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Oxaloacetate translocator in plant mitochondria *

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(1) Mitochondria were prepared from leaves of spinach, green and etiolated seedlings and roots of pea, potato tuber and rat liver and heart. In the case of leaf mitochondria, an improved isolation procedure resulted in high respiratory rates (460–510 nmol/mg protein per min) and good respiratory control ratio (6.8–9.8) with glycine as substrate. (2) In these mitochondria oxaloacetate transport was studied either by following the inhibitory effect of oxaloacetate on the respiration of NADH-linked substrates or by determining the consumption of [4-^{14}C]oxaloacetate. (3) Studies of the competition by other carboxylates and effect of inhibitors on the oxaloacetate transport demonstrate that mitochondria from spinach leaves, green pea seedlings, etiolated pea seedlings and pea roots contain a specific translocator for oxaloacetate with a very high affinity to its substrate (K_m = 3–7 μM) and an even higher sensitivity to its competitive inhibitor phthalonate (K_i = 3–5 μM). The V_{max} values ranged from 150 to 180 nmol/mg protein per min for mitochondria from etiolated pea seedlings and pea roots and from 550 to 570 nmol/mg protein per min for mitochondria from spinach leaves and green pea seedlings. In mitochondria from potato tuber, the K_m was about one order of magnitude higher (V_{max} = 450 nmol/mg protein per min). In mitochondria from rat liver and rat heart, a specific translocator for oxaloacetate was not found. (4) The oxaloacetate translocator enables the functioning of a malate-oxaloacetate shuttle for the transfer of reducing equivalents across the inner mitochondrial membrane. (5) This malate-oxaloacetate shuttle appears to play a role in the photorespiratory cycle in catalyzing the transfer of reducing equivalents generated in the mitochondria during glycine oxydation to the peroxysomal compartment for the reduction of β-hydroxypyruvate. (6) Interaction between the mitochondrial and the chloroplastic malate oxaloacetate shuttles would make it possible for surplus-reducing equivalents, generated by photosynthetic electron transport, to be oxidized by mitochondrial electron transport.

Introduction

In plant mitochondria, the respiration of NADH-linked substrates, such as glycine, has been found to be inhibited by the addition of oxaloacetate [1–6]. Since it has been demonstrated that oxaloacetate does not inhibit the oxidation of glycine and other substrates per se [4], this phe-

* Some of these results have been included in a preliminary report (Chen, J. and Heldt, H.W. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. III, pp. 513–516, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands).

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Abbreviations: Chl, chlorophyll; CCCP, carboxyl cyanide *m*-chlorophylhydrazone; Cyt, cytochrome; PEP, phospho*enol*-pyruvate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine; Hepes, 4-(2-hydroxyethyl-1-piperazineethanesulphonic acid.

nomenon was attributed to withdrawal of reducing equivalents from electron transport by oxaloacetate, which enters the mitochondrial matrix and is reduced to malate by intramitochondrial NADH and malate dehydrogenase. In concurrence with permeability measurements [7] these results indicated that plant mitochondria were capable of transporting oxaloacetate.

The occurrence and physiological significance of oxaloacetate transport in animal mitochondria has been a matter of controversy in the past. Although the general experience is that mitochondria from animal tissues are not sensitive to the addition of oxaloacetate [8], there are reports that in mitochondria from rat liver oxaloacetate was transported at significant rates by the dicarboxylate [9] and also by the 2-oxoglutarate translocator [10]. In contrast to a statement that rat heart mitochondria are virtually impermeable to oxaloacetate [8], other workers reported that in these mitochondria oxaloacetate was transported by the 2-oxoglutarate translocator even more effectively than in mitochondria from rat liver, and a physiological function of a malate-oxaloacetate shuttle [11] has been discussed.

To elucidate the nature of oxaloacetate transport in plant mitochondria, inhibitor studies have been employed. Butylmalonate, an inhibitor of the mitochondrial dicarboxylate translocator [7,12], did not decrease the inhibitory effect of oxaloacetate on mitochondrial respiration, which indicated that oxaloacetate was not transported by the dicarboxylate translocator [13]. The inhibitory effect of oxaloacetate on respiration could be reversed, however, when phthalonate was added [13–16]. Phthalonate, a powerful inhibitor of 2-oxoglutarate transport in rat liver mitochondria [17] has been earlier found also to inhibit oxaloacetate transport in rat heart mitochondria [11], supporting the notion that oxaloacetate was transported by the 2-oxoglutarate translocator. In plant mitochondria, however, an unequivocal conclusion on the nature of oxaloacetate transport could not be drawn from the effect of phthalonate, since contradictory results have been reported on the inhibitory effects of phthalonate on 2-oxoglutarate and glutamate transport [13,15], and phthalonate was also found to inhibit citrate transport [13,15].

A study of the concentration dependence of the inhibitory effect of oxaloacetate on the glycine-dependent respiration of pea leaf mitochondria carried out in our laboratory (for a preliminary report, see Ref. 6), revealed that transport of oxaloacetate with an apparent half saturation of 8–12 μM was not markedly affected even by a 1000-fold excess of 2-oxoglutarate, citrate, malate or aspartate, clearly demonstrating that the transport of oxaloacetate is catalyzed by a translocator of very high specificity, distinct from the dicarboxylate, 2-oxoglutarate and citrate translocator. These conclusions have been supported subsequently by Oliver and Walker [16], who found, by measuring the consumption of oxaloacetate by pea leaf mitochondria in the presence of glycine, an apparent K_m value for oxaloacetate of 15 μM and a maximal rate of over 500 nmol/mg protein per min. These authors also reported that phthalonate inhibited oxaloacetate transport competitively with a K_i value of 60 μM.

The present publication examines the nature and physiological significance of oxaloacetate transport in mitochondria from various plant tissues, and for comparative purposes, from rat liver and heart mitochondria. The uptake of oxaloacetate by mitochondria has been determined by existing procedures and a new method based on the consumption of ^{14}C oxaloacetate [18].

Materials and Methods

Pea seedlings (*Pisum sativum* var. 'Kleine Rheinländerin') were grown in a greenhouse for 12–16 days in moist vermiculite [19]. Spinach (*Spinacea oleracea* var. 'Atlanta') was grown in hydroponic culture [20] and 4–6 week old plants were used. Potatoes (*Solanum tuberosum*) were purchased from local markets. Oxaloacetate, 2-oxoglutarate, α-cyano-4-hydroxycinnamic acid, benzene-1,2,3-tricarboxylate and bovine serum albumin were obtained from Sigma Chemicals Co., St. Louis. Phenylsuccinic acid and *N*-butylmalonic acid were purchased from EGA-Chemie, Steinheim, F.R.G. Other Chemicals were from E. Merck, Darmstadt, F.R.G. Phthalonic acid was synthesized according to Van Braun [21] and generously supplied by Prof. L.-F. Tietze, Göttingen.

Mitochondria from plant tissues were isolated by the method of Douce et al. [22] with the excep-

tion that 0.3 M sucrose instead of 0.3 M mannitol was used throughout the preparation. The washed mitochondrial suspensions obtained with this method were purified on a self-generated density gradient consisting of 32% Percoll in wash-medium. The gradient was spun 30 min at $40\,000 \times g$ in a fixed-angle rotor. With mitochondria from green-plant tissue, this procedure resulted in good separation from contaminating thylakoid material: less than 5% (mitochondria from green pea seedlings) or 10% (spinach leaf mitochondria) of the total protein in the final mitochondrial suspensions was estimated to be thylakoid protein from the determination of chlorophyll content.

