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The roles of malate and aspartate in C_4 photosynthetic metabolism of *Flaveria bidentis* (L.)

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Abstract. In C_4 grasses belonging to the NADP-malic enzyme-type subgroup, malate is considered to be the predominant C_4 acid metabolized during C_4 photosynthesis, and the bundle sheath cell chloroplasts contain very little photosystem-II (PSII) activity. The present studies showed that *Flaveria bidentis* (L.), an NADP-malic enzyme-type C_4 dicotyledon, had substantial PSII activity in bundle sheath cells and that malate and aspartate apparently contributed about equally to the transfer of CO_2 to bundle sheath cells. Preparations of bundle sheath cells and chloroplasts isolated from these cells evolved O_2 at rates between 1.5 and 2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ chlorophyll (Chl) in the light in response to adding either 3-phosphoglycerate plus HCO_3^- or aspartate plus 2-oxoglutarate. Rates of more than 2 $\mu mol\ O_2 \cdot min^{-1} \cdot mg^{-1}$ Chl were recorded for cells provided with both sets of these substrates. With bundle sheath cell preparations the maximum rates of light-dependent CO_2 fixation and malate decarboxylation to pyruvate recorded were about 1.7 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ Chl. Compared with NADP-malic enzyme-type grass species, *F. bidentis* bundle sheath cells contained much higher activities of NADP-malate dehydrogenase and of aspartate and alanine aminotransferases. Time-course and pulse-chase studies following the kinetics of radiolabelling of the C-4 carboxyl of C_4 acids from $^{14}CO_2$ indicated that the photosynthetically active pool of malate was about twice the size of the aspartate pool. However, there was strong evidence for a rapid flux of carbon through both these pools. Possible routes of aspartate metabolism and the relationship between this metabolism and PSII activity in bundle sheath cells are considered.

Key words: Aspartate decarboxylation – Bundle sheath – C_4 photosynthesis – *Flaveria* (C_4 photosynthesis) – Malate decarboxylation

Introduction

One of the three major subgroups of C_4 plants, termed NADP-ME-type, generates CO_2 in bundle sheath cells by decarboxylating malate via NADP-malic enzyme (Hatch 1987). In the grasses like *Zea mays* and *Sorghum bicolor* belonging to this group the bundle sheath cell chloroplasts have very little PSII activity (Ku et al. 1974; Chapman et al. 1980), mostly single unappressed thylakoid membranes and high ratios of chlorophyll *a* to *b* (Woo et al. 1971; Ku et al. 1974). The dicotyledon *Gomphrena celosioides*, which belongs to this same C_4 subgroup, shows substantially higher PSII activity in bundle sheath cells and higher aspartate and alanine aminotransferase activities compared with the grass species (Repo and Hatch 1976). As noted earlier (Repo and Hatch 1976; Chapman and Hatch 1981), if aspartate makes a substantial contribution to transfer of CO_2 to bundle sheath cells then this could be correlated with a requirement for increased bundle sheath cell PSII activity. This argument is based on the fact that the decarboxylative metabolism of malate, but not aspartate, is accompanied by the transfer of reducing power to bundle sheath cells, thus decreasing the need for the generation of NADPH via PSII. However, $^{14}CO_2$ radiolabelling studies with *Gomphrena celosiodes* did not support the view that there was a substantial flux of carbon through the aspartate pool (Repo and Hatch 1976).

The C_4 *Flaveria* species are also dicotyledons belonging to the NADP-ME-type group. Compared with the grass species belonging to this group, the C_4 species *Flavaria trinervia* has been reported to contain higher activities of aspartate and alanine aminotransferases, with substantial proportions of these activities, and also NADP-malate-dehydrogenase (NADP-MDH), in bundle sheath cells (Moore et al. 1984). C_4 *Flaveria* species have

Abbreviations: DHAP = dihydroxyacetone phosphate; NADP-ME(-type) = NADP-malic enzyme (type); NADP-MDH = NADP-malate dehydrogenase; OAA = oxaloacetic acid; 2-OG = 2-oxoglutarate; PEP = phosphoenolpyruvate; PGA = 3-phosphoglycerate; Pi = orthophosphate; Ru5P = ribulose 5-phosphate

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assumed particular importance because one species (*F. bidentis*) can now be routinely transformed (Chitty et al. 1994). Furthermore, the C_4 *Flaveria* species continue to be important in evolutionary studies on C_4 plants and also the ongoing study of naturally occurring *Flaveria* species that have characteristics intermediate between C_3 and C_4 species. We therefore undertook a study of the metabolism of the C_4 acids malate and aspartate occurring during photosynthesis in *F. bidentis*. This paper reports substantial differences in a number of photosynthetic characteristics between *F. bidentis* and grass species belonging to the NADP-ME-type group.

