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Characterization of Dicarboxylate Stimulation of Ammonia, Glutamine, and 2-Oxoglutarate-Dependent O_2 Evolution in Isolated Pea Chloroplasts

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ABSTRACT

Intact isolated chloroplasts from pea (*Pisum sativum*) leaves carried out light-dependent (NH_3, 2-oxoglutarate) and (glutamine, 2-oxoglutarate)-dependent O_2 evolution at rates of 3.3 ± 0.7 (n = 7) and 6.0 ± 0.4 (n = 5) micromoles per milligram chlorophyll per hour, respectively. Malate stimulated the rate of (NH_3, 2-oxoglutarate)-dependent O_2 evolution 2.1 ± 0.5 (n = 7)-fold in the absence of glutamine, and 3.3 ± 0.4 (n = 11)-fold in the presence of glutamine. Malate also stimulated (glutamine, 2-oxoglutarate)-dependent O_2 evolution in the presence of high concentrations of glutamine. The affinity ($K_{1/2}$) of (NH_3, glutamine, 2-oxoglutarate)-dependent O_2 evolution for 2-oxoglutarate was estimated at 200 to 250 micromolar in the absence of malate and 50 to 80 micromolar when malate (0.5 millimolar) was present. In contrast to malate and various other dicarboxylates, aspartate, glutarate, and glutamate did not stimulate (NH_3, glutamine, 2-oxoglutarate)-dependent O_2 evolution in isolated pea chloroplasts. Using both *in vitro* assays and reconstituted chloroplast systems, malate was shown to have no effect on the activities of either glutamine synthetase or glutamate synthase.

The concentration of malate required for maximal stimulation of O_2 evolution was dependent on the concentration of 2-oxoglutarate present. However, the small extent of the competition between malate and 2-oxoglutarate for uptake was not consistent with that predicted by the current 'single carrier' model proposed for the uptake of dicarboxylates into chloroplasts.

It is now widely accepted that refixation of photorespiratory NH_3 takes place via the GS/GOGAT[2] pathway (12). However, while both enzymes are located within the chloroplast, the actual site of refixation of NH_3 by GS is still the subject of some conjecture, because up to 50% of the total cellular GS activity is cytoplasmic in some plant species (22). For refixation by cytoplasmic GS to be a feasible proposition, potential uptake of glutamine into the chloroplast must be sufficiently rapid to maintain the proposed rate of photorespiratory NH_3 recycling estimated at approximately 30 μmol mg^{-1} Chl h^{-1} (24). Evidence to date, however, on glutamine uptake (3) shows this to be unlikely, thereby providing support for the hypothesis that all NH_3 refixation takes place within the chloroplast.

Similarly, 2-OG must also be transported into the chloroplast at rates approaching those cited above. On the basis of ^{14}C uptake experiments with isolated chloroplasts from both spinach (8) and peas (16), it seems that potential rates of 2-OG uptake are sufficient to account for the predicted rate of operation of the photorespiratory cycle. However, despite these findings, attempts to demonstrate similar rates of (NH_3, 2-OG)-dependent O_2 evolution by isolated chloroplasts have not been successful (1). In a recent publication, Woo and Osmond (25) re-examined this problem and showed that the rate of (NH_3, 2-OG)-dependent O_2 evolution by isolated chloroplasts could be significantly enhanced by the addition of certain dicarboxylates, *e.g.* malate, succinate, or fumarate to the assay system.

Given our present understanding of the uptake of dicarboxylate molecules into chloroplasts, *i.e.* it is mediated by a broadly specific dicarboxylate carrier (8), one might predict that malate should in fact inhibit (NH_3, 2-OG)-dependent O_2 evolution, because both malate and 2-OG would compete for entry into the chloroplast on the same carrier. We therefore set out to examine in more detail the phenomenon of dicarboxylate stimulation of (NH_3, 2-OG)-dependent O_2 evolution in isolated pea chloroplasts and have found evidence that malate and 2-OG do indeed compete for uptake into the chloroplast. However, the competition is rather weak and our results are best explained by the operation of two separate carriers in the chloroplast membrane and not that of a single broadly specific dicarboxylate carrier.

MATERIALS AND METHODS

Pea seedlings (*Pisum sativum* vars. Massey Gem, Greenfeast) were grown in vermiculite for 11 to 13 d in a glasshouse. All biochemicals were obtained from Sigma Chemical Co. Spinach ferredoxin was prepared by the method of Rao *et al.* (17).

Isolation of Chloroplasts. Chloroplasts were obtained from pea shoots according to the method of Robinson and Wiskich (18).