For mitochondria from green tissues, 5 mM glycine were added to the isolation and storage media. With this addition, the activity of glycine oxidation remained stable for over 10 h [23]. To the very diluted mitochondrial suspensions employed in the oxaloacetate uptake experiments, 0.5 mM NAD were added in order to preserve the activity of the oxidation of NAD-linked substrates.

The intactness of plant mitochondria was estimated from the measurement of Cyt *c* and ascorbate-dependent O_2-consumption according to Neuburger et al. [24]. The quotient of the rates obtained with osmotically protected and osmotically shocked mitochondria, after correction for non-enzymatic cyanide-insensitive oxidation of Cyt *c*, was used as a measure of the intactness of the outer membrane of the mitochondria. It was found to be better than 95% in all preparations.

Rat liver mitochondria were isolated according to Ref. 25, in a medium consisting of 0.25 M sucrose/10 mM triethanolamine-HCl (pH 7.2)/2.5 mM KH_2PO_4/0.2 mM EGTA, which also served as assay medium. Rat heart mitochondria were prepared in the same medium according to Ref. 26. O_2 uptake was measured as described by Estabrook [27] at 25°C using a Clark-type electrode (Hansatech) with 0.1–0.3 mg mitochondrial protein in 1 ml standard reaction medium (0.3 M sucrose/10 mM potassium phosphate buffer/10 mM KCl/5 mM $MgCl_2$/0.1% bovine serum albumin).

Oxaloacetate uptake by isolated mitochondria was measured according to Ref. 18 as conversion of [4-^{14}C]oxaloacetate into Cu^{2+} stable products in the following assay. Mitochondrial (0.2–0.8 μg) protein were added to 200 μl standard reaction medium containing a NAD-linked substrate (glycine, 2-oxoglutarate) or glutamate and preincubated for 3 min at 25°C. The reaction was started by adding up to 20 nmol [4-^{14}C]oxaloacetate (10–30 nCi per nmol) and terminated after 30–120 s by the addition of 20 μl 10% perchloric acid. From this an aliquot of 100 μl was transferred to a scintillation counting vial containing 200 μl of a freshly mixed solution of 0.12 M KH_2PO_4 and 0.12 M $CuSO_4$, and heated for 10 min at 80°C. This procedure results in complete degradation of oxaloacetate to pyruvate yielding the C_4 carboxyl as CO_2. After removing of the $^{14}CO_2$ by evaporation, the residual radioactivity (representing Cu^{2+} stable products formed from [4-^{14}C]oxaloacetate) was counted. The results were corrected for the activity measured after heating a blank (usually less than 1% of the untreated sample). The procedure for preparing, storing and determining the specific activity of [4-^{14}C] oxaloacetate was as described previously [28]. Protein was estimated according to Lowry et al. [29] using bovine serum albumin as standard, and chlorophyll was determined by the method of Arnon [30]. In mitochondria isolated from green plant tissues, the contamination by thylakoid protein was corrected for by chlorophyll assay and assuming a thylakoid protein to chlorophyll ratio of 7 : 1 [22].

Results

General respiratory properties of isolated plant mitochondria

The previously published method for the isolation of plant mitochondria [22] was subjected to a critical examination. Only slight modifications of this method, e.g., use of sucrose as osmotic agent, and addition of glycine to the isolation and storage media led especially in the case of glycine to a considerable improvement in the respiration rate, and as a result of this, to a large increase of the respiratory control ratio, with values up to 19 (Fig. 1). The addition of glycine led to an apparent stabilization of glycine decarboxylase activity. When glycine was omitted from the isolation and storage media, we observed a rapid decay of

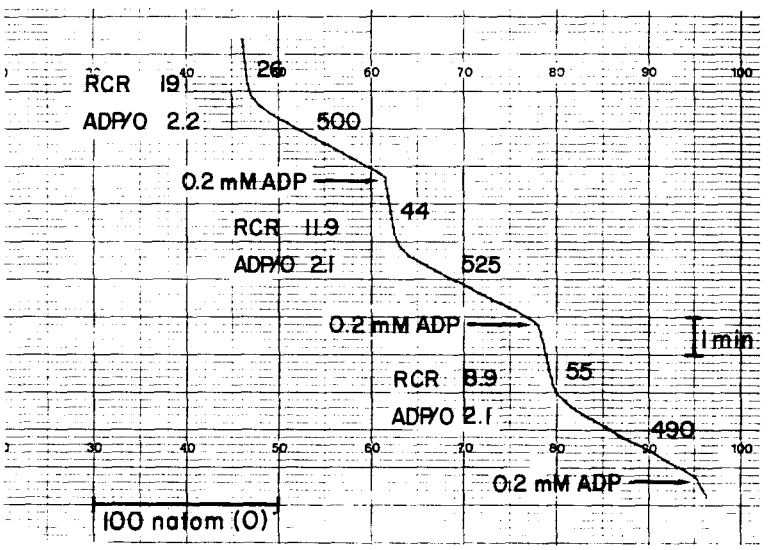

Fig. 1. Glycine-dependent oxygen uptake by spinach leaf mitochondria. Recording of oxygen electrode measurement with 100 μg mitochondrial protein in 1 ml standard reaction medium and 10 mM glycine as substrate. ADP was added where indicated. The numbers represent the respiration rates in natom (O) per mg mitochondrial protein per min. RCR, respiratory control ratios.

mitochondrial glycine oxidation with a half-time of about 1 h (results not shown, see also Ref. 23). The use of sorbitol, which has been used frequently as an osmotic agent for plant mitochondria in the past instead of sucrose, yielded in our experiments less active mitochondria. It may be noted that the respiratory control ratios often obtained a maximum at the third State 3–State 4

TABLE I

RESPIRATORY PROPERTIES OF MITOCHONDRIA ISOLATED FROM VARIOUS SOURCES

Oxygen uptake was measured as described in Materials and Methods with different substrates in concentrations as indicated. When 2-oxoglutarate was used as substrate for plant mitochondria, 250 μM thiaminepyrophosphate was added to get fully active respiration. The rates as well as the respiratory control ratios and ADP/O ratios were determined from the second State 3–State 4 transition in each case. The mean values of spinach and green pea seedlings are of six and of etiolated seedlings, pea roots and potato tuber of at least three preparations ±S.D.

Source of mitochondria	Substrate (mM)	Oxygen consumption (natom (O)/mg protein per min)		Respiratory control ratio	ADP/O
		State 3	State 4		
Spinach leaves	Gly (10)	510± 68	52±16	9.8±2.2	2.2±0.1
	NADH (1)	380± 46	110±24	3.5±0.7	1.4±0.2
Green pea seedlings	Gly (10)	460± 74	68±10	6.9±1.9	2.2±0.2
	NADH (1)	516± 44	136±16	3.8±0.6	1.4±0.1
Etiolated seedlings	2-OG (10)	340± 24	80±20	5.3±0.6	2.5±0.2
	NADH (1)	664±130	196±10	3.5±0.5	1.3±0.1
Pea roots	2-OG (10)	370± 98	56±10	6.7±1.7	2.4±0.2
	NADH (1)	844± 68	156±20	5.5±0.6	1.3±0.1
Potato tuber	2-OG (10)	530± 40	100±16	5.7±1.6	2.6±0.2
	Succ (10)	662± 30	116±24	5.9±1.5	1.4±0.1

transition. Average values for respiratory rates and respiratory control ratios shown in Table I have been determined from the second State 3–State 4 transition. For a comparison, most of the previously published values on glycine-dependent respiration by mitochondria from spinach and pea leaves ranged between 134 and 170 natom (O)/mg protein per min with a respiratory control ratio between 2.2 and 3.4 [4,5,15,23,31,32]. Only Nishimura et al. [33] reported glycine oxidation rates similar to ours (462 natom (O)/mg protein per min) with mitochondria carefully isolated from spinach leaf protoplasts, which, however, showed only low respiratory control ratios (2.7–3.1). Thus, our average respiratory control ratios with glycine as substrate are about 3-fold higher than those obtained by other groups, indicating that plant mitochondria, prepared appropriately, exhibit a remarkably high degree of coupling.