Materials and methods

Materials. *Flaveria bidentis* and *F. trinervia* (Spring.) C. Mohr were grown in soil in a naturally illuminated glasshouse maintained at about 18 and 27 °C night and day in summer and 15 to 25 °C in winter. Biochemicals and enzymes were obtained from either Boehringer-Mannheim, Australia or Sigma Chemical Co., St Louis, Mo., USA. Both $Ba^{14}CO_3$ and $NaH^{14}CO_3$ were obtained from Amersham International and Percoll from Pharmacia-LKB Biotechnology, Australia.

Preparation of bundle sheath cell strands. Plants were darkened for about 20 h to deplete the leaves of starch and then were illuminated for about 40 min prior to harvesting leaves. Upper fully expanded leaves were deribbed and about 10 g of lamina material was sliced into segments of about 1 mm with a sharp razor blade and then blended in a 250-ml cup of a Sorvall Omnimixer with 120 ml of medium containing 0.3 M sorbitol, 20 mM Hepes-KOH (pH 7.7), 2 mM EDTA, 2 mM isoascorbate and 2 mM orthophosphate (Pi). This and subsequent steps were conducted at 0 °C. After blending for 10 s at 60% of line voltage the mixture was blended again in bursts of about 10 s at 40% of line voltage until bundle sheath cell strands were obtained free of mesophyll cells, as judged by microscopic examination. Generally, this required four or five bursts of 10 s. The homogenate was then filtered through a 600-μm net to remove larger pieces of tissue and epidermal material and the cell strands were collected on an 80-μm nylon net and washed twice with 40 ml of a wash-resuspension medium containing 0.3 M sorbitol, 20 mM Hepes-KOH (pH 7.7), 10 mM KCl, 1 mM EDTA and 0.5 mM Pi. The cell strands were then suspended in 40 ml of the same medium in a measuring cylinder and allowed to settle under gravity for about 10 min. The cells settled in a volume of about 8 ml and the upper layer containing much of the epidermal material was removed by suction. The green layer containing bundle sheath cells was diluted to 40 ml with the wash-resuspension medium and stored on ice. Samples were taken by mixing the resuspended cells and then removing aliquots with an Gilson pipette with the tip cut to enlarge the opening.

Preparation of bundle sheath cell chloroplasts. Bundle sheath cell strands, prepared as described above, were collected on an 80-μm net and then suspended in the following mixture to partially digest the cell wall: 0.35 M sorbitol, 10 mM (2-N-morpholino)ethanesulfonic acid (Mes-KOH, pH 6.0), 2 mM EDTA, 1 mM Pi, 10 mM dithiothreitol, 0.2% (w/v) pectinase (from *Rhizopus*; Sigma Chemical Co.), 2% w/v Cellulysin-3S and 0.2% (w/v) Onozuka R-10 cellulase (from Yakult-Honsha Co., Nishinomiya, Japan). The cells were incubated in this mixture for 10 min at 10 °C without stirring and then gently collected on an 80-μm nylon net and immediately washed to remove the digestion mixture by dipping the net several times in 80 ml of the following chloroplast isolation medium: 0.35 M sorbitol, 25 mM Hepes-KOH (pH 7.7), 1 mM EDTA, 2 mM Pi, 2 mM isoascorbate, 1 mg · ml^{-1} bovine serum albumin and 5 mM dithiothreitol (DTT). This and subsequent steps were conducted at 0 °C. The cells, still contained on the 80-μm net were immersed in 40 ml of fresh medium and agitated with fingers against the net for about 90 s. The green material passing through the 80-μm net was then filtered through a 20-μm net to remove intact cells and larger pieces of cell material. This filtrate was centrifuged in a swinging-bucket rotor of a Sorvall centrifuge for 2.5 min at 400 · *g* to pellet chloroplasts. The supernatant was removed by decanting and wiping the inside of the tubes with a tissue. For studies of enzyme activities the pellet was suspended in 3 ml of the chloroplast isolation medium (above) and sub-samples purified on a Percoll step gradient as described below. For studies of chloroplast O_2 exchange and other metabolic activities the chloroplast pellet (about 0.1 ml) was suspended in 4 ml of the chloroplast isolation medium described above and stored at 0 °C.