Assay Procedures. O_2 evolution was measured in a Rank O_2 electrode. The vessel was maintained at 25°C and illuminated with a 150-w tungsten halogen projector lamp giving a light intensity of 550 μE m^{-2} s^{-1} at the center of the vessel. Chloroplasts (50 μg Chl) were illuminated in a 2-ml standard reaction medium containing 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 50 mM Hepes, pH 7.6. For CO_2-dependent O_2 evolution, 4.5 mM $Na_2P_2O_7$, 0.9 mM ATP, 0.18 mM NaH_2PO_4/Na_2HPO_4 were also added. Glyceraldehyde (10 mM) was present during (NH_3, 2-OG) (NH_3, glutamine, 2-OG)- and (glutamine, 2-OG)-dependent O_2 evolution to inhibit endogenous CO_2-dependent O_2 evolution (20). Chloroplast intactness was measured with ferricyanide according to the method of Lilley *et al.* (10). Chl was determined from 80% acetone extracts using the method of Arnon

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[2] Abbreviations: GS, glutamine synthetase; 2-OG, 2-oxoglutarate; Asp, aspartate; OH-Pyr, hydroxypyruvate; Aza, azaserine; MSO, methionine sulfoximine; GOGAT, glutamate synthase.

(2). All dicarboxylate compounds were newly prepared and adjusted to pH 7.0 with NaOH prior to use.

GS was assayed spectrophotometrically according to the method of O'Neal and Joy (14). GOGAT was assayed polarographically (4) using a reconstituted chloroplast system prepared according to the method of Lilley *et al.* (9). The reaction mixture contained envelope-free chloroplasts (250 μg Chl), 100 μl chloroplast extract, 330 mM sorbitol, 2 mM EDTA, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes, 200 units of catalase, 1 mM NH_4Cl, and 180 μg spinach ferredoxin in a final volume of 2 ml at pH 7.6 and 25°C.

RESULTS

Isolated pea chloroplasts carried out light-dependent O_2 evolution in the presence of NH_4Cl and 2-OG (trace A, Fig. 1) and in the absence of PP_i, ADP, and large concentrations of $MgCl_2$, used previously by other researchers for (NH_3, 2-OG)-dependent O_2 evolution (1, 25). Glutamine addition caused only a small increase in the rate of O_2 evolution (trace A, Fig. 1), whereas in the presence of malate it produced a marked stimulation (trace C, Fig. 1). The addition of malate stimulated O_2 evolution, the extent of which was found to be dependent on the presence or absence of added glutamine, *i.e.* while malate was found to stimulate (NH_3, 2-OG)-dependent O_2 evolution in the absence of glutamine, the full extent of malate stimulation was only observed when gluta-

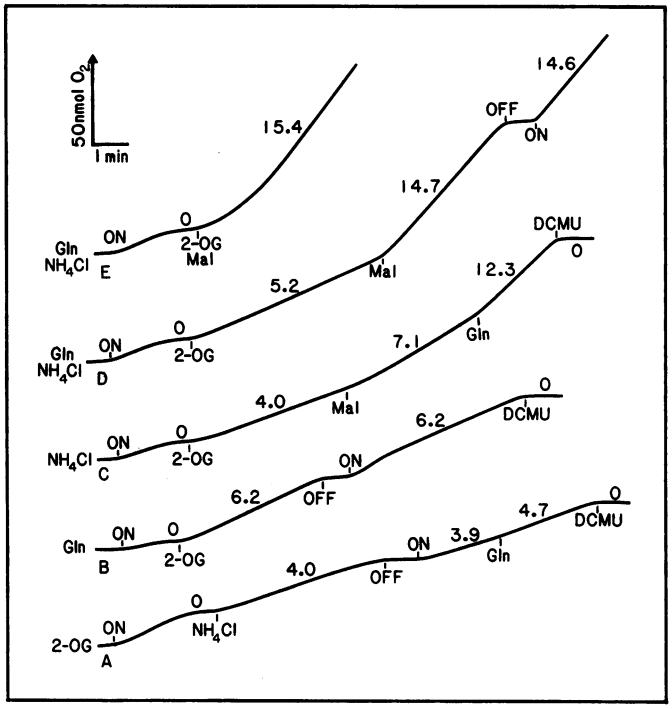

FIG. 1. Effect of glutamine, DCMU and malate, on (NH_3, 2-OG)- and (glutamine, 2-OG)-dependent O_2 evolution by isolated pea chloroplasts. Additions were made to the following concentrations: NH_4Cl, 1 mM; 2-OG, 1 mM; glutamine, 1 mM; malate (Mal), 1 mM; DCMU, 5 μM, Chl concentration: 47 μg ml^{-1}. CO_2-dependent O_2 evolution: 92 μmol mg^{-1} Chl h^{-1}. Intactness: 69%. Values beside the curves represent μmol O_2 evolved mg^{-1} Chl h^{-1}.

