Movement of amino acids into isolated plant mitochondria

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Aspartate, glutamate, serine and glycine all permeate the inner membrane of mitochondria isolated from both etiolated and green plant tissues. No significant difference was found in the transport characteristics shown by mitochondria from either tissue. Influx of each amino acid appears diffusional because substrate saturation was not observed and there was no indication of specific inhibition or a requirement for a compensatory or counter ion for uptake. In contrast, uptake of the keto acid pyruvate did appear saturable. Inhibition by α-cyano-4-hydroxycinnamate, mersalyl and FCCP, but not valinomycin, suggests a carrier and a ΔpH mediate pyruvate transport into the matrix.

Plant mitochondria *Photorespiration* *Centrifugal filtration* *Transport characteristic* *Amino acid* *Keto acid*

1. INTRODUCTION

In the photorespiratory pathway of green leaves the decarboxylation of glycine, to yield serine, CO_2, NH_3 and NADH, occurs in the mitochondrion [1]; requiring, for continuation of the pathway, that both amino acids cross the inner membrane. Results in vitro also suggest that the NADH produced may be oxidised by oxaloacetate, either directly [2] or from aspartate transamination with 2-oxoglutarate [3]. The latter implies the movement of the amino acids aspartate and glutamate into and out of the matrix.

There are results which suggest that the inner membrane of plant mitochondria is permeable to a range of amino acids. Unfortunately, these data are mainly based on indirect evidence, from oxygen uptake experiments [3–5] or from unquantitative methods such as swelling [6–8]. Even direct measurements of uptake, using silicone oil centrifugal filtration as developed for animal mitochondria [9] and later applied to chloroplasts [10], have been limited [8].

For these reasons, we have used the silicone oil technique to directly determine uptake kinetics of certain amino acids by mitochondria, isolated from both etiolated and green plant tissue, as compared to the keto acid pyruvate, a compound which has been reported to involve carrier-mediated uptake in animal [11,12] and etiolated C_4-type mitochondria [13].

Abbreviations: BSA, bovine serum albumin; CHCA, α-cyano-4-hydroxycinnamate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MOPS, 3-(*N*-morpholino)propanesulphonic acid; OAA, oxaloacetate; 2-OG, 2-oxoglutarate; PTA, phthalonic acid; PVP, polyvinylpolypyrrolidone

2. MATERIALS AND METHODS

Imbibed pea seeds (*Pisum sativum* cv. Feltman First; soaked in running tap water for 12–24 h) were planted on saturated John Innes no. 3 compost, covered to a depth of 1 cm with the same and watered on alternate days with tap water. Leaves (~ 100 g) from 13–16-day-old plants, grown in a heated (25°C) greenhouse, were harvested onto ice and subsequently homogenised in 0.8 l of: 0.3 M mannitol; 1 mM EDTA; 0.2% (w/v) BSA; 0.6% (w/v) PVP; 4 mM cysteine and 20 mM MOPS–KOH (pH 7.6). After squeezing through 3 layers cheesecloth/1 layer muslin the filtrate was centrifuged at 3000 × *g* for 5 min. Centrifugation of the supernatant at 14 000 × *g* for 15 min and resuspension of the pellet in 40–50 ml wash medium

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(isolation medium minus cysteine and PVP) was followed by a further centrifugation at 14 000 × *g* for 15 min. The pellet was finally resuspended in 1–2 ml wash medium.

Mung bean hypocotyl mitochondria were prepared as in [4].

Polarographic oxygen consumption was measured in 1 ml reaction medium A [4] using ~0.5–1.0 mg mitochondria protein.

Rates of uptake were estimated from 350 μl incubations containing 0.3 M mannitol; 20 mM MOPS–KOH (pH 7.2); 5 mM $MgCl_2$; 10 mM KCl; 1 mM ATP; 10 μM rotenone; 0.5 μg/ml antimycin A; 10 mM malate; 10 mM phosphate; appropriate concentrations of amino acids and 1.5–2.0 mg mitochondrial protein. The amount of each [^{3}H]amino acid was not considered to contribute significantly to the final concentration of amino acid in the incubation because of their high specific activities ([^{3}H]aspartate, 5.1 Ci/mmol; [^{3}H]-serine, 28 Ci/mmol; [^{3}H]glycine, 30 Ci/mmol; [^{3}H]glutamate, 34 Ci/mmol) and the small quantities used (1 μCi up to 5 mM and 1.5 μCi for greater molarities of amino acid). When pyruvate uptake was measured, the contribution of [^{14}C]pyruvate (0.5 μCi up to 5 mM and 1 μCi for greater pyruvate concentrations; spec. act. 23.8 mCi/mmol) to the total keto acid level in the incubation was taken into account and unlabelled pyruvate was added to give the appropriate final concentration.