The inhibitory effect of oxaloacetate on respiration as a measure of oxaloacetate transport

For an estimation of the half saturation of the oxaloacetate-transporting system, we studied the concentration dependence of the inhibitory effect of oxaloacetate with mitochondria from spinach leaves. The addition of as little as 20 μM oxaloacetate (Fig. 2) was sufficient to cause an immediate decrease in O_2 uptake to about one third of the initial rate. After about 15 s, the O_2 uptake recovered to almost the initial rate, showing that the added amount of oxaloacetate was completely reduced to malate. Subsequent addition of increasing oxaloacetate concentrations resulted in increasing inhibition of O_2 uptake and increasing time spans needed for complete reduction of oxaloacetate. With oxaloacetate concentrations of about 50–100 μM, the inhibition reached a maximum value (90%). As it was shown in earlier investigations [13,15], phthalonate, a structural analogue of oxaloacetate, restored the activity of O_2 uptake almost completely. The difference between the uninhibited and oxoloacetate-inhibited rate of oxygen consumption reflects the rate of oxaloacetate reduction to malate and is thus a minimal estimate of the rate of oxaloacetate transport into the mitochondria. Fig. 3 shows a plot of the decreased rates of respiration obtained

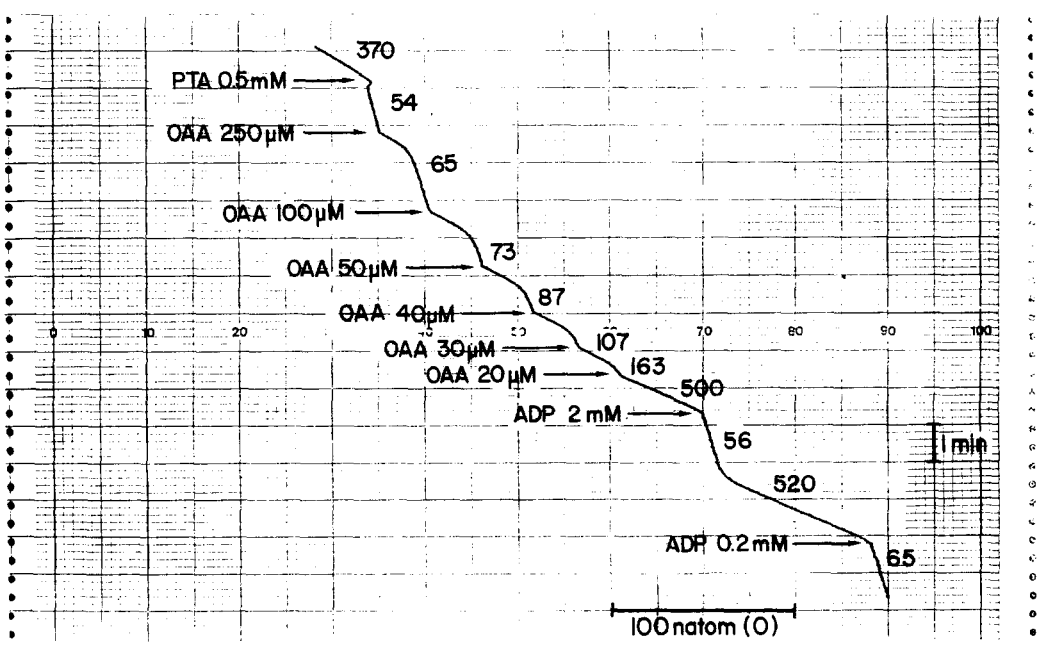

Fig. 2. Respiration of spinach leaf mitochondria and the effects of oxaloacetate (OAA) and phthalonate. The assay conditions were as in Fig. 1, except that 120 μg mitochondrial protein was used. Additions of ADP, oxaloacetate and phthalonate (PTA) were as indicated (rates expressed as natom (O)/mg protein per min).

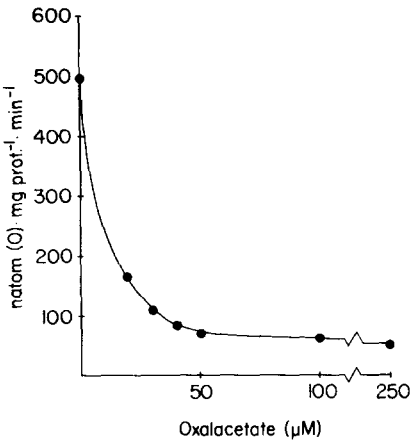

Fig. 3. Effect of oxaloacetate on the glycine-dependent State 3 oxygen uptake of spinach leaf mitochondria. The respiration rates determined in the experiment shown in Fig. 2 are plotted against the added oxaloacetate concentration.

from the experiment in Fig. 2, depending on the concentration of the added oxaloacetate. From the shape of the curve, the oxaloacetate concentration causing 50% inhibition can be roughly estimated as about 10 μM. We obtained almost identical results also with pea leaf mitochondria [6]. For the sake of comparison, we investigated the effect of oxaloacetate on the respiration of mitochondria from rat liver and rat heart. As these mitochondria were not able to oxidize glycine at substantial rates these experiments were carried out with 2-oxoglutarate as substrate. The addition of 500 μM oxaloacetate decreased the 2-oxoglutarate depen-