Where chloroplasts were further purified by Percoll centrifugation, equal parts of the resuspended chloroplasts were layered in two tubes on 2 ml of 25% (v/v) Percoll in the isolation medium (see above) and centrifuged for 4 min at 700 · *g* in a swing-out rotor. After removing the overlaying liquid layers, including a green band at the interface, the pellet was suspended in 0.7 ml of the isolation medium and stored at 0 °C.

Oxygen exchange. Oxygen exchange in bundle sheath cells and chloroplasts was measured at 30 °C using an oxygen electrode (Rank Bros., Cambridge, UK). Reactions of 1 or 2 ml contained cells or chloroplasts equivalent to 30 to 40 μg chlorophyll in the final resuspension medium described above. Reactions were illuminated with a slide projector giving an incident radiation of about 2000 μmol quanta · m^{-2} · s^{-1}.

Light-dependent $^{14}CO_2$ fixation. Reactions containing cells or chloroplasts (about 25 μg chlorophyll) in 2 ml were incubated at 30 °C for 10 min and, where indicated, illuminated with a mercury vapour lamp giving incident PAR of about 1000 μmol quanta · m^{-2} · s^{-1}. The reactions contained the components of the final resuspension medium indicated above for cells or chloroplasts together with 7.5 mM $NaH^{14}CO_3$ (about 5×10^4 Bq · μmol^{-1}) and 3 mM $MgCl_2$. After 10 min, reactions were stopped by the addition of 0.2 ml of 2 M trifluoroacetic acid and then kept at 0° C for 30 min with regular shaking. After centrifugation to remove insoluble material the acid-stable radioactivity in the supernatant was determined by scintillation counting.

Malate decarboxylation to pyruvate. Reactions containing cells or chloroplasts (about 40 μg chlorophyll) in 2 ml were incubated at 30 °C for 10 min. Other details were as described for measurement of $^{14}CO_2$ fixation except that all reactions contained 10 mM malate and 4 mM $MgCl_2$ and were stopped by the addition of 0.2 ml of 2 M HCl. After samples of the clear supernatant were neutralized with KOH, oxaloacetate and pyruvate were measured immediately as previously described (Furbank et al. 1990).

Enzyme activities of mesophyll and bundle sheath cells. Sliced leaf lamina tissue was blended in a Sorvall Omnimixer as described above for 10 s at 40% of line voltage. A sample of the extract obtained by filtering the homogenate through Miracloth (very largely the contents of mesophyll cells) was stored for analysis of enzyme activities. A sample of the residual cell material collected on the Miracloth (bundle sheath cell strands still contaminated with mesophyll cells, as judged by microscocpic examination) was extracted for analysis of enzyme activities; this extract represented preparation 1 in Table 5. The remaining cell material was then subjected to either one or two 40-s periods of blending at 100% of line voltage in a Sorvall Omnimixer to give bundle sheath cell preparations 2 and 3, respectively (see Table 5). Correction of mesophyll cell enzyme activities for contamination by bundle sheath cell contents was based on measurement of the specific activity of the bundle sheath cell marker enzymes [NADP-ME and ribulose-5-phosphate kinase (Ru5P kinase)] in the mesophyll cell extract. It was assumed that mesophyll and bundle sheath cells each contained about 50% of the leaf chlorophyll since the chlorophyll-based specific activities of both phosphoenolpyruvate (PEP) carboxylase in mesophyll extracts

and of NADP-ME in bundle sheath cell extracts were close to twice their specific activities in whole-leaf extracts.