Mitochondria were added to initiate uptake and three 100 μl aliquots were pipetted onto a layer of silicone oil (10:1 (v/v); AR200:AR20). Reactions were terminated after 1 min by centrifuging at 12 000 × *g* for 45 s, mitochondria passing through the oil into 20 μl of 10% (v/v) $HClO_4$. Radioactivity in pellets and 20 μl samples of supernatant was determined by liquid scintillation counting, quenching being corrected for by the channels-ratio method. Controls containing [^{14}C]sorbitol or ^3H_2O were used to calculate label in the matrix volume. This was found to be ~1 μl/mg protein.

Chlorophyll was measured by the method in [15] and protein estimated as in [16]. With green leaf tissue, mitochondrial protein was estimated by assuming a thylakoid protein:chlorophyll of 7:1 [17] and subtracting this value from total protein.

Silicone oils were purchased from Wacker-Chemie (Munich) and radiochemicals from Amersham International (Bucks). All other chemicals were obtained from Sigma (Poole).

3. RESULTS

Isolated pea-leaf mitochondria oxidised glycine and malate, showed good respiratory control for both substrates and gave ADP:O ratios indicating that 3 phosphorylation sites were involved (fig.1(a,b)). Addition of 5 mM 2-oxoglutarate and 5 mM aspartate caused up to 60% inhibition of oxygen uptake rates, as did 5 mM oxaloacetate (not shown). Oxygen uptake could be re-established by 10 mM glutamate, the final rate being equivalent whether 2-oxoglutarate/aspartate or oxaloacetate were added. Fig. 1(b) shows a further reduction in malate oxidation by 5 mM PTA (a potent inhibitor of oxaloacetate efflux across the inner membrane [18]), added after 2-oxoglutarate/aspartate. If PTA is added prior to OAA, when pea-leaf mitochondria are oxidising glycine [18] or malate (state 4, pH 7.2; not shown) little or no inhibition occurs. When included before 2-oxoglutarate/aspartate, however, the final oxidation rate is similar to that seen after a post-addition of PTA [19].

Malate oxidation by isolated mung bean mitochondria was also inhibited by 2-oxoglutarate/aspartate (or OAA; not shown), this being alleviated by a subsequent addition of glutamate (fig.1(c)). These indirect results do therefore suggest that the amino acids glycine, glutamate, aspartate and, by inference, serine all cross the inner mitochondrial membrane.

A direct study of amino acid movement into pea-leaf (fig.2) and mung bean (fig.3) mitochondria supported this suggestion, indicating that glutamate, aspartate, glycine and serine each accumulated in the matrix. Uptake, however, did not appear to be saturable, up to 25 mM, suggesting that carriers were not involved. Omission of phosphate and/or malate from incubations caused no reduction in influx, indicating that the 'cascade' carrier system [20] is not necessary for uptake of these amino acids by pea-leaf or mung bean mitochondria.

Although similarly unaffected by the presence or absence of phosphate and/or malate, the keto acid pyruvate did show saturation kinetics (fig.2,3). Values for V_{max} of 27.8 and 21.8 nmol · mg

