Characterization of the Transport of Oxaloacetate by Pea Leaf Mitochondria

David J. Oliver, University of Idaho
Griffin H. Walker, University of Idaho
Characterization of the Transport of Oxaloacetate by Pea Leaf Mitochondria

Received for publication March 12, 1984 and in revised form June 14, 1984

DAVID J. OLIVER* AND GRIFFIN H. WALKER
Department of Bacteriology and Biochemistry, University of Idaho, Moscow, Idaho 83843

ABSTRACT
Mitochondria isolated from pea (Pisum sativum L.) leaves are able to transport the ketoacid, oxaloacetate, from the reaction medium into the mitochondrial matrix at high rates. The rate of uptake by the mitochondria was measured as the rate of disappearance of oxaloacetate from the reaction medium as it was reduced by matrix malate dehydrogenase using NADH provided by glycine oxidation. The oxaloacetate transporter was identified as being distinct from the dicarboxylate and the α-ketoglutarate transporters because of its inhibitor sensitivities and its inability to interact with other potential substrates. Phthalate and phthalate were competitive inhibitors of oxaloacetate transport with $K_i$ values of 60 micromolar and 2 millimolar, respectively. Butylmalonate, an inhibitor of the dicarboxylate and α-ketoglutarate transporters, did not alter the rate of oxaloacetate transport. In addition, a 1000-fold excess of malate, malonate, succinate, α-ketoglutarate, or phosphate had little effect on the rate of oxaloacetate transport. The $K_m$ for the oxaloacetate transporter was about 15 micromolar with a maximum velocity of over 500 nanomoles per milligram mitochondrial protein/min at 25°C. No requirement for a counter ion to move against oxaloacetate was detected and the highest rates of uptake occurred at alkaline pH values. An equivalent transporter has not been reported in animal mitochondria.

Substrate entry into the matrix of both plant and animal mitochondria is controlled by a family of transporters found in the inner mitochondrial membrane. Transporters have been identified from both types of mitochondria that carry monocarboxylates (18, 21), dicarboxylates (17), α-ketoglutarate (11), tri-carboxylates (3), phosphate (13), and pyruvate (7, 21). In both systems, atractylloside-sensitive adenylate transporters exchange ADP and ATP across the membranes and an atractylloside-insensitive net adenylate accumulation has been noted (1, 2, 20).

There are, however, some differences in the transporters seen in animal and plant mitochondria. Plant mitochondria, in addition to a normal tricarboxylate exchanger, appear to have a transporter capable of exchanging citrate for hydroxyl ions (3, 4). While animal mitochondria have a limited permeability to pyridine nucleotides, NAD is transported by a specific carrier in plant mitochondria (8, 14). Animal mitochondria either do not transport OAA or do so only at slow rates on the dicarboxylate and/or α-ketoglutarate carrier (12, 19). Plant mitochondria, on the other hand, transport OAA at rapid rates (15) by a unique phthalate-sensitive carrier (9, 10). In this paper we explore some of the kinetic capabilities of the OAA transporter in pea leaf mitochondria.

MATERIALS AND METHODS
Mitochondria were isolated from 3-week old greenhouse grown pea (Pisum sativum L.) plants. The peas (300-400 g) were disrupted for 3 s in 1 L of 0.5 M sorbitol, 30 mM Mops-NaOH (pH 7.3), 2 mM EDTA, and 0.2% BSA in a 4-L Waring Blender. After filtering through nylon mesh and Miracloth (CalBiochem), the homogenate was centrifuged at 2000g for 5 min to remove the majority of the chloroplasts and then at 9000g for 10 min to pellet the mitochondria. The pellet was resuspended in the reaction medium, 0.3 M sorbitol, 20 mM Mops-NaOH (pH 7.2), and 0.1% BSA. After clarifying the supernatant at 1000g for 10 min, the mitochondria-enriched pellet was collected at 6000g for 15 min, resuspended in a minimal volume of reaction medium and used without further purification (23, 24).

The chloroplast fragments in this preparation neither reduced OAA nor interfered with its assay.

Measurements of OAA Uptake by Mitochondria. Because of the lability of OAA and the high $V_{max}$ and low $K_m$ of its transporters, conventional techniques for measuring transport by mitochondria proved unsatisfactory. OAA uptake, therefore, was monitored by measuring the disappearance of OAA when mitochondria were supplied with an NADH-generating substrate, usually glycine. Due to the large excess of malate dehydrogenase activity present in the mitochondrial matrix, OAA entering the matrix was immediately reduced to malate. The concentration of OAA was determined spectrophotometrically as the 2,4-dinitrophenylhydrazine derivative (5).