Extraction and assay of enzymes. Samples of bundle sheath cell strands were extracted in a glass homogenizer in the cell resuspension medium (see above) supplemented with 5 mM DTT and 1 mg · ml^{-1} bovine serum albumin. After filtration through Miracloth, the extract was supplemented with Triton X-100 to give a final concentration of 0.2% (v/v) and assayed immediately or after storing at − 80 °C. For isolated chloroplasts, Triton X-100 was added to give a final concentration of 0.2% (v/v) and samples were either assayed immediately or stored at − 80 °C. The activities of NADP-MDH and Ru5P kinase were measured before and after incubating subsamples in a mixture containing 5 mM DTT, 0.2 M KCl and 0.2 M Tricine-KOH (pH 8.8). This treatment activates any component of these enzymes that may exist in the oxidised dark-inactive form (see Ashton et al. 1990). Assays of NADP-MDH, PEP carboxylase, alanine aminotransferase and aspartate aminotransferase (Ashton et al. 1990), and of Ru5P kinase (Leegood 1990) were done as previously described.

Kinetics of metabolite labelling from $^{14}CO_2$ in intact leaves. Top fully expanded leaves were detached and the petiole recut under water. Five leaves were placed in a perspex chamber (6 l volume, air circulated by two high-speed fans) with the petiole protruding through a foam rubber sealing strip of a split lid. The chamber was flushed with humidified air supplemented with a CO_2 gas mixture adjusted to give a CO_2 concentration in the chamber of about 390–400 µl · l^{-1}. The flow rate was about 5 l · min^{-1}. The tips of the petioles were wrapped in tissue and kept saturated with water. Leaves were illuminated at 900 µmol quanta · m^{-2} · s^{-1} of photosynthetically active radiation (PAR). After a period of about 30 min to allow development of a steady-state rate of photosynthesis the $^{14}CO_2$ pulse of pulse-chase experiments was commenced by stopping the air flow and injecting about 1.5×10^7 Bq (approximately 8 µmol) of $^{14}CO_2$. At intervals, leaves were removed through the foam seal and killed in 30 ml of boiling 80% (v/v) ethanol. For the pulse-chase, the chamber lid, with leaves, was removed from the $^{14}CO_2$ pulse chamber after 30 s and transferred to a second chamber maintained at identical light and temperature but with normal air containing about 390 µl · l^{-1} $^{12}CO_2$. Other details of procedures for extraction of leaves (Hatch et al. 1995) and analysis of the distribution of radioactivity in individual compounds or carbon atoms (Hatch 1979) were as previously described.

Chlorophyll (Chl) determinations. For routine studies of bundle sheath cell activities, chlorophyll was extracted in methanol and determined by the procedure of MacKinney (1941). For chloroplasts and leaf extracts, chlorophyll was extracted into 80% (v/v) acetone and determined by the procedure of Porra et al. (1989), including analysis of chlorophyll *a* and *b* ratios.

Results and discussion

Metabolic activities of isolated bundle sheath cells. The light-dependent metabolic activities of *F. bidentis* bundle sheath cells were examined to determine the potential roles of malate and aspartate in photosynthetic metabolism. Isolated bundle sheath cell strands showed substantial rates of light-dependent O_2 evolution when either 3-phosphoglycerate (PGA) plus HCO_3^- or aspartate plus 2-oxoglutarate (2-OG) were provided (Table 1). There was no response with HCO_3^- added alone but adding HCO_3^- after PGA increased the rate of O_2 evolution. Bicarbonate may relieve a limitation on the rate of electron transport imposed by limited ATP consumption. The O_2 evolution resulting from the addition of aspartate plus 2-OG was dependent on adding these compounds together and was presumably due to the oxidation of NADPH accompanying oxaloacetic acid (OAA) reduction via NADP-MDH. Adding OAA also stimulated O_2 production (results not shown). The combined addition of HCO_3^- plus PGA and aspartate plus 2-OG commonly gave rates above 2 µmol O_2 · min^{-1} · mg^{-1} Chl and up to 2.8 (Table 1). This represents a PSII activity about ten times that observed with bundle sheath cells from the NADP-ME-type grass *Zea mays* (Chapman et al. 1980; Chapman and Hatch 1981) and about half of the activity observed with bundle sheath cells considered to contain normal levels of PSII such as those from *Panicum miliaceum* and *Urochloa panicoides* (Agostino et al. 1989). In a single experiment, bundle sheath cells prepared from the C_4 species *F. trinervia* gave rates of 0.73 and 0.9 µmol O_2 · min^{-1} · mg^{-1} Chl with aspartate plus 2-OG and PGA plus HCO_3^-, respectively.