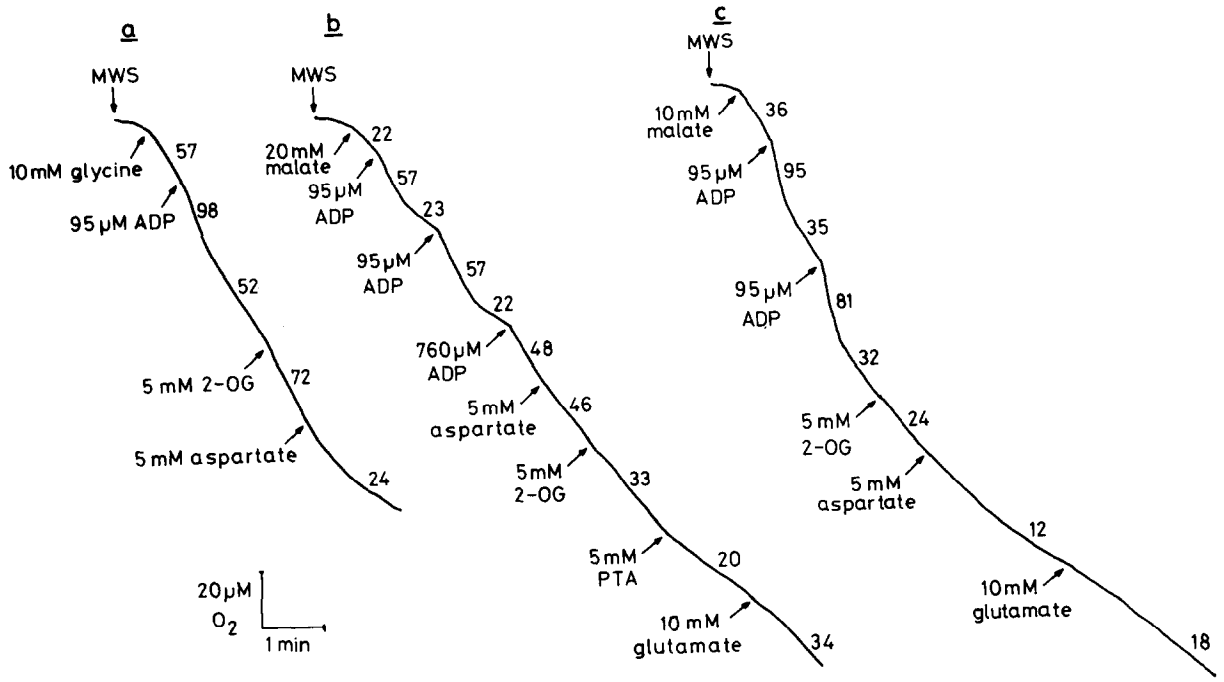

Fig.1. Glycine and malate oxidation by plant mitochondria. Oxygen consumption was measured as in section 2, additions being made as indicated. Numbers by each line represent rate of oxygen uptake, in nmol . mg protein^{-1} . min^{-1}; (a,b) pea-leaf mitochondria; (c) mung bean hypocotyl mitochondria.

protein^{-1} . min^{-1} and for K_m of 5.7 and 4.8 mM were estimated for pea-leaf and mung bean mitochondria, respectively.

Table 1 shows that pyruvate uptake was inhibited by α-cyano-4-hydroxycinnamate, mersalyl and FCCP but not by valinomycin, suggesting that, as with mitochondria from C_4-type plants [13], a carrier protein and a ΔpH are involved in pyruvate transport. Amino acid influx, however, appeared independent of the protonmotive force (table 1), suggesting that each compound crosses the inner membrane in response to diffusional parameters. Glutamate and aspartate were also found not to inhibit each other's uptake (not shown).

Table 1

Uptake of pyruvate, aspartate, glutamate, glycine and serine by pea-leaf mitochondria

Addition	Pyruvate	Aspartate	Glutamate	Glycine	Serine
–	1.6	6.6	6.4	1.8	4.3
FCCP[a]	0.001	6.5	6.4	1.9	4.2
Valinomycin[b]	1.8	6.6	6.5	1.8	4.3
Mersalyl[c]	0.9	6.6	6.6	1.7	4.2
CHCA[d]	0.3	6.5	6.4	1.8	4.4

[a] 1 μM; [b] 100 ng/mg; [c] 200 μM; [d] 100 μM

Uptake, expressed as nmol . mg protein^{-1} . min^{-1}, into the matrix from 1 mM solutions was measured as in section 2

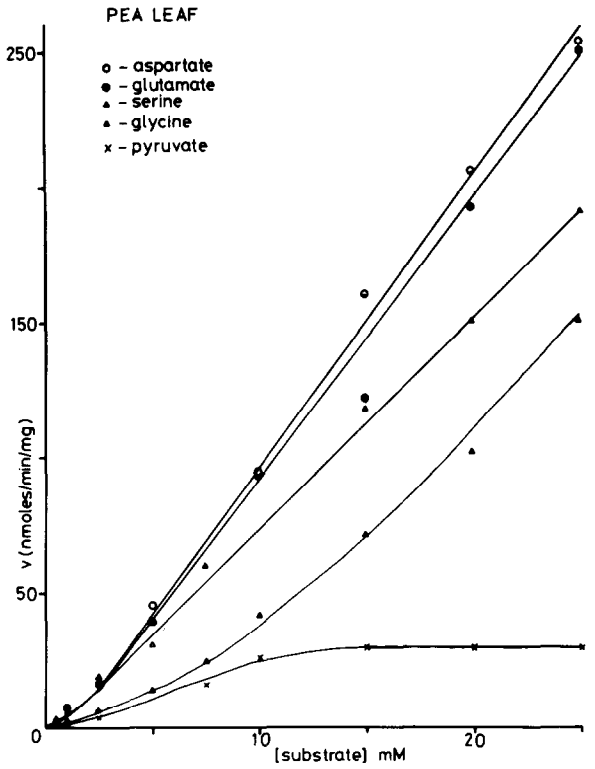

Fig.2. Amino acid and pyruvate uptake by pea-leaf mitochondria. Uptake of each compound was determined as in section 2.

