Glycine Metabolism and Oxalacetate Transport by Pea Leaf Mitochondria

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ABSTRACT

Isolated pea leaf mitochondria oxidatively decarboxylate added glycine. This decarboxylation could be linked to the respiratory chain (in which case it was coupled to three phosphorylations) or to mitochondrial malate dehydrogenase when oxalacetate was supplied. Decarboxylation rates measured as O2 uptake, or CO2 and NH3 release were adequate to account for whole leaf photosynthesis. Oxalacetate-supported glycine decarboxylation, measured by linking malate efflux to added malic enzyme, yielded rates considerably less than the electron transport rates. Butylmalonate inhibited malate efflux but not oxalacetate entry; phthalonate inhibited oxalacetate entry but had little effect on malate or a-ketoglutarate oxidation. It is suggested that oxalacetate and malate transport are catalyzed by separate carrier systems of the mitochondrial membrane.

Mitochondria from the leaves of higher plants are capable of oxidatively decarboxylating glycine (2 mol glycine being converted to one of serine), and this reaction is considered to be the primary source of CO2 released during photosynthesis (15, 30). Conversion of glycine to serine is accompanied by release of CO2 and NH3 and is catalyzed by glycine decarboxylase (EC 2.1.2.10) plus serine hydroxymethyl transferase (EC 2.1.2.1). NADH is also generated within the mitochondria by this reaction and must be reoxidized to allow decarboxylation to continue. Reoxidation in isolated mitochondria can occur in two ways: via the respiratory chain linked to ATP formation (4, 25) or via malate dehydrogenase when OAA5 is supplied (25, 35). The latter system may involve an OAA-malate shuttle between mitochondria and peroxisomes in vivo (35) and has the advantage of being unaffected by high phosphate potential.

Much of the above information has been gained from studies with isolated spinach mitochondria. The present investigation sought to extend these studies to pea leaf mitochondria and to answer some unsolved questions, viz, what are the relative potentials of the two NADH oxidizing systems and by what mechanism do malate and oxalacetate cross the mitochondrial membrane?

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3Abbreviations: CCP, carboxyl cyanide m-chlorophenyl-hydrazone; OAA, oxalacetate; MDH, malate dehydrogenase.

MATERIALS AND METHODS

Pea seedlings (Pisum sativum L.) were grown for 2 to 3 weeks in vermiculite (supplemented with Hoagland solution) in a glasshouse. [14C]Glycine was purchased from the Radiochemical Centre (Amersham), and other biochemicals came from Sigma or Calbiochem (Sydney, Australia). 2-n-Butylmalonate was prepared by the Organic Chemistry Department of Adelaide University (Adelaide, Australia), and phthalonate acid was prepared by Dr. Roger Harris, Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry (Canberra, Australia), using published techniques (31). Piericidin A was generously provided by Dr. J. M. Palmer (Imperial College, London, England).

Mitochondria were isolated from 20 to 40 g of young pea leaves, as described previously (11). Protein was estimated according to Lowry et al. (21) with BSA as standard, and Chl was estimated by the method of Arnon (1). Mitochondrial protein was corrected for the contribution by broken thylakoids by assuming a thylakoid protein-to-Chl ratio of 7:1 (13). On this basis, thylakoids contributed 30 to 40% of the total protein in the mitochondrial fraction, and specific activities shown in figures and tables have been corrected for this contribution. 'Whole cell' rates of glycine decarboxylation, expressed as μmol h−1 mg Chl−1 were estimated by taking into account the yield of isolated mitochondria and the total Chl extracted from the leaves (19).

O2 consumption was measured using a Rank O2 electrode (Rank Bros., Cambridge, U. K.) in 2 to 3 ml of standard reaction medium (0.3 M sorbitol, 10 mM Tes buffer, 10 mM KH2PO4, 2 mM MgCl2, 0.1% BSA [pH 7.2]) at 30 C. O2 in air-saturated medium was assumed to be 240 μM.

Enzyme assays were performed spectrophotometrically at room temperature. Mitochondria were disrupted either by incubating for 5 min with 0.5% digitonin (and then treating on a column of Sephadex G-25) (16) or by diluting with reaction medium and sonicating. Malate dehydrogenase was assayed according to Ochoa (26), NAD-malic enzyme according to Chapman and Hatch (6), and fumarase by the method of Huang and Beevers (18).