Table 1. Light-dependent O_2 evolution from isolated bundle sheath cells of *Flaveria bidentis*. Abbreviations not previously defined and the concentration of additions were: HCO_3^-, 10 mM; PGA, 5 mM; malate, 12.5 mM; aspartate (Asp), 12.5 mM; 2-OG, 12.5 mM. For other details see *Materials and methods*. The results are shown for two different preparations of cells

Cell preparation	Sequential additions and activity (µmol O_2 · min^{-1} · mg^{-1} Chl)			
	Expt. 1		Expt. 2	
1.	Light	0	Light	0.06
	HCO_3^-	0	Asp	0.10
	PGA	2.0	2-OG	1.1
	Asp	2.4	PGA	1.5
	2-OG	2.8	HCO_3^-	1.8
	Dark	− 0.03	Dark	− 0.03
2.	Light	0.03	Light	0
	PGA	1.0	Asp	0.03
	HCO_3^-	1.5	2-OG	1.3
	Asp	1.6	HCO_3^-	1.2
	2-OG	2.0	PGA	1.9
	Dark	− 0.3	Malate	1.6

Light-dependent CO_2 fixation by *F. bidentis* bundle sheath cells was low in the absence of other additions but was stimulated by the inclusion of 3-PGA or dihydroxyacetone phospate (DHAP) as carbon sources (Table 2). The rate of 1.15 µmol O_2 · min^{-1} · mg^{-1} Chl with DHAP was increased to 1.66 by including malate. More than 90% of the fixed radioactivity was located in PGA, triose phosphates and hexose phosphates (results not shown). In contrast to *Z. mays* bundle sheath cells (Chapman et al. 1980), ribose-5-phosphate did not stimulate CO_2 fixation. The factors limiting CO_2 fixation were not apparent. Adding AMP did not increase activity.

Malate decarboxylation to pyruvate was light-dependent and required the addition of PGA. Adding HCO_3^- gave no additional increase. Maximum rates of between 1.5 and 2 µmol pyruvate · min^{-1} · mg^{-1} Chl were recorded (Table 2 and other results not shown). Presumably, the oxidation of NADPH to NADP, necessary to sustain malate decarboxylation, occurs during the reduction of PGA to triose-phosphate. Light would be required to generate the ATP necessary for the prerequisite

Table 2. Fixation of $^{14}CO_2$ and decarboxylation of malate to pyruvate by bundle sheath cells of *F. bidentis*. Abbreviations as in Table 1. For other details see *Materials and methods*

$^{14}CO_2$ fixation Additions	CO_2 fixed ($\mu mol\ CO_2 \cdot min^{-1} \cdot mg^{-1}$ Chl)	
	Light	Dark
$H^{14}CO_3^-$ (6.7 mM) alone	0.15	0.02
$H^{14}CO_3^-$ + 10 mM malate	0.35	–
$H^{14}CO_3^-$ + 5 mM PGA	0.72	–
$H^{14}CO_3^-$ + 3.3 mM DHAP	1.15	0.11
$H^{14}CO_3^-$ + DHAP, malate	1.66	0.40
Malate decarboxylation to pyruvate Additions	Pyruvate ($\mu mol \cdot min^{-1} \cdot mg^{-1}$ Chl)	
	Light	Dark
Malate (10 mM) alone	0.12	–
Malate + 5 mM PGA	1.6	–
Malate + PGA, 5 mM HCO_3^-	1.7	0.24
Malate + 10 mM Asp, 10 mM 2-OG[a]	0.35	0.26
Malate + PGA, HCO_3, Asp, 2-OG[a]	1.7	0.36

[a] With aspartate plus 2-OG added, OAA was produced at a rate of about 1.3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ Chl

Table 3. Preparation of chloroplasts from bundle sheath cells of *F. bidentis*: recovery and intactness. The procedure for preparing bundle sheath cells and chloroplasts is described in *Materials and methods*. Bundle sheath cells were isolated from 10 g of leaf lamina. Chloroplast intactness was determined from the specific activity of the bundle sheath chloroplast marker enzymes NADP-ME and Ru5P kinase

Fraction	Chlorophyll content		Chloroplast intactness (%)
	μg	%	
Original bundle sheath cells	1490	100	–
Bundle sheath cell extract	1180	79	–
Chloroplasts (400·*g* pellet)	350	24	76
Chloroplasts (after Percoll purification)	80	5.4	100

