GLYCINE TRANSPORT BY PEA LEAF MITOCHONDRIA

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1. Introduction

Mitochondria are the site of glycine decarboxylation in the leaf during photorespiration [1–4]. The NADH produced by this decarboxylation can be reoxidized in the mitochondrial matrix by 2 pathways [3]; either via malate dehydrogenase when OAA is supplied, or by the respiratory chain. In spinach mitochondria glycine is oxidized rapidly by the respiratory chain and is coupled to 3 phosphorylations [4]. Addition of OAA inhibits oxygen uptake with glycine but stimulates its decarboxylation [3,4], and it has been suggested that an OAA_{in}/$malate_{out}$ shuttle may operate to oxidize mitochondrial NADH when the respiratory chain is restricted by high energy charge [3]. Whichever system operates glycine must cross the inner mitochondrial membrane, and very little is known about this process in plants. Oxidative studies with spinach mitochondria have hinted at a selective carrier for glycine [5], but non-specific diffusion has been suggested for mammalian mitochondria [6]. This paper reports a more direct investigation of glycine transport in pea leaf mitochondria.

2. Materials and methods

Pea seedlings (*Pisum sativum* L.) were grown for 2–3 weeks in vermiculite (supplemented with Hoagland's solution) in a glasshouse. Biochemicals were obtained from Sigma Chemical Co. (St Louis, MO) and Calbiochem (Sydney) except for [^{14}C]glycine which was purchased from The Radiochemical Centre, Amersham.

To isolate mitochondria, 40–50 g pea leaves were disrupted with a Polytron PTA-35 probe for 2 s at setting 7, in 200 ml cold (2°C) medium containing 0.3 M sorbitol, 50 mM TES buffer (pH 7.6), 10 mM KH_2PO_4, 1 mM EDTA, 10 mM isoascorbate, 0.2% (w/v) BSA and 0.5% (w/v) PVP-40. The homogenate was filtered through 2 layers of miracloth and centrifuged for 5 min at 1000 × *g* in a Sorvall RC-2B centrifuge. The supernatant was centrifuged at 9000 × *g* for 15 min and the pellets washed by resuspending in ~60 ml 0.3 M sorbitol containing 20 mM TES buffer (pH 7.4) and 0.1% BSA, and recentrifuging at 9000 × *g* for 15 min. Final resuspension was in 3–4 ml wash medium.

Protein was estimated by the method in [7] with BSA as standard, and chl according to [8]. Mitochondrial protein was corrected for the contribution by broken thylakoids by assuming a thylakoid protein: chl ratio of 7:1 [9]. On this basis, thylakoids contributed 30–40% of the total protein in the 9000 × *g* pellet. Specific activities shown in figures and tables have been corrected for this contribution.

Oxygen consumption was measured polarographically in 2 ml standard reaction medium (0.3 M sorbitol, 10 mM TES buffer, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 0.1% BSA, pH 7.2) using a Rank oxygen electrode at 30°C. Oxygen was 240 μM in the air-saturated medium.

Swelling of the mitochondria was measured as a decrease in A_{520} [10] using a Beckman Acta C III spectrophotometer and 1 cm lightpath cuvettes, at room temperature. Mitochondria (1 mg protein) were added to a medium containing 10 mM TES buffer (pH 7.2), 1 mM EGTA, 5 μM antimycin A and isoosmolar solute (see fig.1 for other details).

[^{14}C]Glycine uptake was measured using the sili-

Abbreviations: BSA, bovine serum albumin; chl, chlorophyll; CCP, carbonyl cyanide *m*-chlorophenylhydrazone; INH, isonicotinyl hydrazide; NEM, *N*-ethylmaleimide; PVP, polyvinylpyrrolidone; SHAM, salicyl hydroxamic acid

cone oil technique in [11]. Mitochondria (~2 mg protein) were allowed to accumulate glycine for 1 min at room temperature in 1 ml standard reaction medium (see above) containing 0.1 M INH (to inhibit glycine decarboxylase), 20 μM rotenone (to inhibit internal NADH oxidation), 2 mM cold glycine, 1 μCi [^{14}C]glycine and 2 mM NADH (except where indicated). Samples of 200 μl were layered over 50 μl silicone oil (a 7:2 mixture of Dowcorning 550 and 200 oils) which had in turn been layered over 50 μl 0.4 M sucrose. The reaction was terminated by centrifuging for 1 min in a Beckman model B microfuge. The tubes were cut 1 mm below the oil–sucrose interface and the pellet (and remaining sucrose) resuspended in 1 N HCl. Radioactivity was measured by scintillation counting using the channel-ratios method.