Table I. *Effect of Malate on in Vitro Glutamate Synthase Activity Measured with a Reconstituted Chloroplast System*

The reaction mixture containing 5 mM glutamine was illuminated for 2 min prior to the first addition.

Sequential Additions	Glutamate Synthase Activity
	nmol O_2 evolved min^{-1}
2-Oxoglutarate (1 mM)	34.6
Malate (1 mM)	34.6
Malate (4 mM)	34.6
Chloroplast extract (150 μl)	59.3
Azaserine (0.5 mM)	0

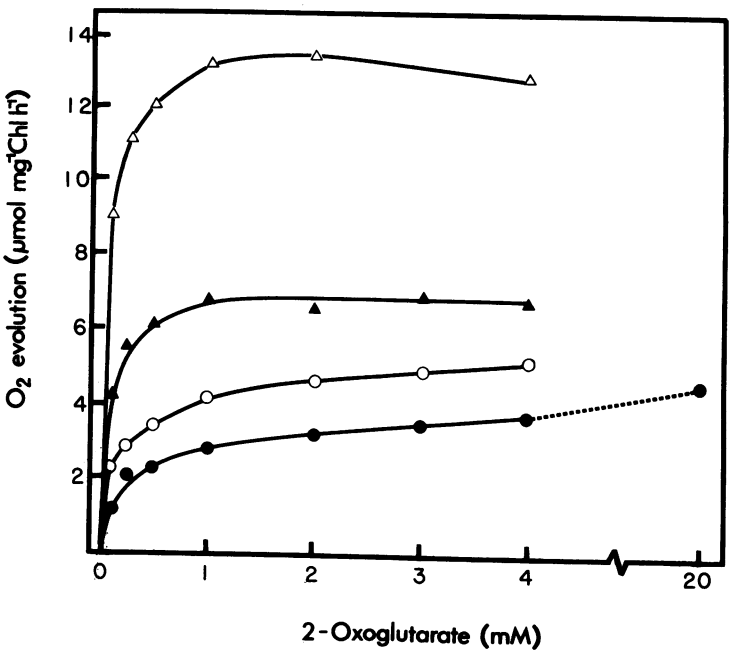

FIG. 2. Effect of 2-OG concentration on (NH_3, 2-OG)-dependent O_2 evolution in the presence and absence of glutamine and malate. Where added, NH_4Cl, glutamine, and malate were present at a concentration of 1 mM. O_2 evolution was initiated by the addition of 2-OG (± malate) and the recorded rates represent the steady state rates of O_2 evolution after 5 min. (NH_3, 2-OG), (●); (NH_3, glutamine, 2-OG), (○); (NH_3, 2-OG, malate), (▲); (NH_3, glutamine, 2-OG, malate), (△). Chl concentration: 45 μg ml^{-1}. CO_2-dependent O_2 evolution: 70 μmol mg^{-1} Chl h^{-1}. Intactness: 75%.

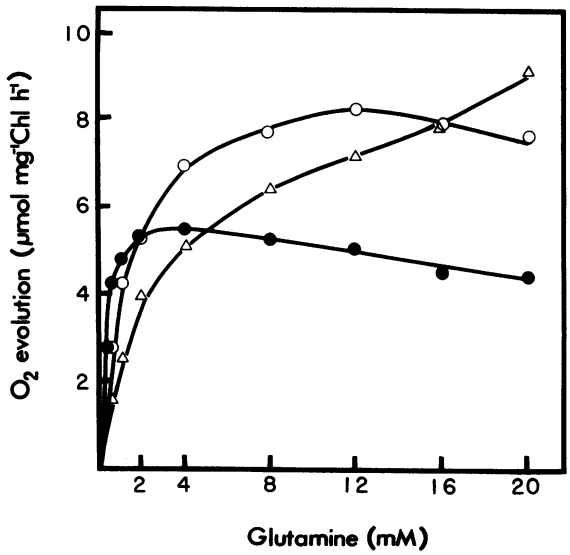

FIG. 3. Effect of malate on (glutamine, 2-OG)-dependent O_2 evolution by isolated pea chloroplasts in the presence of varying glutamine concentrations. 2-OG was present at 1 mM. Malate was added at a concentration of either 0.1 (○) or 1.0 mM (△) following the establishment of a steady state rate of O_2 evolution. Chl concentration: 50 μg ml^{-1}. CO_2-dependent O_2 evolution: 64 μmol mg^{-1} Chl h^{-1}. Intactness: 66%.