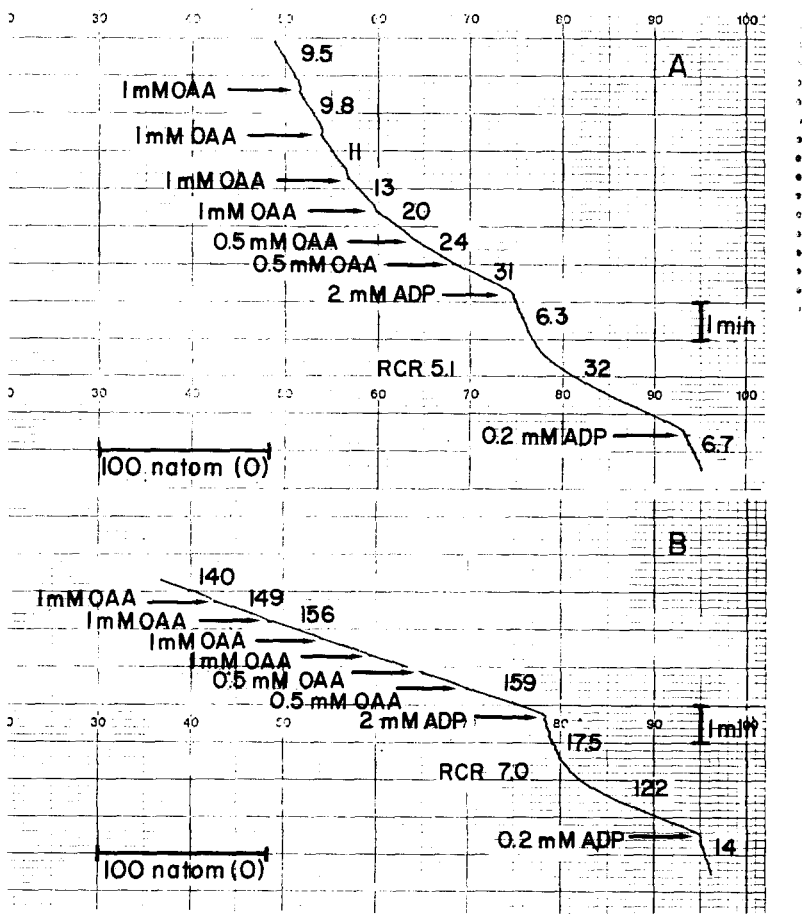

Fig. 4. Respiration of rat liver and rat heart mitochondria and the effect of oxaloacetate (OAA). Mitochondria isolated from (A) rat liver (1.58 mg protein) and (B) rat heart (0.45 mg protein) were incubated in 1 ml assay medium (0.25 M sucrose, 10 mM triethanolamine, 2.5 mM KH_2PO_4, 0.2 mM EGTA, pH 7.2) in the presence of 5 mM 2-oxoglutarate. Where indicated, ADP and oxaloacetate were added. RCR, respiratory control ratio.

dent respiration in rat liver mitochondria by 21% only, and in rat heart mitochondria not at all (Fig. 4). This insensitivity of respiration towards oxaloacetate in animal mitochondria is in a sharp contrast to the strong effect of oxaloacetate on respiration in plant mitochondria shown.

The conversion of ^{14}C-oxaloacetate as a measure of oxaloacetate transport

In order to increase the sensitivity of our measurements, we employed an alternative approach by measuring the conversion of radioactively labelled oxaloacetate. The assay procedure recently developed in our laboratory [18] takes advantage of the fact, that [4-^{14}C]oxaloacetate is rapidly decarboxylated in the presence of Cu^{2+}, releasing the label as CO_2, whereas the products of oxaloacetate conversion such as malate or aspartate remain stable under these conditions. In this way, the formation of Cu^{2+}-stable ^{14}C-label can be used as a measure of oxaloacetate conversion. This method makes it possible not only to follow the conversion of oxaloacetate to malate by NADH generated from substrate oxidation, but also to measure the consumption of oxaloacetate by transamination of glutamate to aspartate (Fig. 5). It may be noted, that mitochondria from non-green plant tissues (e.g., etiolated seedlings or roots) are not able to oxidize glycine [34].

Because of the high sensitivity of the method, very low concentrations of mitochondria (1–4 μg protein/ml) were employed. Fig. 6 shows a typical time-course for the conversion of oxaloacetate by mitochondria from green pea seedlings oxidizing glycine. Even with oxaloacetate concentrations as low as 2.4 μM, the reaction was linear for 1 min. Very similar time-courses were also obtained with mitochondria converting oxaloacetate by transamination in the presence of glutamate (data not shown here).

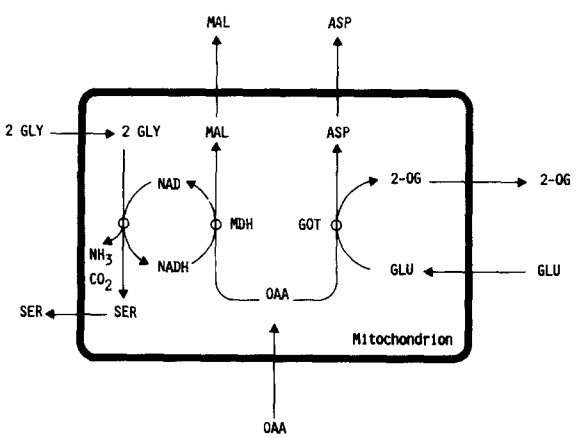

Fig. 5. Sequence of reactions for measurement of oxaloacetate uptake. (MDH, malic dehydrogenase; 2-OG, 2-oxoglutarate; GOT, glutamate oxaloacetate transaminase. MAL, malate).

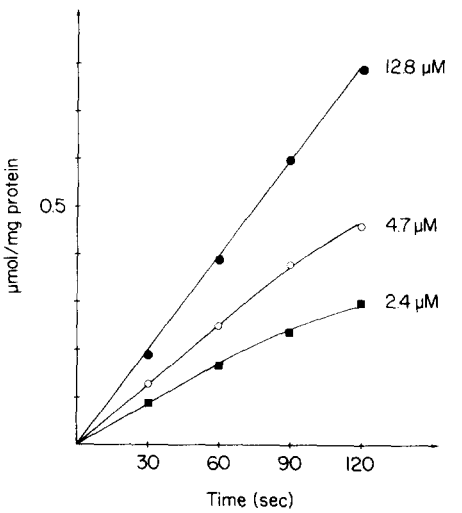

Fig. 6. Time course for the conversion of [4-^{14}C]oxaloacetate into Cu^{2+}-stable products by spinach leaf mitochondria. The reaction was carried out in the standard assay using 10 mM glycine as substrate.

In order to investigate the location of the stable products of oxaloacetate conversion, we incubated mitochondria isolated from green pea seedlings (at 10-fold higher concentration than employed in the standard assays) with glycine, added 80 μM [^{14}]oxaloacetate and stopped the reaction by adding 1 mM of phthalonate. 30 and 60 s after the addition of oxaloacetate 86% and 98% of the total stable oxoloacetate conversion products were found in the supernatant medium. Apparently, the malate formed from oxaloacetate reduction during glycine oxidation in the mitochondria was immediately released by them.

Concentration dependence of oxaloacetate uptake

The oxaloacetate uptake, as evaluated from initial rates of oxaloacetate conversion by reduction and transamination (Fig. 6) followed conventional Michaelis–Menten characteristics (Figs. 7 and 8)

with apparent K_m values ranging between 3 and 7 μM, with the exception of mitochondria of potato tuber ($K_m = 44$ μM) (Table II). In mitochondria from etiolated pea seedlings and pea roots the maximal rates of oxaloacetate uptake were less than one third of the corresponding rates of green pea seedlings or spinach leaves, whereas the maximal rate in mitochondria from potato tuber was relatively high.

In mitochondria from rat liver a similar conversion of [^{14}C]oxaloacetate could not be detected with oxaloacetate concentrations up to 100 μM and citrate, 2-oxoglutarate or glutamate as substrate. This concurs with the insensitivity of respiration in these mitochondria towards the addition of similar oxaloacetate concentrations. When incubating mitochondria from rat liver and rat heart in the presence of 26 μM [^{14}C]oxaloacetate, 290 μM malate and 350 μM 2-oxoglutarate, concentrations which have been earlier analyzed for the cytosol of perfused liver [35], in both cases the rate of oxaloacetate conversion were below our limit of detection (5 nmol/mg protein per min).

TABLE II

KINETIC CONSTANTS FOR OXALOACETATE UPTAKE WITH MITOCHONDRIA ISOLATED FROM DIFFERENT PLANT TISSUES

The kinetic constants were determined as described in Figs. 7 and 8. As the oxaloacetate uptake is competitively inhibited by 2-OG, the K_m value for oxaloacetate uptake had to be determined indirectly in experiments where 2-OG was used as substrate. For this, the apparent K_m values of oxaloacetate uptake at various 2-OG concentrations (7.5–15 mM) were determined and plotted versus the 2-OG concentrations. Extrapolation of the resulting straight line to a 2-OG concentration of zero yields the true K_m value. The mean values for spinach leaves and green pea seedlings are of at least five determinations $\pm$S.D.