Mitochondria were incubated in 0.5 ml of the reaction medium containing 10 mM glycine at 25°C. After a 1-min preincubation, the OAA at the desired concentration was added and mixed by vortexing. The reaction was stopped at the time specified (usually 5 or 10 s) by adding 0.5 ml of 0.05% (w/v) 2,4-dinitrophenylhydrazine in 2 M HCl (made fresh daily). The dinitrophenylhydrazine-derivative of OAA was allowed to form at 25°C for 30 min before it was extracted into 1 ml of ethyl acetate by vortexing for 20 s. After the liquid phases were separated by centrifuging at 1500g for 1 min, 0.5 ml of the ethyl acetate layer was removed. The hydrazide derivative was extracted into 0.5 ml of 10% Na2CO3 by vortexing for 20 s. The absorbitivity of the aqueous phase at 380 nm was then measured and compared with a standard curve. The assay as used could measure 0.2 to 100 nmol of OAA.

Alternatively, OAA levels were measured using HPLC. A 100 μl sample of the reaction medium was transferred to 100 μl of acetonitrile to stop the reaction. The OAA was separated on a HPX-87H ion exclusion column (Bio-Rad) and measured by its absorption at 210 nm (23).

Synthesis of Inhibitors. Butylmalonate was synthesized from

---

1 Funded by a grant from the National Science Foundation (PCM-8204186) and is publication No. 8364 of the Idaho Agricultural Experiment Station.

2 Abbreviations: OAA, oxaloacetate; Mops, 3-(N-morpholino)propane-sulfonic acid.
its diethylester (Aldrich Chem. Co.). Diethylbutyralonate (25 g or 0.116 mol) was stirred vigorously with 50 ml of NaOH (0.250 mol) overnight until the two phases joined. The solution was extracted twice with 100 ml of diethylether. Sufficient concentrated H₂SO₄ was added to lower the pH to 2.0 and the butyralonate acid was extracted into 500 ml of ether. After the ether was removed by evaporation, the disodium salt was formed by adding sufficient NaOH to bring the pH to 7.0. The solution was extracted twice with two volumes of ether and the aqueous phase collected and lyophilized. The solid disodium butyralonate was stored at room temperature.

Phthalonate was synthesized using the method of Von Braun (22) and was estimated to be about 90% pure using HPLC. The major contaminant was phthalate as noted by Day and Wiskich (9).

RESULTS AND DISCUSSION

Several authors have noted that O₂ uptake by plant mitochondria oxidizing NAD-linked substrates could be inhibited by adding exogenous OAA (9, 10, 15). The inhibition was transient with O₂ uptake resuming after the OAA in the medium was depleted. Such is the case when the glycine decarboxylase complex in pea leaf mitochondria oxidizes glycine in CO₂, NH₃, and a one-carbon fragment with the obligate reduction of NAD to NADH (24, 25). Oxygen uptake is prevented when the NADH is reoxidized by an excess of the enzyme malate dehydrogenase in order to reduce added OAA to malate before the electrons can be donated to the electron transport chain. In Figure 1, pea leaf mitochondria were incubated with 1 mM OAA and the concentration of OAA in the medium and the rate of O₂ uptake were monitored. In the absence of an electron donor, little O₂ consumption and O₂ uptake were observed. After the addition of 10 mM glycine, the O₂ level in the medium decreased rapidly and within 4 min was no longer detectable. O₂ uptake continued at a low level for 3 min after the glycine addition. At this point, approximately 95% of the OAA had been consumed and within the next minute glycine-dependent O₂ uptake had reached a maximum rate.

When pea leaf mitochondria were supplied with glycine as a source of matrix NADH production, the rate of OAA depletion from the reaction medium was dependent on the OAA concentration (Fig. 2). Under the conditions employed, when 50 or 100 μM OAA were supplied to the mitochondria, OAA depletion was linear for 60 s. At lower OAA concentrations, O₂ uptake was linear for 20 s (25 μM OAA) or 10 s (10 μM OAA). At lower OAA concentrations (10 or 25 μM) and at higher concentrations and longer times (Fig. 1), OAA was depleted below the lowest detection limit, 0.2 nmol.

When the initial rates of O₂ uptake at varying OAA concentrations were presented as a double reciprocal plot, Michaelis-Menten kinetics were observed (Fig. 3). For five experiments the apparent Kₘ was 15 ± 1 μM and the Vₘₐₓ was 550 ± 50 nmol/mg protein-min at 25°C. Because of the indirect method used for measuring O₂ uptake, we were concerned that OAA reduction to malate at high OAA levels rather than the rate of O₂ transport may have limited the rate of depletion. Malate dehydrogenase activity in freeze-thawed mitochondia was over 2000 nmol/mg protein-min (also see [9]). Its activity, therefore, was not limiting. In addition, when 10 mM α-ketoglutarate and citrate were provided to increase the potential for matrix NADH synthesis, the maximum rate of OAA disappearance only increased 540 to 570 nmol/mg-min. The addition of 5 mM citrate did not change the rate of O₂ transport, suggesting that OAA was not moving on the dicarboxylate transporter (Fig. 3).