Table II. *Summary of Mean Values for Various Forms of O_2 Evolution by Isolated Chloroplasts over n Preparations*

Rates corrected for chloroplast breakage during preparation (CFB) are also given (mean intactness: 68%; $n = 13$). Assay of A, B, D, and E were always carried out with a 1 mM concentration of all substrates involved. Assay of C involved glutamine, 2 mM, 2-oxoglutarate, 1 mM. Values represent μmol O_2 evolved mg^{-1} Chl h^{-1}.

Type of O_2 Evolution	*n*	Mean	SD	CFB
A. NH_3, 2-OG	7	3.3	0.7	4.8
B. NH_3, glutamine, 2-OG	12	4.4	0.9	6.4
C. Glutamine, 2-OG	5	6.0	0.4	8.8
D. NH_3, 2-OG, Malate	7	6.9	1.6	10.0
E. NH_3, Glutamine, 2-OG, Malate	11	14.6	2.2	21.5
D/A	7	2.1	0.5	
D/E	7	0.5	0.1	
E/B	11	3.3	0.4	

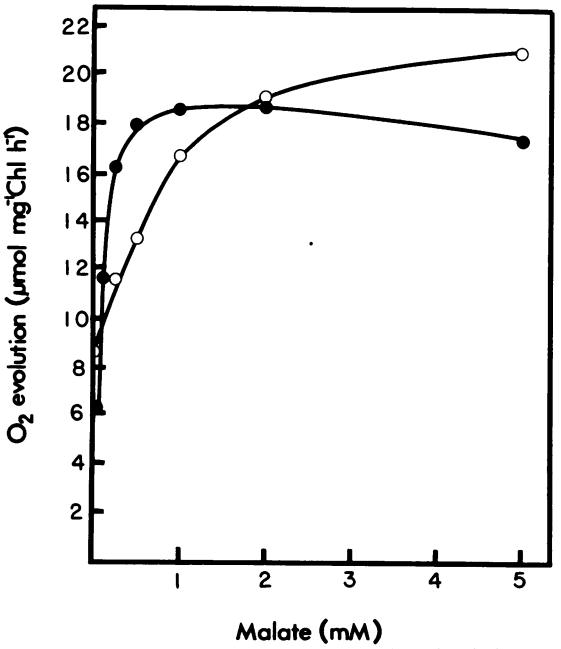

FIG. 4. Effect of malate concentration on the stimulation of (NH_3, glutamine, 2-OG)-dependent O_2 evolution by isolated pea chloroplasts in the presence of two different 2-OG concentrations. Standard assay contained NH_4Cl, 1 mM; glutamine, 1 mM, and 2-OG at a concentration of either 1 (●) or 10 mM (○). Malate, at varying concentrations, was added at the same time as 2-OG which was used to initiate O_2 evolution. Chl concentration: 40 μg ml^{-1}. CO_2-dependent O_2 evolution: 85 μmol mg^{-1} Chl h^{-1}. Intactness: 70%.

mine was also present (traces C, D, and E, Fig. 1). Isolated pea chloroplasts also supported light-dependent O_2 evolution in the presence of glutamine and 2-OG, and, like (NH_3, 2-OG)-dependent O_2 evolution, this activity was inhibited by DCMU (traces A and B, Fig. 1). MSO or Aza inhibited (NH_3, 2-OG)-dependent O_2 evolution, both in the presence and absence of malate, at concentrations of 2.5 and 0.25 mM, respectively (data not shown), indicating that O_2 evolution resulted from the operation of the GS/GOGAT pathway (19, 21) and not from the metabolism of malate within the chloroplast.