Source of mitochondra	Substrate (mM)	K_m (μM)	V_{max} (nmol/mg protein per min)
Spinach leaves	Gly (10)	6 ± 3	550 ± 80
Green pea seedlings	Gly (10)	7 ± 1	570 ± 50
Etiolated pea seedlings	Glu (10)	3	150
	2-OG (10)	4 [a]	180
Pea roots	Glu (10)	5	160
Potato tuber	Glu (10)	44	450

[a] Estimated (see above).

As the estimated maximal rates of oxaloacetate uptake in mitochondria isolated from green plant tissue were in the same range as the rates of glycine oxidation in the appropriate preparation, the question arose whether the measured rates of formation of Cu^{2+} stable products (here: malate) were limited by the NADH yielding reaction of oxidative glycine decarboxylation rather than by oxaloacetate uptake. In order to answer this, oxaloacetate uptake was measured with saturating concentrations of oxaloacetate (50 μM) in the presence of glycine, glutamate and both glycine and glutamate (Table III). The activity in the presence of both substrates was substantially lower than the sum of the single activities in the presence of glycine or glutamate. This indicates that in the presence of both substrates the oxaloacetate uptake was now the rate limiting step. However, kinetic experiments showed that the apparent K_m value and the V_{max} value only slightly increased in the two-substrate system compared to the one-substrate experiment (data not shown). This was not surprising, as for K_m determinations, the reaction rates were measured with oxaloacetate concentrations far below saturation. In doing, so, the corresponding oxaloacetate uptake rate could not exceed the maximum glycine oxidation rates, and thus, the apparent K_m values estimated on the base of the rates measured with only glycine as substrate were in the true range. As the addition of glutamate complicated the experimental system (for example, the reaction rates had to be cor-

TABLE III

OXALOACETATE-UPTAKE RATES WITH DIFFERENT SUBSTRATES AT SATURATING OXALOACETATE CONCENTRATIONS BY SPINACH LEAF MITOCHONDRIA

Oxaloacetate uptake was measured in standard assay and with a [4-^{14}C]oxoloacetate concentration of 50 μM. The concentration of substrates was as indicated.

Experiment	Substrate (mM)	Oxaloacetate uptake (nmol/mg protein per min)
1	none	10
2	glycine (10)	550
3	glutamate (5)	400
4	glycine (10) and glutamate (5)	670

rected for external glutamate oxaloactate aminotransferase activity found in most mitochondrial preparations) all other kinetic experiments were performed with glycine as substrate, ensuring that in no case a limitation by the oxaloacetate conversion to malate could occur.

In mitochondria from non-green tissue, a limitation of oxaloacetate conversion to aspartate in the presence of glutamate is not to be expected as the glutamate oxaloacetate-transaminase activity was in all preparations far above the maximal oxaloacetate uptake rates (300–800 nmol/mg protein per min, measured with 50 μM oxaloacetate, 5 mM glutamate under the same experimental conditions as for oxaloacetate uptake, but after treating the mitochondria with 0.1% Triton X-100).

Specificiy and mechanism of oxaloacetate uptake

In order to differentiate the oxaloacetate translocator found in plant mitochondria from other known mitochondrial transport systems, the effect of several metabolites and specific inhibitors on the oxaloacetate uptake rates was tested.

2-Oxoglutarate, citrate and malonate inhibited oxaloacetate uptake competitively. The K_i values range between 1 and 11 mM, depending on the metabolite, and are therefore two to three orders of magnitude higher than the apparent K_m value for oxaloacetate (Table IV, Figs. 7 and 8), and more than 10-fold higher than the corresponding K_m values for citrate, dicarboxylate and 2-oxoglutarate transport in rat liver mitochondria [12,36,37], indicating that the binding site for oxaloacetate transport is different from the binding sites of the citrate, dicarboxylate and 2-oxoglutarate translocators. These results concur with our earlier observations [6] on the effect of malate, 2-oxoglutarate and citrate on the inhibition of glycine dependent respiration by oxaloacetate, and similar observations by Oliver and Walker with pea leaf mitochondria [16].

Among the inhibitors tested, the strongest effect was caused by phthalonate which acted as a competitive inhibitor with a K_i value being even lower than the K_m for the substrate oxaloacetate (Figs. 7 and 8 Table IV). Apparently, the oxaloacetate translocator has a higher affinity to phthalonate than to its natural substrate. Benzene-1,2,3-tricarboxylate, an inhibitor of the tricarboxylate-carrier [7,36], had no significant effect in concentrations which were up to 1000-fold higher than the

TABLE IV

K_i VALUES FOR COMPETITIVE INHIBITORS OF OXALOACETATE UPTAKE

The K_i values for different competitive inhibitors of oxaloacetate uptake were determined by replotting the data obtained from Lineweaver-Burk plots similar to those presented in Fig. 7 and 8 according to Dixon. Each value represents an average of at least three determinations.

Source of mitochondria	Inhibitor	K_i (mM)
Spinach leaves	citrate	11
	malonate	5
	2-oxoglutarate	2
	phthalonate	0.005
Green pea seedlings	citrate	9
	malonate	3
	2-oxoglutarate	1
	phthalonate	0.003

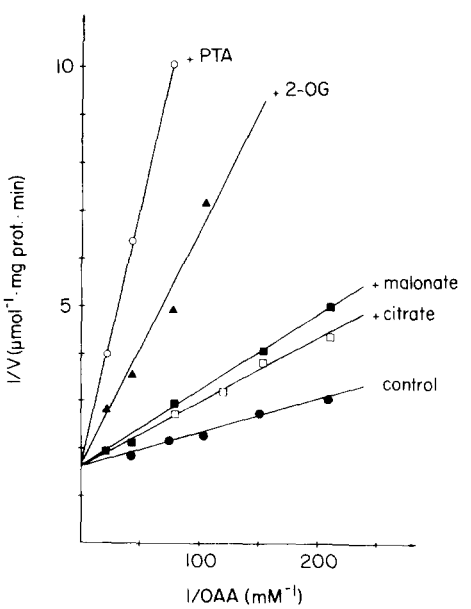

Fig. 7. Effect of varying concentrations of oxaloacetate (OAA) on the conversion of oxaloacetate to products and inhibition by different metabolites (spinach leaf mitochondria). Experiments were performed in standard assay with 10 mM glycine as substrate. Citrate, malonate and 2-oxoglutarate were added in concentrations of each 5 mM, whereas the phthalonate concentration was 30 μM.

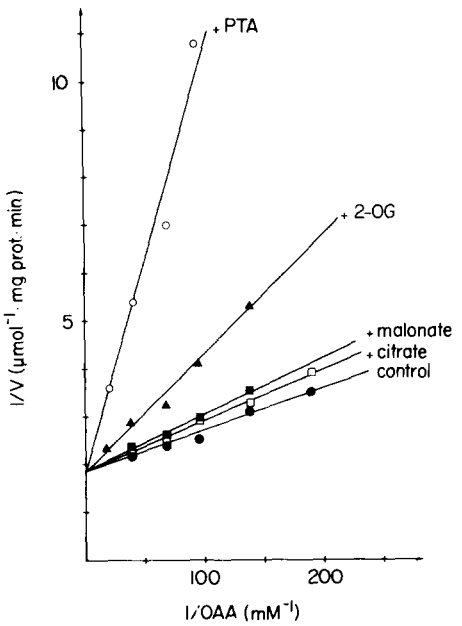

Fig. 8. Effect of varying concentrations of oxaloacetate on the conversion of oxaloacetate to products by mitochondria isolated from green pea seedlings and inhibition by different metabolites. Assay conditions were as in Fig. 7, except that citrate, malonate and 2-oxoglutarate (2-OG) concentrations were only 1 mM. PTA, phthalonic acid.