Table 4. Light-dependent O_2 evolution by chloroplasts isolated from bundle sheath cells of *F. bidentis*. All reactions contained the components of the chloroplast isolation medium described in *Materials and methods*. Abbreviations as in Table 1. The data were corrected on the basis that only 80% of the chloroplasts were intact. Data shown are for two sequences of substrate addition with the same chloroplast preparation. They are representative of the results obtained with several different preparations

Sequential additions and activity ($\mu mol\ O_2 \cdot min^{-1} \cdot mg^{-1}$ Chl)			
Light	−0.35	Light	−0.40
HCO_3^- (10 mM)	−0.32	Asp (10 mM)	−0.37
PGA (5 mM)	1.4	2-OG (10 mM)	0.73
Pi (extra 1 mM)	1.6	PGA (5 mM)	1.6
DHAP (2 mM)	1.3	malate (10 mM)	1.4
Dark	−0.3	Dark	−0.4
Light	1.3	Light	1.6

conversion of PGA to 1,3-diphosphoglycerate. When aspartate plus 2-OG was added, OAA was produced at a rate of up to 1.3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ Chl (see Table 2 footnote), and OAA can be reduced in the light at similar rates (O_2-exchange data in Table 1). In view of this we could not explain why adding aspartate with 2-OG did not support rapid rates of pyruvate production from malate in place of PGA (Table 2). Reducing the concentrations of aspartate and 2-OG to 5 mM had no effect. Furthermore, these compounds apparently did not inhibit malate uptake into the chloroplasts since the addition of aspartate plus 2-OG did not inhibit PGA-dependent decarboxylation of malate (Table 2). We also showed that pyruvate was not converted to alanine in these reactions. Both CO_2 fixation and malate decarboxylation showed an absolute requirement for Mg^{2+} (2 mM optimal) but it is interesting that there was no Mg^{2+} requirement for PGA-dependent O_2 evolution.

Preparation of chloroplasts from bundle sheath cells. Isolating intact chloroplast from bundle sheath cells of C_4 plants has proved to be difficult. For some species, modest yields of chloroplasts have been obtained by first isolating protoplasts (Edwards et al. 1979; Moore et al. 1984). There is only one report describing the preparation of chloroplasts from bundle sheath cell strands (from *Z. mays*) but the yields were very low (Jenkins and Boag 1985). During the present studies we developed a procedure for isolating highly intact chloroplasts from *F. bidentis* bundle sheath cell strands in good yields using a procedure involving brief preliminary digestion of cells with a cellulase-pectinase mixture. For the preparation described in Table 3 about 24% of the original cell chlorophyll was recovered in the first 400 · *g* pellet and the chloroplasts in this pellet were 76% intact. Recoveries in other preparations were usually around 25% and chloroplast intactness ranged between 70 and 85%. Chloroplast preparations showing between 90 and 100% intactness could be obtained by centrifuging the resuspended chloroplasts through a layer of medium containing 25% (v/v) Percoll (see *Materials and methods*) (Table 3). However, there was a substantial loss of chloroplasts during this procedure, possibly due to variations in the density of the intact chloroplasts in the first pellet. Chloroplasts prepared by simple differential centrifugation were used for the studies of metabolic activities described below.

Metabolic activities of chloroplasts isolated from bundle sheath cells. Chloroplasts isolated from bundle sheath cells evolved O_2 when either PGA or aspartate plus 2-OG were added (Table 4). As was the case with bundle sheath cells (see Table 1) there was no response to adding HCO_3^- to chloroplasts with or without DHAP but HCO_3^- increased PGA-dependent O_2 (results not shown). Oxygen evolution was only marginally inhibited by adding malate

Table 5. Enzyme activities in mesophyll and bundle sheath cells of *F. bidentis*. Details of the procedure for preparing mesophyll and bundle sheath cell fractions and for calculating the specific activity in mesophyll cells are provided in the *Materials and methods* section. Microscopic examination of bundle sheath cell preparation 1 showed substantial contamination with mesophyll cells. Mesophyll cells were not evident in preparations 2 and 3 obtained by additional blending of the cell strands in preparation 1. Similar leaf and bundle sheath cell activities were obtained in several different experiments