The possibility that malate stimulated O_2 evolution by activating either GS or GOGAT was investigated using broken chloroplasts (GS) or a reconstituted assay system (GOGAT). Malate had no

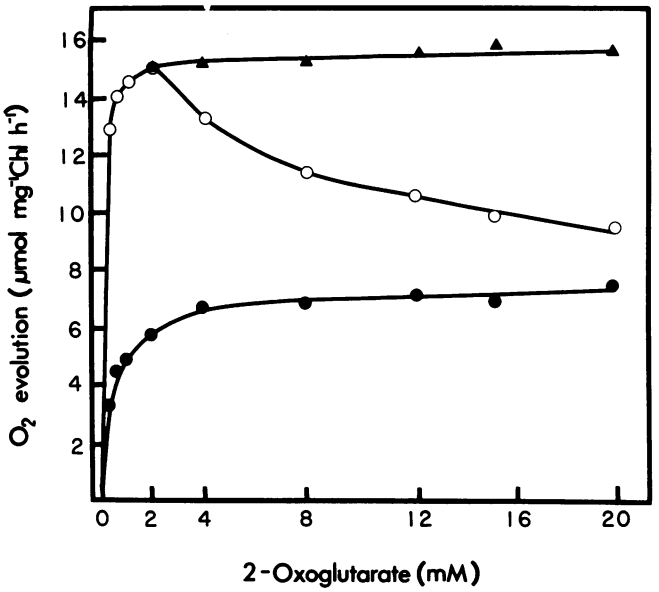

FIG. 5. Effect of 2-OG concentration on the stimulatory effect of malate on (NH_3, glutamine, 2-OG)-dependent O_2 evolution by isolated pea chloroplasts. Standard assay contained NH_4Cl, 1 mM, glutamine, 1 mM (●), and malate, 0.5 mM (○), or at a concentration equaling the 2-OG concentration used in the assay (▲). Where present, malate addition was made with 2-OG, which was used to initiate O_2 evolution. Chl concentration: 38 μg ml^{-1}. CO_2-dependent O_2 evolution: 98 μmol mg^{-1} Chl h^{-1}. Intactness: 72%.

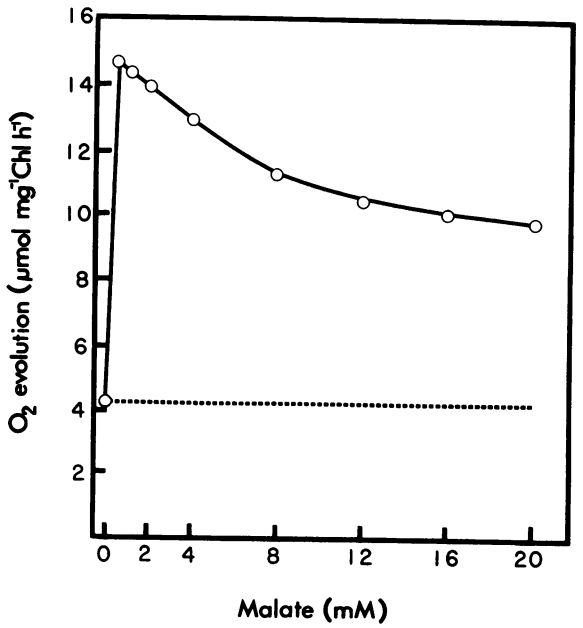

FIG. 6. Effect of increasing malate concentration on its stimulatory effect on (NH_3, glutamine, 2-OG)-dependent O_2 evolution by isolated pea chloroplasts. Standard assay contained NH_4Cl, 1 mM, glutamine, 1 mM, 2-OG, 0.5 mM. Malate addition was made with 2-OG, which was used to initiate O_2 evolution. The dotted line represents the rate of O_2 evolution in the absence of added malate. Chl concentration: 43 μg ml^{-1}. CO_2-dependent O_2 evolution: 100 μmol mg^{-1} Chl h^{-1}. Intactness: 71%.

effect on the *in vitro* activities of either GS (data not shown) or GOGAT (Table I) over the wide range of substrate concentration tested, indicating that the stimulation of (NH_3, glutamine, 2-OG)-dependent O_2 evolution by malate required the presence of an intact chloroplast membrane.

Table III. *Specificity of Stimulation by Various Decarboxylates of (NH_3, Glutamine, 2-OG)-Dependent O_2 Evolution by Isolated Chloroplasts*

Standard assay contained NH_4Cl, 1 mM; glutamine, 1 mM; 2-OG, 1 mM; and dicarboxylate, 2 or 5 mM. Dicarboxylate addition was made with 2-OG which was used to initiate O_2 evolution. Aminooxyacetic acid (5 mM) was used to inhibit (Asp, 2-OG)-dependent O_2 evolution in assays involving Asp. There was no effect of aminooxyacetic acid on the rate of (NH_3, glutamine, 2-OG)-dependent O_2 evolution. The control rate represents the rate of O_2 evolution in the absence of added dicarboxylate.