TABLE V

INHIBITION OF OXALOACETATE UPTAKE AND GLYCINE-DEPENDENT STATE 3 O_2 UPTAKE BY VARIOUS TRANSPORT INHIBITORS (SPINACH LEAF MITOCHONDRIA)

The effect of different inhibitors on oxaloacetate uptake was tested in the standard assay with 10 mM glycine as substrate and saturating concentrations of [4-^{14}C]oxaloacetate (50–60 μM). Oxygen uptake was measured as described in 'Materials and Methods' in the presence of 10 mM glycine and 1 mM ADP.

Inhibitor	mM	% Inhibition oxaloacetate uptake	% Inhibition O_2 uptake
N-ethylmaleimide	0.1	65	not determined
	0.5	81	38
α-Cyano-4-hydroxy-cinnamic acid	0.01	0	0
	0.05	4	6
Benzene-1,2,3-tricarboxylate	1	5	0
	5	8	5
2-*n*-Butylmalonate	5	42	26
	10	56	32
Phenylsuccinate	5	24	5
	10	54	13

apparent K_m value. Also α-cyano-4-hydroxy-cinnamic acid, which inhibits the pyruvate translocator of corn mitochondria specifically in concentrations of about 10 μM [38], showed virtually no inhibition (Table V).

In contrast, low concentrations of the SH reagent *N*-ethylmaleimide (NEM) caused a strong inhibition of oxaloacetate uptake (Table V). Although part of this inhibition was due to the effects of NEM on glycine oxidation, oxaloacetate transport was inhibited twice as much as glycine oxidation. This indicated that either the oxaloacetate translocator itself contains an SH-group essential for transport, or that the oxaloacetate transport is coupled with another NEM-sensitive reaction, e.g., phosphate or dicarboxylate exchange. A similar pattern was seen with phenylsuccinate, an inhibitor of the dicarboxylate and 2-oxoglutarate carrier [7,12]. The small inhibition of State 3 glycine oxidation (probably due to phosphate uptake inhibition) was greatly exceeded by the inhibition of oxaloacetate uptake (Table V). With *n*-butyl-malonate, another inhibitor of the dicarboxylate carrier [7,12], the picture was not so clear. Although the extent of *n*-butylmalonate inhibition of oxaloacetate uptake was similar to phenyl succinate, glycine oxidation was more sensitive to *n*-butylmalonate than to phenyl succinate. Thus it was not possible to attribute the observed inhibition to oxaloacetate uptake.

These inhibitor studies clearly indicate that the tricarboxylate or pyruvate translocator does not participate in oxaloacetate uptake, although the effects of phenyl succinate and *n*-butylmalonate did not allow a decisive conclusion with regard to the 2-oxoglutarate and dicarboxylate carrier. Thus, further experiments were performed to investigate the effects of phthalonate on the uptake of 2-oxoglutarate and malate. For that purpose the rates of 2-oxoglutarate and malate dependent oxygen consumption were measured in the presence of increasing concentrations of phthalonate. As shown in Table VI, malate and 2-oxoglutarate oxidation was unaffected by a phthalonate concentration of

TABLE VI

INHIBITION OF STATE 3-O_2 UPTAKE WITH DIFFERENT SUBSTRATES BY PHTHALONATE (SPINACH LEAF MITOCHONDRIA)

Oxygen uptake was measured as described above with 10 mM glycine, 10 mM malate or 10 mM 2-oxoglutarate as substrate and in the presence of 1 mM ADP. The control rates were: glycine: 480; malate: 290; 2-oxoglutarate: 370 (rates expressed in natom (O)/mg prot. min)

Substrate	Phthalonate (mM)	% Inhibition
Glycine	1	0
	3	0
	5	13
Malate	1	0
	3	3
	5	16
2-Oxoglutarate	1	0
	3	17
	5	35

1 mM which inhibits oxaloacetate uptake completely. Only higher concentrations were inhibitory.

It may therefore be concluded, that the oxaloacetate-transporting system is distinct from the 2-oxoglutarate and also from dicarboxylate translocators, as was indicated by the competition experiments of Figs. 7 and 8. Despite of this, there may be a link between oxaloacetate and malate transport. As shown in Table VII, the uptake of oxaloacetate by the mitochondria can be increased when these have been preincubated with millimolar concentrations of malate. It should be noted

TABLE VII

STIMULATION OF OXALOACETATE UPTAKE BY MALATE (SPINACH LEAF MITOCHONDRIA)

Oxaloacetate uptake was measured in the standard assay with 10 mM glycine as substrate at saturating concentrations of [4-^{14}C]oxaloacetate (50–60 μM). Intramitochondrial malate concentrations were allowed to equilibrate with the external concentrations by a 3 min preincubation.

Addition	uptake Oxaloacetate (nmol/mg protein per min)
None	480
1 mM malate	560
5 mM malate	650

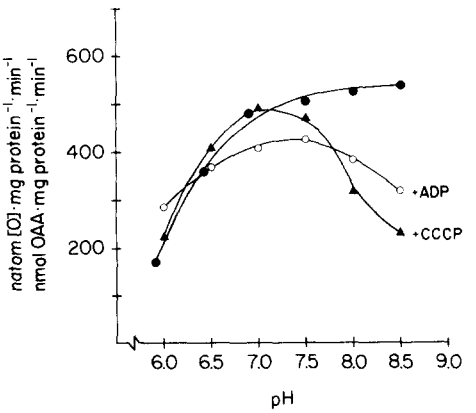

Fig. 9. Effect of pH on oxaloacetate uptake and on glycine-dependent oxygen uptake (mitochondria from green pea seedlings). The standard reaction medium was modified by adding 10 mM of each Tricine and Hepes buffer and adjusting the pH to the values indicated. Oxygen uptake was measured polarographically in the presence of 5 μM CCCP (uncoupled; ▲) or 1 mm ADP (State 3; ○) using 10 mM glycine as substrate. Oxaloacetate uptake was measured with saturating oxaloacetate concentrations (70–80 μM) using the standard procedure and 10 mM glycine as substrate (●).

that the mitochondrial preparation used in this experiment was substantially free of extramitochondrial malate dehydrogenase. An insensitivity of oxaloacetate/malate exchange towards butylmalonate could be due to the fact that butylmalonate does not penetrate the mitochondria, and this does not reach the internal binding site for malate export.

Fig. 9 shows the pH dependence of oxaloacetate uptake, as measured with mitochondria from green pea seedling. In concurrence with earlier results by Oliver and Walker [16], the rate of oxaloacetate uptake showed a steady increase up to pH 8.5, whereas the oxygen consumption showed an optimum at about pH 7.5 measured in the presence of ADP (State 3) and at about 7.0 with uncoupled mitochondria. In the experiments of Oliver and Walker, also carried out with uncoupled pea leaf mitochondria, this optimum occurred at pH 6.5.

