between internal isocitrate and external citrate (Table III). The lack of citrate/isocitrate exchange is in agreement with earlier work (D.J. Oliver, unpublished data) that mitochondria supplied with citrate do not excrete isocitrate into the medium. Citrate/isocitrate exchange is usually considered to be a property of

#### Table II. Effects of Different Compounds on the n-Butylmalonate-Sensitive Citrate/Citrate Transport in Proteolipid Vesicles

<table>
<thead>
<tr>
<th>Compound</th>
<th>mM</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-Butylmalonate</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>1,2,3-Benzenetricarboxylate</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>p-Hydroxyglyoxal</td>
<td>20</td>
<td>96</td>
</tr>
<tr>
<td>Pyridoxal 5-phosphate</td>
<td>20</td>
<td>63</td>
</tr>
<tr>
<td>α-Cyano-4-hydroxycinnamate</td>
<td>20</td>
<td>58</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>n-Ethylmaleimide</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>Mersalyl</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Substrates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>5</td>
<td>84</td>
</tr>
<tr>
<td>Malate</td>
<td>5</td>
<td>64</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>5</td>
<td>43</td>
</tr>
<tr>
<td>d,l-Isocitrate</td>
<td>10</td>
<td>28</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Phosphate</td>
<td>5</td>
<td>-4</td>
</tr>
<tr>
<td>Phosphoenol/pyruvate</td>
<td>5</td>
<td>-13</td>
</tr>
<tr>
<td>ATP</td>
<td>5</td>
<td>-37</td>
</tr>
<tr>
<td>Malonate</td>
<td>5</td>
<td>-136</td>
</tr>
</tbody>
</table>

#### Table III. Exchange of Different Preloaded Organic Acids for External [14C]Citrate

Proteoliposomes were prepared with the hydroxyapatite-purified transporter fraction and incorporated into liposomes. The liposomes were then preloaded with one of the organic acids (6 mM). [14C]Citrate (1 mM) was provided externally and the reaction was terminated after 5 min.

<table>
<thead>
<tr>
<th>Internal Compound</th>
<th>n-Butylmalonate-Sensitive Citrate Uptake % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>100</td>
</tr>
<tr>
<td>d,l-Isocitrate</td>
<td>19</td>
</tr>
<tr>
<td>Malate</td>
<td>62</td>
</tr>
<tr>
<td>Malonate</td>
<td>109</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>0</td>
</tr>
</tbody>
</table>
the tricarboxylate transporter (30), and the reason for the
difference between these data with the isolated transporter
and the results with intact mitochondria is not obvious.

The function of the tricarboxylate transporter is expected
to be different in plants and animals. In animal cells, the
tricarboxylate transporter exchanges citrate to the cytosol,
where citrate lyase produces acetyl CoA for lipid synthesis
and oxaloacetate that is reduced to malate and reenters the
mitochondria. In plants, citrate is synthesized exclusively
in the mitochondria and exported to the cytosol, where cytosolic
aconitase and NADP+ citrate dehydrogenase form α-keto-
toglutarate needed for NH4+ fixation by glutamine synthetase
(18). Malate, the counter ion for citrate exchange by the
tricarboxylate transporter, is likely to be synthesized in the
cytosol by phosphoenolpyruvate carboxylase and malate de-
hydrogenase (5). As suggested by Douce and Neuburger (9),
the citrate3-/malate2- electrogenic exchange may couple this
exchange to the electrochemical gradient and provide direc-
tional transport. The lack of isocitrate exchange suggests that
the mitochondrial and cytosolic pools of this intermediate are
separate, thereby allowing more independent regulation of the
isocitrate dehydrogenase activities in both compartments.

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