Enzyme	Activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$ Chl)				
	Whole leaf	Mesophyll cell extract	Bundle sheath cell preparations		
			1	2	3
PEP carboxylase	16	30	6.8	1.3	1.3
NADP-MDH	53	88	31	13	11
Aspartate aminotransferase	17	29	10	5.4	5.2
Alanine aminotransferase	25	40	15	10.1	9.6
NADP-ME	13	< 1	20	25	24

Table 6. Enzyme activities associated with chloroplasts isolated from bundle sheath cells of *F. bidentis*. Activities are shown for the 400·*g* chloroplast pellet before and after centrifugation through 25% (v/v) Percoll to remove broken chloroplasts; see *Materials and methods* for details. Very similar results were obtained in two other experiments

Enzyme	Specific activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$ Chl)		
	Bundle sheath cells	Isolated chloroplasts	
		Before Percoll	After Percoll
Ru5P kinase	95	66	96 (1.01)[a]
NADP-ME	27	22	28 (1.05)
NADP-MDH	12.2	8.3	9.5 (0.78)
Aspartate aminotransferase	5.5	2.1	2.7 (0.49)
Alanine aminotransferase	8.4	0.4	0.3 (0.03)

[a] In parentheses is the ratio of the specific activity of the enzymes (on chlorophyll basis) in the post-Percoll chloroplast pellet compared with the specific activity in bundle sheath cell extracts

which, through NADP-ME, could provide an alternative route for NADP reduction.

The rates of O_2 evolution of up to 1.6 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ Chl (Table 4) indicated PSII activity in isolated chloroplasts comparable to that seen with bundle sheath cell preparations. Rates of $^{14}CO_2$ fixation (with PGA or DHAP plus malate) and pyruvate production from malate (with malate plus PGA) recorded with isolated chloroplats were about 1.1 and 0.5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ Chl, respectively (data not shown).

Enzyme activities in mesophyll and bundle sheath cells. Phosphoenolpyruvate carboxylase, normally regarded as a marker enzyme for C_4 mesophyll cells, was substantially more active in mesophyll cell extracts than whole-leaf extracts when expressed on a chlorophyll basis (Table 5). Preparations of bundle sheath strands essentially free of mesophyll cells were obtained by repeated blending of leaf tissue (cell preparations 2 and 3 in Table 5). The PEP carboxylase activity in these preparations was only about 4% of that in mesophyll cells. The fact that this activity declined to a constant low value suggests that bundle sheath cells may contain a small amount of this enzyme or that it is present in the associated vascular tissue. The activity of NADP-ME, considered to be exclusive to bundle sheath cells in NADP-ME-type C_4 species, was much higher in bundle sheath cells compared with leaf extracts (Table 5).

Extracts of *F. bidentis* leaves contained very high activities of NADP-MDH (Table 5). The activity of more than 50 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ Chl is about three times those commonly seen in the NADP-ME-type grass species like *Z. mays* and about 25 times those seen in leaves of C_4 plants from other C_4 sub-groups or from C_3 plants (Hatch et al. 1975; Hatch 1987). Unlike grass species such as *Z. mays* in which essentially all the NADP-MDH activity is confined to mesophyll cells, the bundle sheath cells of *F. bidentis* contained activities greater than 10 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ Chl (Table 5, also see Table 6). While much less than the activity in *F. bidentis* mesophyll cells, this activity is not much lower than that associated with mesophyll cells of NADP-ME-type grasses.

Leaves from *F. bidentis* also contained much higher aspartate and alanine aminotransferase activities than the leaves of C_4 grasses belonging to the NADP-ME-type group (Hatch et al. 1975; Hatch 1987) and, unlike the grass species (Gutierrez et al. 1974), there were substantial amounts of these enzymes in bundle sheath cells (Table 5). Notably, the activity of these enzymes, and also NADP-MDH, in bundle sheath cell equals or exceeds the maximum photosynthesis rates for C_4 species including *F. bidentis* (4–6 $\mu mol\ CO_2 \cdot min^{-1} \cdot mg^{-1}$ Chl, see below and Lunn and Hatch 1995). In an earlier study with *F. trinervia*, substantially lower activities were recorded for these three enzymes in extracts of mesophyll and bundle sheath protoplast (Moore et al. 1984).