Day and Wiskich (9, 10) have reported that the inhibition of glycine-dependent O₂ uptake by OAA was prevented by phthalonate but not butyralonate. Phthalonate and phthalate were both competitive inhibitors of O₂ uptake (Fig. 4) while butyralonate had little effect (data not shown). The Kᵣ for phthalonate was 0.06 mM and for phthalate 2.1 mM. Day and Wiskich (9) have reported that phthalonate, in addition to inhibiting O₂ transport, can inhibit glutamate and citrate (but not α-ketoglutarate and dicarboxylate) transport by plant mitochondria. Although it inhibited malic enzyme activity, it was not an effective inhibitor of malate dehydrogenase.

Butyralonate was an effective inhibitor of both malate and succinate oxidation. In both cases the inhibition was competitive with an apparent Kᵣ of 2 to 3 mM (Table I). Both malate and succinate enter the matrix via the dicarboxylate transporter. Since the dicarboxylate transporter of pea leaf mitochondria was sensitive to butyralonate (butyralonate does not cross the membrane [17]) and this inhibitor did not alter O₂ uptake, this again supports the contention that O₂ transport does not occur on the dicarboxylate carrier. In addition, 10 mM malate, malonate, α-ketoglutarate, or succinate were shown to have little effect on the rate of uptake of 10 μM OAA.

The pH response for O₂ uptake is presented in Figure 5. The rate of O₂ uptake was maximal at pH 8.0 to 8.5. The rate of OAA consumption was greater at the high pH values despite the observation that glycine-dependent O₂ uptake was maximal at pH 6.5. The apparent saturation in the rate of O₂ uptake and reduction at pH 8.5 may have resulted from the decrease in NADH available from glycine decarboxylation. OAA consumption was not limited by matrix malate dehydrogenase activity at low pH values, because the addition of higher concentrations of OAA under these conditions substantially increases the rate of

---

Fig. 1. Glycine oxidation and OAA reduction by pea leaf mitochondria. Mitochondria (1 mg protein) were incubated 1 ml of the reaction medium (pH 7.2) for 2 min before the addition of 1 mM OAA (time 0). O₂ uptake was measured continuously through the course of the assay. At the times indicated, samples were taken and the OAA concentration determined by HPLC. Two min after the addition of OAA, 10 mM glycine was added to the reaction.
OXALOACETATE TRANSPORT IN PEA MITOCHONDRIA

FIG. 2. Oxaloacetate uptake and reduction by pea leaf mitochondria. Mitochondria (46 μg protein) were incubated in 0.5 ml of reaction medium (pH 7.2) containing 10 mM glycine for 1 min. OAA at the concentrations indicated was then added with rapid mixing. At the appropriate time, the reaction was terminated by adding the dinitrophenylhydrazine reagent and the remaining OAA determined spectrophotometrically.

FIG. 3. Kinetic parameters of OAA uptake and reduction by pea leaf mitochondria. The experiment was similar to that in Figure 2 except 40 μg of mitochondrial protein were used and the reaction was terminated 10 s after the addition of the OAA. Where indicated, 5 mM K-phosphate was included in the reaction medium.

OAA disappearance.

Because of the potential lability of OAA at high pH values, controls were performed at each pH by incubating the OAA at the desired pH for 10 s before adding the 2,4-dinitrophenyl hydrazone reagent and mitochondria.

CONCLUSIONS

Pea leaf mitochondria contain a unique transporter for OAA that differs significantly from their animal counterparts. The OAA transporter has an apparent $K_m$ of 15 ± 1 μM and a maximal velocity of at least 500 nmol/mg protein · min at 25°C. Phthalate and phthalonate are competitive inhibitors of OAA transport with $K_i$ values of 60 μM and 2 mM, respectively. Other transported anions, including malate, malonate, succinate, α-ketoglutarate, and phosphate, had little effect on OAA transport. In addition, butyraldehyde, an inhibitor of the dicarboxylate and α-ketoglutarate transporters, was without effect. This confirmed earlier work by Day and Wiskich (9, 10) who showed that phthalonate, but not butyraldehyde, was capable of preventing added OAA from inhibiting glycine-dependent O2 uptake. Taken as a whole, the data presented show that pea leaf mitochondria possess a specific carrier with a high affinity and turnover rate for OAA.