Dicarboxylate	Experiment 1		Experiment 2	
	O_2 Evolution	Stimulation by Malate	O_2 Evolution	Stimulation by Malate
	$\mu mol\ mg^{-1}\ Chl\ h^{-1}$	%	$\mu mol\ mg^{-1}\ Chl^{-1}$	%
Control	4.6		5.2	
Malate (2 mM)	16.6	100	15.0	100
Succinate (2 mM)	16.3	98		
Fumarate (2 mM)	16.6	100		
L-Tartrate (2 mM)	12.8	68	11.1	60
L-Tartrate (5 mM)	13.4	73	12.3	72
Aspartate				
2 mM	5.4	6	6.0	8
5 mM	6.1	12	6.5	13
Glutarate				
2 mM	6.4	15		
5 mM	7.1	21	6.5	13
Glutamate				
2 mM	2.0			
5 mM	1.0			
Thiomalate				
2 mM			8.9	38
5 mM			11.4	63
Malonate				
2 mM	4.6	0		
5 mM	4.1			

The dependence of (NH_3, 2-OG)-dependent O_2 evolution by isolated pea chloroplasts on 2-OG concentration, both in the presence and absence of glutamine and malate, is shown in Figure 2. Again it can be seen that malate stimulates (NH_3, 2-OG)-dependent O_2 evolution both with and without added glutamine, over the range of 2-OG concentrations tested. The $K_{1/2}$ for 2-OG in the absence of malate, was estimated at 200 to 250 μM. Glutamine addition had no effect on this value, while the presence of malate reduced the $K_{1/2}$ estimate to 50 to 80 μM. Malate was also found to stimulate (glutamine, 2-OG)-dependent O_2 evolution (*i.e.* no added NH_4Cl) when isolated pea chloroplasts were incubated with high concentrations of glutamine (Fig. 3).

Table II gives the mean values of the various types of O_2 evolution shown in Figure 2 as well as (glutamine, 2-OG)-dependent O_2 evolution, measured with a number of separate chloroplast preparations. It also includes the estimated rates of O_2 evolution when corrected for chloroplast breakage during isolation. It should be noted that the most variable parameter listed in Table II was found to be the ratio D/E, *i.e.* the rate of malate-stimulated (NH_3, 2-OG)-dependent O_2 evolution in the absence of glutamine, compared to the maximal rate of malate-stimulated O_2 evolution in the presence of glutamine. This was not only true for chloroplast preparations within the variety Massey Gem, but also between pea varieties, with the variety Greenfeast consistently having a much smaller value for this ratio (*e.g.* 0.20 to 0.25) than Massey Gem. This most probably reflects differences in the levels of endogenous intermediates of the GS/GOGAT pathway, within chloroplasts isolated from these different pea varieties. The optimum pH for (NH_3 glutamine, 2-OG)-dependent O_2 evolution, both with and without added malate, was 7.4.

The relationship between malate concentration and the degree of stimulation of (NH_3 glutamine, 2-OG)-dependent O_2 evolution is illustrated in Figure 4. In the presence of 1 mM 2-OG, half-maximal stimulation of O_2 evolution was achieved on addition of malate to a concentration of 150 μM and maximal stimulation was reached when the value of the ratio of concentrations of malate to 2-OG was in the range of 1 to 2. However, when malate was added in the presence of an increased concentration of 2-OG (10 mM), stimulation at each malate concentration was found to be reduced and the $K_{1/2}$ for malate increased to approximately 700 μM.

These results indicated that while malate could significantly stimulate (NH_3, glutamine, 2-OG)-dependent O_2 evolution, the degree of stimulation produced by a particular malate concentration was dependent on the concentration of 2-OG also present. This is further illustrated in Figure 5. Here, the malate concentration was kept constant (0.5 mM) while the 2-OG concentration was varied between 0 and 20 mM. The resultant stimulation of (NH_3 glutamine, 2-OG)-dependent O_2 evolution, was compared to that produced by malate, when present at the same concentration as 2-OG, *i.e.* a malate/2-OG concentration ratio of 1. Again, the results show that the degree of stimulation of (NH_3 glutamine, 2-OG-)-dependent O_2 evolution, produced by a particular malate concentration, was dependent on the concentration of 2-OG present. Increasing the amount of malate added in the presence of a constant 2-OG concentration was also found to lead to a decrease in the rate of malate-stimulated (NH_3, glutamine, 2-OG)-dependent O_2 evolution (Fig. 6).