Discussion

Evaluation of the method of oxaloacetate uptake measurement

The recent development of a method for the

preparation of [4-^{14}C]oxaloacetate from phosphoenolpyruvate and [^{14}C]bicarbonate by PEP-carboxylase [28] and the demonstration that this radioisotope is stable under experimental conditions opened the possibility of more sensitive measurement of oxaloacetate transport in isolated cell organelles. It would have been desirable to measure oxaloacetate uptake directly by silicone layer filtering centrifugation, but this did not appear possible because of the very high rate of oxaloacetate uptake and the rapid release of the malate produced. We therefore used the conversion of [^{14}C]oxaloacetate to stable products as a measure of oxaloacetate uptake. However, there are two critical conditions which must be met to obtain valid uptake rates. First, it has to be ensured that the measured rates are in fact limited by oxaloacetate-uptake rather than by the oxaloacetate-converting activity (see results). A limitation by the latter reaction would lead to an underestimation of apparent kinetic constants (K_M and V_{max}). The other condition is that the substrate which furnishes reducing equivalents or amino groups, should not interfere with the oxaloacetate transport system. Such an interference, however, was found for 2-oxoglutarate, which competitively inhibited oxaloacetate uptake with a K_i value in the range of 1 mM. Thus, in an uptake experiment carried out with 2-oxoglutarate as NAD-linked substrate, the determined Michaelis constant was far above the expected value. However, a recalculation in consideration of the K_i for 2-oxoglutarate resulted in a value similar to that obtained with glutamate as substrate (Table II).

Properties of the oxaloacetate translocator

Our results indicate that a very active oxaloacetate translocator occurs in plant mitochondria. This system shows a very high specificity for its substrate with an apparent K_M value of 6–7 μM and a maximum velocity of 500–650 nmol/mg protein per min (Table II). This is in agreement with our previous estimation from the inhibitory effect of oxaloacetate on glycine-dependent respiration, yielding a K_m value of about 10 μM [6] and with the results obtained by Oliver and Walker [16] who estimated a K_m value of 15 μM and a V_{max} of 550 nmol/mg protein min for unpurified pea-leaf of mitochondria based on measurements of the disappearence of oxaloacetate added to mitochondria oxidizing glycine. Our K_m value is lower than that for half saturation of the malate-oxaloacetate shuttle by oxaloacetate (42 μM) found earlier with a reconstituted system of supernatant and mitochondria from spinach leaves [39].

This oxaloacetate transport has to be attributed to a separate translocator binding site. None of the known carboxylate translocators in plant mitochondria (for tricarboxylates, pyruvate, dicarboxylates and 2-oxoglutarates show properties similars to oxaloacetate transport, which is insensitive to α-cyano-4-hydroxy-cinnamic acid, benzene-1,2,3-tricarboxylate, only partially sensitive to phenyl succinate, and completely inhibited by micromolar concentrations of phthalonate (Tables IV and V). De Santis et al. [7] reported evidence for transport of oxaloacetate by the 2-oxoglutarate and dicarboxylate translocator in mitochondria from bean. The fact that phthalonate does not inhibit 2-oxoglutarate or malate uptake (as judged by the effect of phthalonate on 2-oxoglutarate or malate oxidation (Table VI, see also Ref. 13) in concentrations that are much higher than are needed for complete inhibition of oxaloacetate uptake, strongly argue against participation of the 2-oxoglutarate translocator in high-affinity-transport of oxaloacetate. It may be noted that the experiments of De Santis et al. [7] were performed with millimolar oxaloacetate concentrations far above possible physiological concentrations. Some exchange of oxaloacetate for 2-oxoglutarate or malate in these concentrations may occur via the appropriate carriers, but the physiological significance of such exchanges is questionable.

Phthalonate is a very potent competitive inhibitor (K_i, 3–5 μM), with a higher affinity to the binding site than oxaloacetate. This differs strongly from the data of Oliver and Walker [16] who measured a K_i value for phthalonate of 60 μM. These authors mentioned that their phthalonate preparation contained about 10% impurities. Differences in phthalonate preparations have already been held responsible for varying effects of phthalonate on 2-oxoglutarate uptake [15].

Based on the inhibition by CCCP of a reconstituted malate/oxaloacetate shuttle, Day and Wiskich [4] suggested an energy-dependent oxaloacatete/H^+ symport or oxaloacatete/OH^-

exchange. Oliver and Walker have pointed out that such a mechanism should result in an increase of oxaloacetate uptake with the decrease of external pH. In fact Oliver and Walker and ourselves (Fig. 9) observed a decrease of oxaloacetate transport with decreasing pH. On the other hand, the results of Table VII strongly indicate that oxaloacetate might be transported in counterexchange with malate. Further experiments are required to elucidate the matter.

Physiological function of the malate-oxaloactate shuttle

Because the equilibrium of the malate dehydrogenase reaction lies far towards malate formation ($K_{eq} = 3 \cdot 10^{-5}$ [40]), cytosolic concentrations of oxaloacetate are expected to be in the order of 10 μM, and the malate concentration to be very much higher. For this reason, an oxaloacetate-transporting system participating in a malate/oxaloacetate shuttle must not only be saturated at very low oxaloacetate concentrations, but must also be highly specific in order to avoid competitive inhibition by other dicarboxylates, such as 2-oxoglutarate and malate, that are present in large excess over oxaloacetate.

In animal mitochondria, a suitable system for a malate-oxaloacetate shuttle does not appear to exist. Although in mitochondria from rat liver and rat heart transport of oxaloacetate via the dicarboxylate and 2-oxoglutarate translocator has been shown to be possible in principle [9–11], the corresponding K_m values for oxaloacetate were higher than those for the transport of either 2-oxoglutarate or malate. It is therefore not surprising that under physiological concentrations of

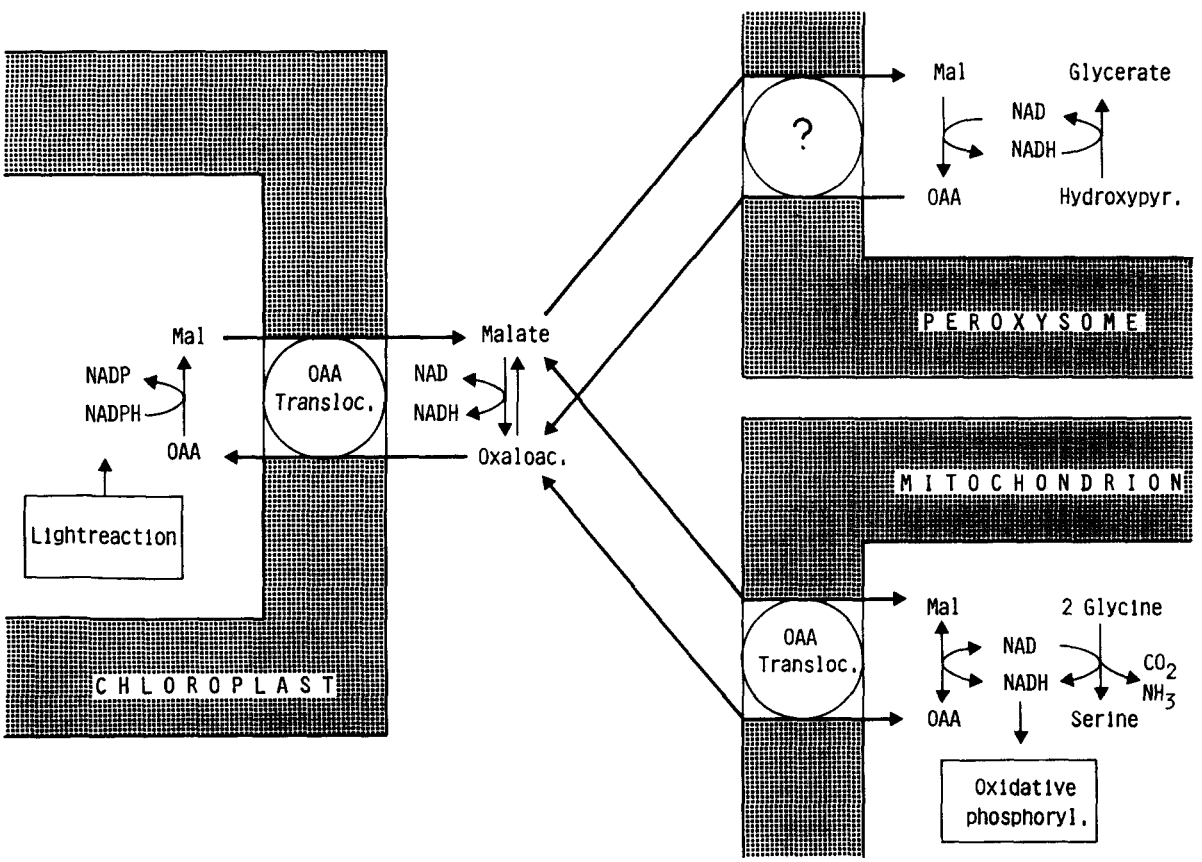

Fig. 10. Proposed interaction of mitochondria, chloroplasts and peroxysomes via the malate-oxaloacetate shuttle system in a green plant cell in the light.