Glycine decarboxylation via a reconstituted OAA-malate shuttle was measured as follows. Mitochondria (approximately 1 mg protein) were added to 0.9 ml standard reaction medium which also contained 5 μM antimycin A, 0.1 mM OAA, 4 mM NADP, and 2 to 3 units chicken-liver NADP-malic enzyme. The reaction was initiated by adding 10 mM glycine, and the reduction of NADP followed at 340 nm.

NH3 release was measured simultaneously with O2 consumption in a sealed Perspex vessel into which were fitted an Orion ammonia electrode (connected to a Beckman pH meter) and a Clarke-type O2 electrode (Yellow-Springs, OH). The signals from both electrodes were monitored with a twin-channel Honeywell recorder. The capacity of the vessel was 9 ml. Mitochondria (0.5 ml, 3 to 7 mg protein) were added to 8 ml standard reaction
medium (see above). Other additions were made as indicated in the table legends. NH$_3$ release was calibrated by injecting known amounts of NH$_4$Cl.

Release of $^{14}$CO$_2$ from $[^{14}]$glycine was measured essentially as described in (35). Mitochondria (0.1 ml) were added to 1.32 ml standard reaction medium which also contained either 5 mm oxalacetate or 4 mm ADP plus 10 mm glucose and 10 μl (1 mg/ml) hexokinase. The reaction was performed in 50-ml conical flasks fitted with serum stoppers; a GS-A filter disc soaked with 0.1 ml 20% NaOH was suspended from the stopper, and the flasks were shaken at 25 C. [1-$^{14}$C]Glycine (20 mM, with a specific radioactivity of 3 nCi/μmol) was injected to start the reaction. The reaction was terminated by adding 0.5 ml 8 N formic acid; the flasks were shaken for a further 15 min, and the filter discs removed and placed in scintillation fluid (35). Radioactivity was determined using external standards.

RESULTS

General Respiratory Properties of Isolated Pea Leaf Mitochondria. Respiratory rates with several different substrates are shown in Table I. In general, good respiratory control and high ADP to O ratios were observed, with inhibitor sensitivity much the same as that of spinach mitochondria (13). Alternative path activity was only about 20% of the Cyt path activity, as judged by antimycin inhibition (Fig. 1A). In contrast to spinach mitochondria (results not shown), pea leaf mitochondria readily oxidize external NADPH via the respiratory chain, and this oxidation is coupled to two phosphorylations and is insensitive to piericidin A (Table I) and rotenone. External NADPH oxidation has recently been observed with mitochondria from a number of different plant species and is thought to involve an externally located dehydrogenase distinct from that which catalyzes NADH oxidation (2). Pea mitochondria also show substantial rates of O$_2$ consumption with glutamate as substrate (Table I), but this required addition of thiamine pyrophosphate and was inhibited by arsenite (not shown). This implies that some (at least 50%) of the O$_2$ uptake observed is due to oxidation of α-ketoglutarate (formed by glutamate dehydrogenase).

NAD-linked substrate oxidation is sensitive to the site I inhibitor, piericidin A (Table I), but this inhibition can be partly relieved by adding NAD. This is shown for glycine in Figure 1B but was also observed with other substrates, the relief being most pronounced with malate. Similar observations with other plant mitochondria have been interpreted as evidence for direct transfer of reducing equivalents out of the mitochondria (10). Adding NAD did not significantly stimulate rotenone-inhibited O$_2$ uptake by spinach mitochondria (data not shown; see Ref. 13).

Glycine Oxidation and Decarboxylation. O$_2$ consumption with glycine as substrate is rapid and coupled to three sites of phosphorylation (Fig. 1A). Glycine oxidation followed conventional Michaelis-Menten kinetics, with an apparent $K_m$ of 2 mM. Inhibitor sensitivity was the same as that of other NAD-linked substrates (Table I and Fig. 1), showing that, under these conditions, the NADH generated during glycine decarboxylation is reoxidized via the internal NADH dehydrogenase of the respiratory chain. Addition of OAA dramatically restricts O$_2$ consumption (Fig. 1C), as matrix NADH is oxidized via malate dehydrogenase. However $^{14}$CO$_2$ release from labeled glycine is sustained under these conditions (Table II). In our hands, the OAA (MDH)-supported glycine decarboxylation is approximately as fast as the ADP (respiratory chain)-supported system (Table II).