3. Results

Isolated pea leaf mitochondria rapidly oxidize glycine via the respiratory chain with good respiratory control; typical state 3 rates of oxygen consumption were 60–100 nmol . min^{-1} . mg protein^{-1}, and respiratory control ratios 2.0–3.0. Glycine oxidation exhibited similar characteristics to those of other NAD-linked substrates, being partially inhibited by rotenone (an inhibitor of internal NADH oxidation) and antimycin A (which blocks electron transport between cytochrome *b* and *c*), and displayed ADP/O ratios of 2.4–2.7. These results confirm those in [4] with spinach, and show that glycine oxidation is linked to 3 phosphorylations.

Pea leaf mitochondria swelled spontaneously when added to a 300 mM glycine solution (fig.1A), and this swelling was not influenced by addition of NADH (as respiratory substrate) or the uncoupler CCP (not shown). Including the SH-group inhibitors, mersalyl (fig.1a) or NEM in the medium did not inhibit swelling in glycine, although these reagents are potent inhibitors of most mitochondrial transport systems [16]. Swelling in other solutes is also shown in fig.1 for comparison. The extent of glycine-induced swelling was similar to that in iso-osmolar ammonium acetate, a known permeant [10], but the rate with glycine was considerably slower (fig.1b). The mitochondria also swelled spontaneously in proline (fig.1c); however, with KH_2PO_4 as solute, the mitochondria slowly contracted until valinomycin was added to facilitate

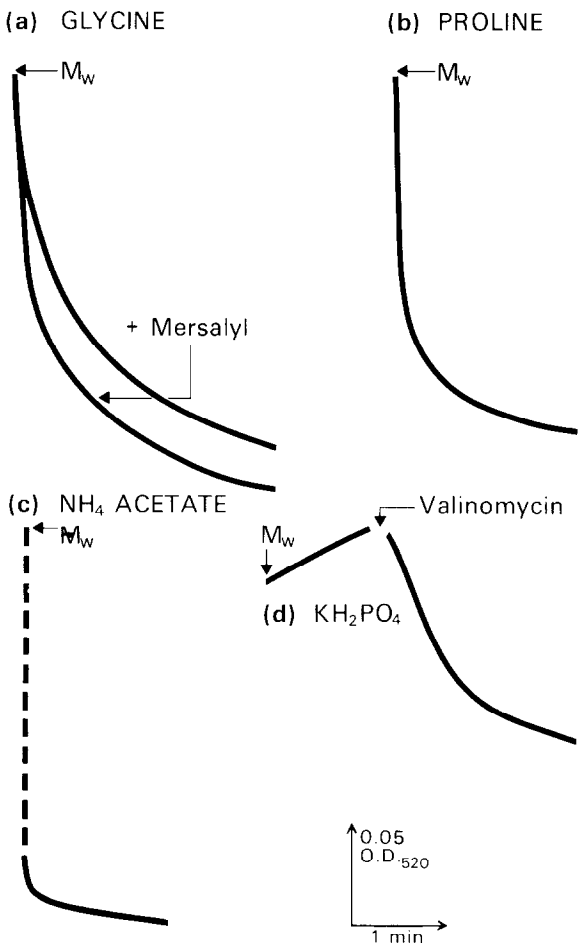

Fig.1. Swelling of pea leaf mitochondria. Swelling was measured spectrophotometrically as in section 2. (a) 300 mM glycine; (b) 150 mM ammonium acetate; (c) 300 mM proline; (d) 150 mM KH_2PO_4. Where indicated, 0.6 mg mitochondrial protein (Mw) and 0.5 μg valinomycin were added. Mersalyl (0.2 mM) was included in the reaction medium, when used. A downward deflection indicates swelling.

K^+ entry (fig.1d). Under these conditions P_i probably enters the matrix electrogenically but its movement is restricted by K^+ entry. These results suggest that glycine enters the matrix as the neutral amino acid down a concentration gradient [6], and this movement probably does not involve a carrier. The data in fig.2 support the latter suggestion; the rate of mitochondrial swelling increased linearly with the glycine concentration of the medium, up to 300 mM. If a carrier had been responsible for glycine uptake, saturation kinetics might have been expected [6].

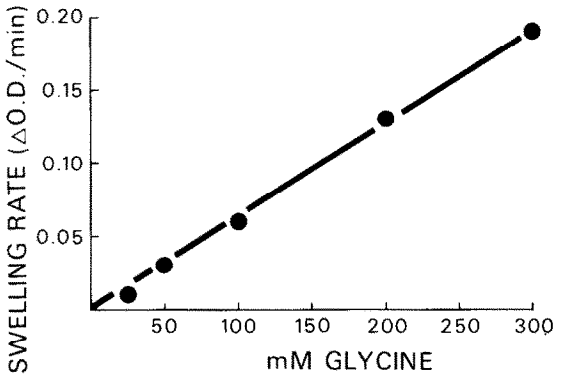

Fig.2. Effect of glycine concentration on the rate of mitochondrial swelling. A final osmolarity of 300 mM was maintained by adding appropriate volumes of sorbitol as the glycine concentration of the medium was varied. A small degree of swelling occurred in the absence of glycine and this value was subtracted from the other rates to yield those shown. About 1 mg mitochondrial protein was added to 1 ml 20 mM Hepes buffer (pH 7.5) containing 1 mM EDTA, 5 μM antimycin A, and varying amounts of glycine and sorbitol.