oxaloacetate, malate and 2-oxoglutarate, only minimal rates of oxaloacetate transport are observed, as shown here. Oxaloacetate transport by the existing 2-oxoglutarate or dicarboxylate translocators is apparently not suited for catalyzing significant fluxes of a malate-oxaloacetate shuttle across the mitochondrial inner membrane under physiological conditions.

Plant mitochondria, on the other hand, posess a specific translocator for oxaloacetate, with a very low K_m, and of such high specificity that the transport of oxaloacetate is practically unaffected by even a 100-fold excess of other dicarboxylates. The very high activity of this translocator together with the high activity of mitochondrial malate dehydrogenase enables the malate-oxaloacetate shuttle to maintain large fluxes of redox equivalents. In green plant cells, this shuttle appears to play an essential role in the photorespiratory pathway, by which glycolate, a byproduct of carbon fixation, is converted to phosphoglycerate, and thus reutilized in the CO_2 fixation cycle [41]. In the photorespiratory cycle, every two molecules of glycine that are oxidized in the mitochondria yield one NADH and one serine, and the latter, after transamination to β-hydroxypyruvate, is reduced in the peroxysomes by NADH to glycerate (Fig. 10). Thus, all NADH generated in the mitochondria from glycine oxidation is required again in the peroxysomes for the reduction of β-hydroxypyruvate. A malate-oxaloacetate shuttle is a likely candidate for transferring redox equivalents between the intracellular compartments [3–5]. This shuttle remained undefined due to lack of identification of the translocator involved. Our results clearly show that plant mitochondria are indeed able to catalyze under physiological conditions the uptake of oxaloacetate and reduction to malate with rates similar to the rates of glycine oxidation required in photorespiration. In the spinach leaves employed in our experiments with a usual net rate of CO_2 fixation in air of 200 μmol CO_2/mg Chl per h, the rate of photorespiration can be estimated as 55 μmol O_2/g Chl per h [42] involving rates of glycine oxidation or β-hydroxypyruvate reduction of 27.5 μmoles/mg Chl per h. Using citrate synthase as a mitochondrial marker enzyme, these spinach leaves were found to contain 1.54 mg mitochondrial protein/mg Chl (Ebbighausen, unpublished data). From this and the kinetic data shown in Table II, with 10 μM oxaloacetate present in the cytosol, the rate of oxaloacetate transport into the mitochondria is calculated as 32 μmol/mg Chl per h, corresponding well with the rate of photorespiration in these leaves shown above. In view of the very high activity of malate dehydrogenase in the peroxysomes [43], one might expect that malate and oxaloacetate are also specifically transported across the peroxysomal membrane, although experimental evidence for this is lacking. Since the equilibrium of β-hydroxypyruvate reduction is far on the side of glycerate ($K_{eq} = 3 \cdot 10^{-5}$ [44]), this reaction is expected to act as a drain, sequestering the redox equivalents from the mitochondria. Thus, in spite of the very high rates of glycine oxidation by the mitochondria in the light, the NADH so generated might not be available for mitochondrial electron transport, unless another source of redox equivalents were able to serve the reduction of β-hydroxypyruvate.

It appears now that photosynthetic electron transport can be such an alternative source of redox equivalents. It had been earlier shown that chloroplasts are able to photoreduce oxaloacetate via NADP malate dehydrogenase located in the chloroplast stroma [45–47]. It was found in our laboratory that chloroplasts from spinach leaves posses a very active and specific oxaloacetate translocator [18], distinct from the known dicarboxylate translocator transporting malate [48]. This oxaloacetate translocator with an average maximal velocity of 39 μmol/mg Chl per h (Dröscher, L., unpublished results) is half saturated by very low concentrations of oxaloacetate (K_m, 9 μM), is very little affected by other dicarboxylates (malate, K_i 1,4 mM; 2-oxoglutarate, K_i, 0.4 mM) and is thus well suited for catalyzing an efficient malate-oxaloacetate shuttle [18]. Since the chloroplast malate dehydrogenase is NADP specific, and the redox potential of the NADPH pool in the chloroplast stroma is much more negative than the redox potential of the cytosolic (or peroxysomal) NADH, an uncontrolled shuttle would drain redox equivalents from the chloroplasts. Evidence has been presented that such a control is exerted by regulation of the chloroplast malate dehydrogenase. This enzyme is regulated by light via the

thioredoxin system but, additionally, is only active in the light in the presence of a high NADPH/NADP quotient [49]. Therefore the chloroplast malate-oxaloacetate shuttle will operate only when the chloroplast NADPH/NADP quotient is very high. In this way, any excess of redox equivalents generated by photosynthetic electron transport not required in the chloroplasts could be utilized for the reduction of β-hydroxypyruvate in the peroxysomes, allowing the equivalent of NADH generated by mitochondrial glycine oxidation to remain available for mitochondrial electron transport. Thus, in balance, any excess of reducing equivalents from the chloroplasts could be eliminated by mitochondrial oxidation. According to the ATP demand, this may proceed either by coupled or uncoupled electron transport. It has been suggested that in plant mitochondria, depending on the metabolic situation, the number of energy transduction sites involved in the oxidation of malate can vary from three to zero, in the latter case involving the rotenone-resistant and cyanide-resistant pathways [50]. The interplay of this flexible electron transport with the chloroplast and mitochondrial malate-oxaloacetate shuttles may provide an efficient mechanism to prevent any overreduction of chloroplast redox carriers during illumination. It may be noted, however, that mitochondria from non-green plant tissues, such as roots, are also capable of catalyzing a malate-oxaloacetate shuttle. Although many questions concerning its general function still remain unanswered, it appears that the mitochondrial malate-oxaloacetate shuttle is not a specific feature of green plant cells in particular, but a more general constituent of plant cells altogether.

These considerations may illustrate the function of mitochondria in a green plant cell. Whereas in the dark period these mitochondria fulfil their usual role as a power plant-generating ATP for cellular metabolism, this function may be less important in the light, where ATP can also be delivered by the chloroplasts to the cell through a triosephosphate-phosphoglycerate shuttle [51]. During illumination these mitochondria carry out two other vital functions, namely catalyzing the glycine conversion required in phosphorespiration and providing a means for a regulation of the redox balance during photosynthesis. The latter function may be of special significance under high light intensities, if photosynthesis is limited by carbon fixation or utilization and there is a surplus of electron-transport capacity. In this case, the oxidation of light-generated reducing equivalents by the mitochondria may be one mechanism for preventing possible irreversible damage of the photosynthetic apparatus due to the overreduction of photoelectron carriers [52].

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