Glycine decarboxylation can also be followed by measuring the release of NH$_3$. We followed NH$_3$ release using an ammonia electrode in a vessel which also allowed the use of an O$_2$ electrode; such simultaneous measurements allow a more dynamic picture to be obtained than is possible with the $^{14}$CO$_2$ measurements. State three-state four transitions can be observed with both O$_2$ uptake and NH$_3$ release (Table III, Exp. 1), illustrating the close coupling between glycine decarboxylase and the respiratory chain when ADP is supplied. Addition of OAA, on the other hand, uncouples the two systems as O$_2$ uptake is inhibited and NH$_3$ release stimulated (Table III, Exp. 2). The latter stimulation by OAA in the absence of ADP shows that, under these conditions, glycine decarboxylation is independent of phosphate potential.

Table I. General Respiratory Properties of Pea Leaf Mitochondria

<table>
<thead>
<tr>
<th>Substrate</th>
<th>State Three</th>
<th>State Four</th>
<th>+Piericidin A</th>
<th>ADP/O</th>
<th>nmol·min$^{-1}$mg$^{-1}$ protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate + glutamate</td>
<td>140</td>
<td>50</td>
<td>50</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>90</td>
<td>30</td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>69</td>
<td>29</td>
<td>2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td>150</td>
<td>82</td>
<td>152</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>100</td>
<td>62</td>
<td>95</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>

Table II. $^{14}$C/Glycine Decarboxylation by Pea Leaf Mitochondria

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Substrates</th>
<th>State Three O$_2$ Uptake (+ADP +OAA)</th>
<th>$^{14}$CO$_2$ Release (nmol·min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glycine</td>
<td>65</td>
<td>109</td>
</tr>
<tr>
<td>2</td>
<td>Glycine</td>
<td>58</td>
<td>82</td>
</tr>
<tr>
<td>3</td>
<td>Glycine</td>
<td>210</td>
<td>181</td>
</tr>
<tr>
<td>4</td>
<td>Glycine + malate</td>
<td>215</td>
<td>170</td>
</tr>
<tr>
<td>5</td>
<td>Glycine + succinate</td>
<td>239</td>
<td>190</td>
</tr>
<tr>
<td>6</td>
<td>Glycine + α-ketoglutarate</td>
<td>219</td>
<td>210</td>
</tr>
</tbody>
</table>
The ratio of O₂ consumed to NH₃ or CO₂ released was 1:2 (Tables II and III), in agreement with the conversion of 2 mol of glycine to 1 mol each of serine, NH₃, CO₂, and NADH₂, which reduces 0.5 mol of O₂ (3, 4).

Addition of malate, α-ketoglutarate or succinate to mitochondria oxidizing glycine had very little effect on the release of NH₃ or CO₂ (Tables II and III, Exp. 3), showing that glycine oxidative decarboxylation was not inhibited by operation of the tricarboxylic acid cycle. O₂ uptake, on the other hand, was stimulated considerably, as observed previously with cauliflower bud mitochondria (9), indicating that concurrent oxidation of substrates occurred. These results suggest that, in pea leaf mitochondria, the respiratory chain has the capacity to oxidize two substrates simultaneously and that the other NAD-linked substrates do not compete with glycine for matrix NAD. The slight effect of α-ketoglutarate also suggests that mitochondrial glutamate dehydrogenase is not involved in the reassimilation of NH₃ released during the photosynthetic nitrogen cycle (20), although this enzyme is present in pea mitochondria (Table 1).

Oxalacetate-Malate Shuttle. The above results show that OAA can readily enter pea leaf mitochondria and reoxidize the NADH formed by glycine decarboxylation; however, they tell us nothing about the fate of the malate formed. We attempted to reconstitute the proposed OAAₐₐ/malateₚₚ shuttle (for the transfer of reducing equivalents between mitochondria and peroxisomes) (35) by adding NADP-malic enzyme to the medium and following the reduction of extramitochondrial NADP upon efflux of malate (Fig. 2). OAA inhibits NADP-malic enzyme appreciably at higher concentrations, and, therefore, the OAA concentration was kept below 0.25 mm and excess malic enzyme added. Under these conditions, a substantial, linear rate of NADP reduction after a slight lag was observed. Adding small quantities of malate to the medium stimulated the reduction (Fig. 2A), showing that malic enzyme activity was not rate-limiting. Rather, it seems that the rate of efflux of malate from the mitochondria limits the system.