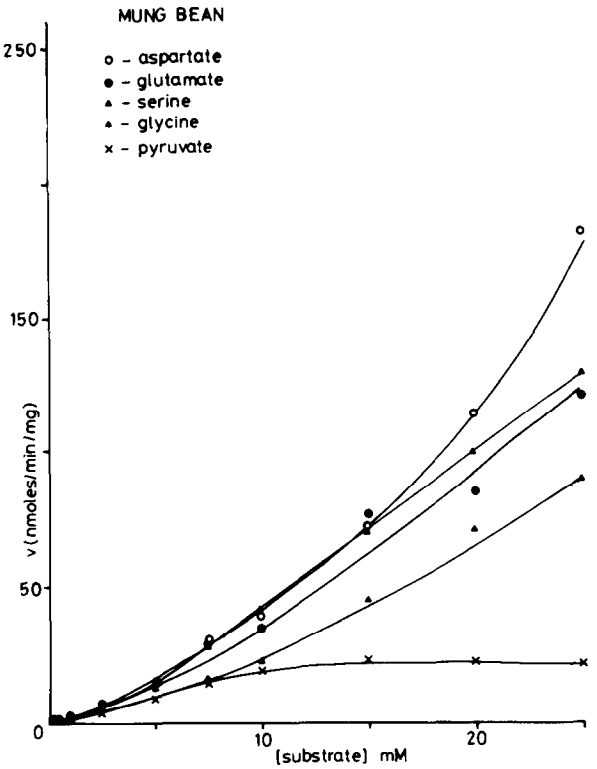

Fig.3. Amino acid and pyruvate uptake by mung bean hypocotyl mitochondria. Uptake of each compound was determined as in section 2.

4. DISCUSSION

These results suggest that, in addition to malate, the keto acids 2-oxoglutarate, oxaloacetate and pyruvate and the amino acids aspartate, glutamate, glycine and serine rapidly permeate the inner mitochondrial membrane of etiolated and green leaf tissue.

Oxaloacetate inhibition of malate and glycine oxidation via the electron-transport chain (caused by the removal of NADH by a reversal of malate dehydrogenase [2], may be achieved directly or indirectly – through transamination of aspartate with 2-oxoglutarate. That this transamination occurs in the matrix is evidenced by the inhibitory effects of phthalonic acid. Such data also show that this inhibitor of oxaloacetate transport has no effect on 2-oxoglutarate or aspartate movement in these mitochondria.

Uptake experiments for each amino acid confirmed the report in [8], that glycine influx was energy-independent, but were in contrast to others [6,7] which proposed that movement of a range of amino acids was carrier-mediated, with glutamate requiring the presence of phosphate and malate. It must be stressed that these latter results were obtained indirectly.

As glutamate did not inhibit aspartate transport, and vice versa, and uptake of both amino acids was insensitive to inhibitors of energy transduction, such as FCCP (cf. pyruvate), and to the relatively non-specific transport inhibitor, mersalyl, it seems unlikely that there exists a carrier which catalyses their exchange (see for instance [3]). Other results [19] have also shown that phthalonic acid has no effect on influx of glutamate or aspartate, as measured directly by silicone oil centrifugal filtration.

That plant mitochondria do not exhibit either a glutamate uniporter and/or glutamate/aspartate

antiporter, as found in mammalian mitochondria [20], can be explained by the different characteristics shown by each. Only the former organelles have an NADH dehydrogenase located on the outer surface of the inner membrane. Consequently they do not require complex exchange systems for the transfer of reducing equivalents from cytosol to matrix, as is the case in mammalian mitochondria. Instead, they may be seen to fulfil a role in the transfer and recycling of amino groups and carbon skeletons between plant cell organelles. Fluxes of these, and possibly other amino acids, will be determined by movement of their corresponding keto acids (these do appear to be carrier-mediated) and also on the local concentrations of each throughout the cell.

Similarly, fluxes of glycine and serine, amino acids which are important intermediates in photorespiratory nitrogen and carbon cycling, would show 'source/sink' responses, dependent on production of glycine and removal, by transamination, of serine (processes which occur in the peroxisomes) as well as glycine decarboxylation in the mitochondria. It is interesting to note that there appeared to be very little difference in the rates of uptake between etiolated and green leaf tissue mitochondria. This suggests that although these organelles may be involved in regulation of keto acid levels it is probably the chloroplast which has ultimate control over concentrations of amino acids within the cell.

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