The validity of the values presented for the kinetics of OAA transport are dependent on a number of assumptions. First, it is essential that OAA reduction occurred within the matrix. Malate dehydrogenase activity (about 200 nmol/mg·min) was measurable on the outer surface of these mitochondria, but was not sufficient to reduce OAA at the rates observed. More importantly, the reduction of OAA was dependent on the addition of glycine (Fig. 1). NADH production by the glycine decarboxylase complex occurs in the mitochondria matrix and no mechanism is known to account for NADH export at the rates required (8). Second, it is important to show that the rate of OAA depletion by mitochondria was limited by the rate of OAA uptake and not the rate of its reduction in the matrix. As noted above, total malate dehydrogenase activity and the potential for NADH
Fig. 4. The effect of phthalonate and phthalate on OAA uptake and reduction by pea leaf mitochondria. The conditions were the same as those in Figure 2 except 38 μM of mitochondrial protein were used per assay and where indicated either 0.5 mM phthalonate or 10 mM phthalate were included during the 1-min incubation before OAA addition.

Table 1. Kinetics ofDicarboxylate Oxidation and Inhibition by Butylmalonate in Pea Leaf Mitochondria

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K_m (mM)</th>
<th>Butylmalonate</th>
<th>Type of inhibition</th>
<th>K_i (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate</td>
<td>7.7</td>
<td>Competitive</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>2.4</td>
<td>Competitive</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>α-KG</td>
<td>2.1</td>
<td>Competitive</td>
<td>2.2</td>
<td></td>
</tr>
</tbody>
</table>

production are not limiting. Phthalonate can inhibit OAA reduction by inhibiting its transport and K_i values calculated with 50 μM and 0.5 μM phthalonate were the same. Together this suggests that OAA reduction is transport limited. It is somewhat surprising that OAA addition almost completely inhibited glycine-dependent O_2 uptake (Fig. 1) if OAA transport was indeed limiting. This may indicate that the decrease in matrix NADH/NAD ratio following OAA addition resulted in a disproportionate loss of electron transport capacity. Recently, Chen and Heldt (6) reported half-maximal inhibition of glycine-dependent O_2 uptake in spinach mitochondria by approximately the same concentration of OAA that gave us half-maximal uptake.

Our results differ sharply from reports of OAA transport in animal mitochondria. In these organelles, OAA transport is generally reported to be less than 100 nmol/mg protein-min with K_m values ranging between 0.1 and 10 mM (12, 19). Although some disagreement still exists in the literature, OAA uptake into the matrix of animal mitochondria appears to involve both the dicarboxylate and α-ketoglutarate transporters of these organelles. Animal mitochondria show no indication of a unique carrier with kinetic and specificity properties equivalent to that from pea leaf mitochondria.

The mechanism of the OAA transporter in pea leaf mitochondria is unresolved. Mitochondrial membrane transporters generally function by exchanging an anion on one side of the membrane for an anion on the other side (antiport mechanism). Those carriers that do not exchange for a substrate anion (i.e. the F,, transporter) either exchange for hydroxyl ions or cotransport a proton along with the anion (synport mechanism). It is difficult to differentiate between these two options experimentally. We have been unable to identify a substrate anion that transports against OAA. The most likely mechanism, therefore, would be exchange for hydroxyl ions or cotransport with protons. In this case, OAA transport should be increased at lower pH. This was not the case (Fig. 5). The high pH optimum may reflect a direct effect of pH on the activity of the transporter that more than compensates for the reduced concentration of exchangeable protons in the medium. More work will need be done before a mechanism is confirmed.

The physiological role of this OAA transporter in pea leaves had yet to be determined. In combination with the dicarboxylate transporter it could be used to move reducing equivalents either into or out of the mitochondrial matrix. The existence of an external NADH dehydrogenase in these organelles decreases the need for an NADH shuttle system designed to carry cytosolic reducing equivalents into the matrix. Woo and Osmond (25) have suggested that a malate/OAA shuttle may be involved in shuttling NADH produced during photorespiration by the glycine decarboxylase complex out of the mitochondria. Inhibitor studies, however, suggest that such a shuttle is not important during respiratory glycine oxidation (16). In addition, it is unlikely that this is the unique role of this transporter because mitochondria isolated from etiolated corn, a plant with low
photorespiration, exhibited an OAA transporter with $K_m$ of 16 $\mu$M and a $V_{max}$ of over 200 $\mu$mol/mg protein-min (data not presented). Irrespective of its role, the low $K_m$ of the transporter should allow it to compete successfully with cytosolic or matrix malate dehydrogenases.

**LITERATURE CITED**


5. BONTING SL 1955 Colorimetric determination of pyruvic acid and other $\alpha$-ketocids in submicrogram quantities. Arch Biochem Biophys 58: 100–111


20. PFAPP E, M KLINGENBERG, HW HELDT 1965 Unspecific permeation and specific exchange of adenine nucleotides in liver mitochondria. Biochim Biophys Acta 104: 312–315