Woo et al. (34) have recently demonstrated a similar shuttle in spinach using nitrate reductase and malate dehydrogenase to monitor malate efflux. However, no estimate has been made to date of the potential in vivo rate of such shuttle systems compared with that of the respiratory chain. An estimate of whole cell rates can be made by calculating the percentage of recovery of mitochondria from measurements of a suitable marker enzyme activity (e.g., fumarase) in the leaf homogenate and in the mitochondrial pellet (19). Rates can then be expressed on the basis of the Chl content of the original, crude leaf homogenate. Examples of such rates are shown in Table IV. We consistently found the shuttle rates to be less than one-half of those of the respiratory chain-supported rates with both spinach and pea leaf mitochondria. The respiratory-linked rates in Table IV are approximately equivalent to the estimates of whole leaf rates of photosynthesis that are considered the most reliable (35); that is, approximately 30 μmol h⁻¹ mg⁻¹ Chl (8, 22). Although such comparisons must be made with caution when different species are involved, the OAA/malate shuttle rates we measure are barely adequate to account for the most modest estimates of whole leaf photosynthesis.

Oxalacetate Transport. It is well established that plant mitochondria possess a dicarboxylate carrier which catalyzes the transport of malate, succinate, and Pi across the inner membrane (33). This carrier has been implicated in the reconstituted OAA/malate shuttle-supported nitrate reductase system of spinach (34), by virtue of this system's inhibition by butyramalate (a selective inhibitor of the dicarboxylate carrier). However, malate apparently can utilize other transporters, including the α-ketoglutarate carrier which is also sensitive to butyramalate (12). Involvement of the α-ketoglutarate carrier in OAA/malate exchange was suggested by phthalonic acid inhibition of OAA-supported glycine decarboxylation in spinach mitochondria (24). Phthalonic acid is...
a selective inhibitor of α-ketoglutarate transport in rat liver mitochondria (23). In an effort to shed more light on this matter, we tested the effect of phthalonic acid on pea leaf mitochondria. Phthalonate has very little effect on glycine oxidation itself, but it does diminish the inhibition of O₂ uptake by OAA (Fig. 3A); when added after OAA, phthalonate stimulates O₂ uptake (Fig. 3B and Table V). The effect of phthalonate on NH₃ release is

shown in Table V; OAA stimulates NH₃ release and inhibits O₂ uptake, but subsequent addition of phthalonate reverses these effects. Yet phthalonate has no effect on solubilized mitochondrial malate dehydrogenase and, in fact, does not appear to penetrate the inner membrane (Day and Wiskich, unpublished results). Furthermore, phthalonate has very little effect on α-ketoglutarate oxidation by pea mitochondria (Fig. 3D) or on malate oxidation (Fig. 3C). These results imply that OAA entry occurs on a carrier which is sensitive to phthalonic acid but distinct from the dicarboxylate and α-ketoglutarate carriers.

As mentioned previously, malate transport and oxidation is inhibited by butyramalonate (Fig. 4B). Our reconstituted OAA-malate shuttle is also sensitive to this inhibitor (Fig. 2, B and C), which has little effect on glycine decarboxylation per se (Fig. 4A) and none on malate dehydrogenase (not shown) or on the NADP-malic enzyme used here (Fig. 2, B and C; rates after addition of malate). Yet, butyramalonate does not prevent OAA inhibition of O₂ uptake (Fig. 4A). These results also imply that OAA entry and malate efflux occur on separate transport systems. Consistent with this idea is the inference of the shuttle system by CCP, an uncoupler of oxidative phosphorylation and a protonophore (Fig. 2D). Again, CCP has no effect on glycine decarboxylation (11), malate dehydrogenase, or malic enzyme. This implies that operation of the shuttle requires expenditure of energy (in the form of membrane gradients), and this would not be expected of a simple OAA-malate exchange via a single carrier. Unfortunately, the effect of phthalonate on the shuttle could not be tested because of the extreme sensitivity of NADP-malic enzyme to the inhibitor.

**DISCUSSION**

The results presented show that isolated pea leaf mitochondria readily oxidize glycine via the respiratory chain at rates that are adequate to account for observed rates of whole leaf photorespiration. The properties of this system are similar to those of spinach mitochondria (25, 35). Glycine decarboxylation can also be maintained by a reconstituted OAAmalate shuttle, but these rates are lower than those linked to respiration. In the shuttle system, malate obviously is formed at the same rate as that at which OAA is utilized, and this is similar to the respiratory rate (Table II). It seems that the rate at which malate accumulates in the external medium limits the shuttle. Under the somewhat artificial conditions employed, malate effluxes into a large external volume and, hence, its concentration in the medium at a given time will be low. In vivo, this may not be the case, since the extramitochondrial volume is much smaller, particularly if there is strict coupling between the mitochondria and peroxisomes (where the malate could be used to again reduce NAD formed in the conversion of hydroxy pyruvate to glyceraldehyde) (35). Close interaction between these organelles must also be postulated to avoid oxidation of cytoplasmic nucleotides (and, of course, loss of OAA) as the OAA formed in the peroxisomes finds its way back to the mitochondria. Such a reaction is probable because of high activity of malate dehydrogenase in the cytoplasm (28). This problem could be avoided if a shuttle, such as the malate-aspartate cycle which operates in liver cells (7), were used to transfer reducing power between mitochondria and peroxisomes.