The effects of a range of dicarboxylates on (NH_3, glutamine, 2-OG)-dependent O_2 evolution are shown in Table III. Malate, succinate, fumarate, L-tartrate and thiomalate were all found to stimulate O_2 evolution markedly. In contrast, Asp (in the presence

of aminooxyacetic acid to inhibit GOT) and glutarate produced only very minor increases, while glutamate addition resulted in an inhibition of (NH_3, glutamine, 2-oxoglutarate)-dependent O_2 evolution. Malonate which is not thought to be transported into the chloroplast (8) had no effect.

DISCUSSION

Our results demonstrate that isolated pea chloroplasts are capable of supporting both (NH_3, 2-OG)- and (glutamine, 2-OG)-dependent O_2 evolution in the absence of PP_i, ADP, or excessively high concentrations of $MgCl_2$ (10 mM) as used previously (1, 25). This is especially significant with respect to $MgCl_2$, inasmuch as recent reports (6, 11) have shown that the presence of $MgCl_2$ at concentrations as low as 4 mM can cause significant acidification of the chloroplast stroma and an inhibition of CO_2-dependent O_2 evolution.

In contrast to the observations of Woo and Osmond (25), malate was found to stimulate (NH_3, 2-OG)-dependent O_2 evolution in isolated pea chloroplasts in the absence of added glutamine. However, the full stimulatory effect of malate was only seen when glutamine was also present, with NH_4Cl. This requirement for glutamine most probably results from a deficiency of glutamate for GS, due to the unusually high K_m of this enzyme for this substrate, *e.g.* 3.5 to 13 mM (15), and the presence, in isolated pea chloroplasts, of endogenous glutamate at concentrations of only 5 to 6 mM (13). This is in contrast to spinach chloroplasts, which did not require glutamine for maximal stimulation of (NH_3, 2-OG)-dependent O_2 evolution by malate (25), and which have been found to contain glutamate at concentrations of up to 15 mM (8).

The demonstration of malate-stimulated (glutamine, 2-OG)-dependent O_2 evolution (Fig. 3) indicates that the effect of malate is on GOGAT-dependent O_2 evolution. It is also clear that the extent of the stimulation is dependent on the relative availability of glutamine to the GOGAT enzyme. When the only source of glutamine is via uptake (*e.g.* Fig. 3), malate may be seen to have little or no effect, due to the slow transport of this amino acid across chloroplast membranes (3). When isolated pea chloroplasts are incubated with both glutamine and NH_4Cl, however, the availability of glutamine within the chloroplast is markedly increased through the operation of GS, thereby allowing the maximal stimulatory effect of malate on GOGAT-dependent O_2 evolution to be observed (Figs. 1 and 2).

No evidence was found to suggest that the observed stimulation of (NH_3, glutamine, 2-OG)-dependent O_2 evolution by malate resulted from the metabolism of malate within the chloroplast, or that the addition of malate led to any activation of the pathway enzymes. Because malate can be shown to stimulate both (NH_3, 2-OG)- and (glutamine, 2-OG)-dependent O_2 evolution, it is unlikely that the malate effect is mediated through direct effects on glutamine or NH_3 uptake. More probably, malate increases the availability to GOGAT of its other substrate (2-OG) either by stimulating 2-OG uptake or increasing the affinity of the GOGAT enzyme for this substrate. Woo and Osmond (25) observed a dramatic decrease in the $K_{1/2}$ for 2-OG in (NH_3, 2-OG)-dependent O_2 evolution from 6 mM to 73 μM on the addition of malate to spinach chloroplasts. Our measurements with pea chloroplasts, however, showed the $K_{1/2}$ estimates for 2-OG, both in the presence and absence of malate (50 to 250 μM), to be in the range expected from previous studies on chloroplast transporters (8) and the GOGAT enzyme (21). Thus, the stimulatory effect of malate on (NH_3, glutamine, 2-OG)-dependent O_2 evolution probably results from an increase in the rate of 2-OG uptake, via exchange reactions with malate across the chloroplast membrane.

It is important to note that glutamate, a dicarboxylate known to exchange across the chloroplast membrane and produced during (NH_3 glutamine, 2-OG)-dependent O_2 evolution, could be expected to exchange for 2-OG *in vivo*, in order to maintain the stoichiometric balance of the photorespiratory cycle. However, it seems from the limited rate of (NH_3, glutamine, 2-OG)-dependent O_2 evolution in the absence of malate that glutamate (in contrast to malate) may not be the optimal substrate for exchange reactions involving 2-OG.