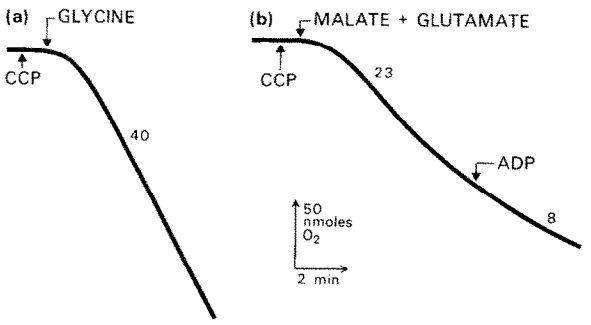

Fig.3. Effect of uncoupler on malate and glycine oxidation. Oxygen consumption was measured as in section 2, except that ADP (0.3 μmol) was included in the reaction medium. Other additions were 10 mM malate, 10 mM glutamate, 10 mM glycine, 2.5 μM CCP, 0.3 μmol ADP. Rates are expressed as nmol O_2 . min^{-1} . mg $protein^{-1}$. State 3 rates of oxygen uptake were 50 and 60 nmol . min^{-1} . mg $protein^{-1}$ for glycine and malate, respectively.

Oxygen electrode measurements also suggest that glycine uptake by pea mitochondria is electroneutral and independent of protonmotive force. Oxygen consumption rates with glycine in the presence of uncoupler are linear and similar to those in the presence of ADP (fig.3a). Malate oxidation, on the other hand, is inhibited by prior addition of uncoupler, and this inhibition increases with time (fig.3b). It should be noted that ADP was present in both cases to avoid complications due to secondary effects of adenine nucleotides on the respiratory chain [12]. Inhibition of substrate oxidation by uncoupler has been observed with other plant mitochondria and has been attributed to inhibition of substrate transport upon collapse of the protonmotive force [13]. Rapid malate oxidation by plant mitochondria requires concomitant P_i transport [13,14] which in turn is driven by the pH gradient across the inner membrane [10]. Fig.3a shows that glycine oxidation is not dependent on these energy-related processes.

Direct measurement of glycine uptake by pea mitochondria supported the swelling and oxygen uptake data (table 1). A small amount of glycine was taken up by the mitochondria but this was not affected by dispersion of the membrane protonmotive force (omitting substrate, presence of CCP) nor by SH-poisons, in agreement with the data of fig.1. Oxygen consumption with glycine, on the other hand, was severely inhibited by NEM and mersalyl (table 1).

Table 1
Glycine uptake and oxidation by pea leaf mitochondria

Assay conditions	Glycine accumulated (nmol/mg protein)	Oxygen consumption (nmol/min)
Control[a]	2.7	30
NADH omitted	3.3	–
+ 5 μM CCP	2.7	65
+ 1 mM NEM	3.1	15
+ 0.2 mM mersalyl	4.0	7

[a] Assay conditions are given in section 2. Control rate of oxygen consumption was measured in the absence of ADP. NEM and mersalyl were added in the presence of CPP

Concentrations of these reagents were 10-fold those in table 1 had no effect on glycine accumulation.

4. Discussion

These results demonstrate that glycine permeates isolated pea leaf mitochondria as the neutral amino acid, and that its uptake is not related to the energy status of the mitochondria. The same was concluded [6] from swelling studies with mammalian mitochondria, discounting suggestions of carrier-mediated glycine transport [15] on the grounds of lack of specificity and non-saturation kinetics of swelling. We also failed to observe saturation kinetics of swelling (fig.3), and did not see any inhibition of uptake by NEM or mersalyl. It is therefore unlikely that pea leaf mitochondria possess a specific carrier for glycine. The inhibition of glycine oxidation by SH-poisons, observed with spinach mitochondria [5] and here with peas, is probably due to inhibition of the enzyme glycine decarboxylase.

The ability of amino acids to form rings with intramolecular hydrogen bonds may permit them to rapidly penetrate the inner membrane of rat liver mitochondria by diffusion [6]. This may also be the case with glycine in leaf mitochondria, and may account for the rapid oxidation of glycine by these organelles.

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