If the rates shown in Table IV are an accurate estimate of in vivo rates, then, clearly, the OAA-supported system must be supplemented by the respiratory chain. It has been suggested that a high cytoplasmic phosphate potential during photosynthesis severely limits mitochondrial electron transport (17, 29), although turnover of the tricarboxylic acid cycle continues (5), perhaps because of NADH turnover via substrate shuttles (34). However, preliminary experiments (Wiskich, unpublished) with pea mitochondria suggest that respiratory chain activity remains substantial (although less than the state three rate) even at high external ATP to ADP ratios. Thus, the two NADH oxidizing systems may operate in tandem. However, if this is the case, then there must be an alternative mechanism for generating NADH within the per-
oxisome.

Since turnover of organic acids via the tricarboxylic acid cycle continues in the light (5), it is important to determine to what extent glycine decarboxylase and the tricarboxylic acid cycle interact or interfere with each other. In cauliflower bud mitochondria, oxidation of a given substrate was inhibited by oxidation of another, although overall O2 consumption was stimulated (9). That is, the two substrates competed for a common respiratory chain component (and, with malate and succinate, for the dicarboxylate carrier). However, in pea leaf mitochondria, concurrent oxidation of tricarboxylic acid cycle intermediates does not influence glycine oxidative decarboxylation (Tables II and III). This suggests that the respiratory chain has the capacity to cope with reducing equivalents from two substrates concurrently. Furthermore, it shows that malate and α-ketoglutarate do not compete with glycine for matrix NAD. This could be due either to a large NAD pool size (plus a high activity of the internal NADH dehydrogenase) or to compartmentation (discrete pools) of matrix NAD and the enzymes involved. The latter could be achieved by one or more of the enzymes being bound to the inner membrane. In this context, it is worth noting that O2 consumption with glycine was never completely inhibited by OAA (e.g., Fig. 1), even at high OAA concentrations, implying that not all of the NADH produced by glycine decarboxylation was available to malate dehydrogenase.

The slight effect of α-ketoglutarate on NH3 release confirms previous reports with spinach mitochondria (20, 32) and suggests that mitochondrial glutamate dehydrogenase is not involved in the reoxidation of NH3 during photorespiration, despite the fact that it readily converts glutamate to α-ketoglutarate (Table I). Since the ammonia electrode requires that NH3 be free in the medium, it is obvious that the gas readily diffuses out of the mitochondria.

The results in Figures 3 and 4 and in Table V suggest that malate efflux and OAA influx occur on separate carriers. Malate transport is sensitive to butylmalonate while that of OAA apparently is not; conversely, phthalonate has little effect on malate oxidation but severely restricts OAA transport. Furthermore, malate does not inhibit OAA-supported glycine decarboxylation (Tables II and III). At first glance, the phthalonate effects suggest the involvement of the α-ketoglutarate carrier (24). This is clearly not so, however, since phthalonate has virtually no effect on α-ketoglutarate oxidation by pea leaf mitochondria (Fig. 3). The inhibition of the reconstituted OAA/malate shuttle by CCP (Fig. 2D) indicates an energy-requiring step which could well reflect the nature of the transport involved. For example, malate could exchange for external Pi (supplied in all media) while OAA could enter at the expense of the transmembrane pH gradient. A more detailed investigation of the effect of phthalonate on plant mitochondria will be published elsewhere (Day and Wischik, manuscript in preparation).

Finally, we wish to point out that even the highest estimates of glycine decarboxylation in the cell (Table IV) (35) are only just sufficient to account for the whole leaf photorespiratory rates. This is unusual. In general, enzyme activities in cell extracts are substantially higher than the in vivo rate of the process they are involved in. While it is feasible that some glycine decarboxylase is inactivated during mitochondrial isolation (the enzyme is quite labile) (25), the low rates also remind us that there may be more than one site of CO2 release during photorespiration (14, 27).

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