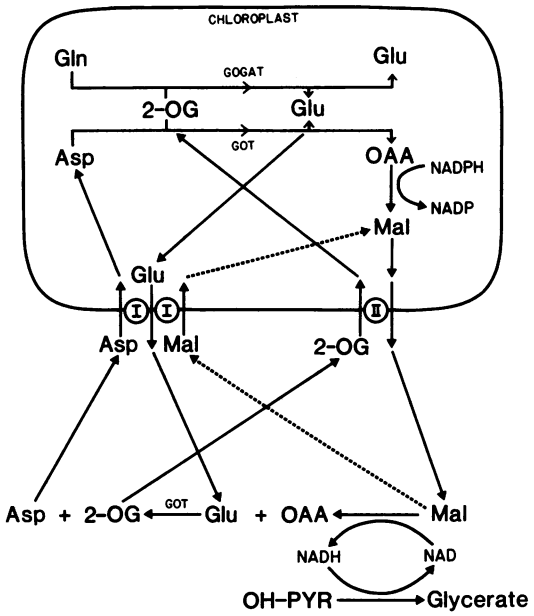

FIG. 7. Movement of dicarboxylate molecules during (a) the operation of a malate/Asp shuttle (solid lines) and (b) malate-stimulated uptake of 2-OG for GOGAT-catalyzed O_2 evolution (broken lines). See text for explanation of symbols I and II. GOT, glutamate oxaloacetate transaminase; Mal, malate.

Isolated chloroplasts would not contain sufficient endogenous dicarboxylates (8), such as malate, to maintain this type of exchange for any length of time following the addition of 2-OG. This may explain the apparent anomaly between the estimated rates of 2-OG uptake by isolated chloroplasts as measured in ^{14}C uptake experiments over 10 to 20 s, *i.e.* 26 to 32 μmol mg^{-1} Chl h^{-1} (8, 16), and those rates approximated from polarographic measurements, as done here, of between 8 and 12 μmol mg^{-1} Chl h^{-1} (Table I), which represent steady state rates calculated over 5–10 min.

Asp is rapidly taken up in isolated pea chloroplast as indicated by the high rates of (Asp, 2-OG)-dependent O_2 evolution which can be observed (20–25 μmol mg^{-1} Chl h^{-1}). However, this amino acid has little effect on (NH_3, glutamine, 2-OG)-dependent O_2 evolution (Table III). This suggests that under the experimental conditions used internal Asp does not exchange for external 2-OG, as readily as do the C_4-type dicarboxylates such as malate, succinate, or L-tartrate.

Another major point regarding malate-stimulated (NH_3, glutamine, 2-OG)-dependent O_2 evolution is that it is not consistent with a single dicarboxylate carrier model. If the chloroplast membrane contained only one type of dicarboxylate carrier, the addition of malate during (NH_3, glutamine, 2-OG)-dependent O_2 evolution should result in strong competition between malate and 2-OG for uptake and consequently inhibit, not stimulate, O_2 evolution. Figure 6 illustrates that while some competition is observed at very high malate 2-OG ratios, the rate is always well above the rate achieved in the absence of malate, *i.e.* full inhibition of 2-OG uptake is never approached, as one might expect at malate/2-OG ratios of 40:1. Conversely, high concentrations of 2-OG should inhibit the uptake of malate into the chloroplast, thereby reducing its stimulatory effect on O_2 evolution. The results of Figures 4 and 5 confirm this; however, again the extent of the competition or inhibition at high 2-OG/malate ratios is much

lower than would be predicted from the similarity in affinity of the dicarboxylate carrier for these two substrates (23). For example, Lehner and Heldt (8) showed that the uptake of malate into spinach chloroplasts could be inhibited 60% by the presence of a 3-fold higher concentration of succinate or fumarate. Our results show that the presence of a 3-fold higher concentration of 2-OG had virtually no effect on the stimulation of (NH_3, glutamine, 2-OG)-dependent O_2 evolution by malate.

We feel that results of this type strongly suggest the possible existence of separate carriers in the chloroplast membrane as depicted in Figure 7. Using this model, it can be seen that the explanation for malate-stimulated 2-OG uptake lies in the ability of malate to 'short circuit' the reaction and transport sequence which can support rapid rates of 2-OG uptake during (Asp, 2-OG)-dependent O_2 evolution, via the operation of a malate-Asp shuttle proposed originally by Heber (5) as a method for transferring reducing equivalents out of the chloroplast. *In vivo* uptake of 2-OG may be coupled to an OAA-malate shuttle (25) which could supply reducing equivalents for the hydroxypyruvate reduction step in peroxisomes (Fig. 7). However, the feasibility of such a shuttle is under question from suggestions that it is the mitochondrion and not the chloroplast which supplies the reducing equivalents for the hydroxypyruvate reaction (7).

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