Enzyyme activities associated with bundle sheath cell chloroplasts. The proportion of intact chloroplasts in the pre and post-Percoll treatment pellets can be calculated from the ratio of the specific activity of the marker enzymes Ru5P kinase and NADP-ME in chloroplast preparations to that in the original bundle sheath cell extracts. The chloroplasts in the initial 400·*g* pellet were about 75 intact and essentially all the chloroplasts pelleted through Percoll were intact, i.e. the ratio of specific activities was about 1.0 (Table 6). The enzyme NADH-MDH behaved in a similar manner, indicating that most or all of this activity was associated with chloroplasts (confirmed in other experiments showing up to 100% in chloroplasts). This contrasted with alanine aminotransferase which showed essentially no association with chloroplasts. The specific activity of aspartate aminotransferase in the purified chloroplast pellet was about half of that in cell extracts, suggesting that about half of this enzyme is associated with chloroplasts. A very similar result was obtained in two other experiments.

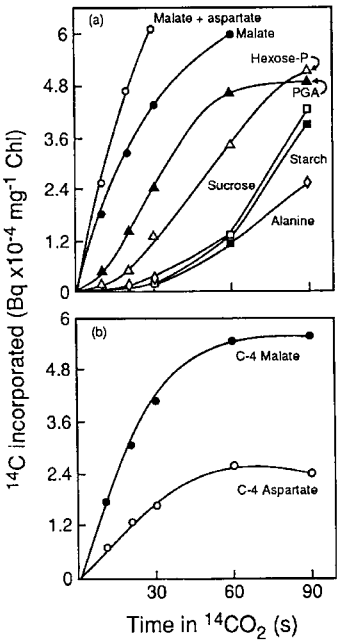

Fig. 1a, b. Time-course labelling of compounds in *F. bidentis* leaves during a pulse in $^{14}CO_2$. Part **b** of the figure shows the kinetics of labelling of the C-4 carboxyl of malate and aspartate. Experiments were conductded under steady-state conditions with incident PAR of 900 μmol quanta·m^{-2}·s^{-1} and a CO_2 concentration of about 390 μl·l^{-1} CO_2. Other details are provided in the *Materials and methods* section. The photosynthesis rate, measured as $^{14}CO_2$ fixed, was 4.7 μmol·min^{-1}·mg^{-1} Chl

Chloroplast thylakoid structure and ratios of chlorophyll a and b. Photosystem II is associated only with the stacked (appressed) regions of the chloroplast thylakoid membranes, i.e. the granal regions (Anderson 1986). In NADP-ME-type C_4 grasses like *Z. mays* and *S. bicolor* the bundle sheath chloroplasts show very little PSII activity (Ku et al. 1974; Chapman et al. 1980), few regions of thylakoid appression and no granal stacking (Woo et al. 1971), and a high ratio of chlorophyll *a* and *b* typical of PSI (Woo et al. 1971; Ku et al. 1974).

From electron micrographs of *F. bidentis* bundle sheath cell chloroplasts we assessed that about 30% of the total thylakoid length was appressed to at least one other adjacent membrane. However, most of this appresssion occurred by pairing of thylakoids and only occasionally were grana-like structures with three to five appressed membranes seen.

Chlorophyll *a* and *b* ratios were determined using the modified and more accurate procedure described by Porra et al. (1989). This procedure gives substantially higher ratios compared with determinations based on the equations of Arnon (1949). For *F. bidentis* the ratios were 5.0 for whole-leaf extracts, 4.4 for mesophyll cells and 5.4 for bundle sheath cells. It should be noted that these ratios would be 3.4, 3.1 and 3.6 when calculated from the Arnon equations (see Porra et al. 1989). For comparison, the ratios we obtained for *Z. mays* leaf extracts and bundle sheath cells were 5.4 and 9.8, respectively. These results are consistent with our conclusion that bundle sheath cells of *F. bidentis* contain much more PSII than NADP-ME-type grass species like *Z. mays*.

Kinetics of labelling from $^{14}CO_2$. The kinetics of $^{14}CO_2$ labelling of C_4 acids and other intermediates in leaves was examined in an attempt to further define the relative roles of malate and aspartate in the photosynthetic metabolism of *F. bidentis*. During a pulse in $^{14}CO_2$ under steady-state conditions a classical C_4 labelling pattern was observed (Fig. 1). The ^{14}C labelling of malate and aspartate was linear from zero time while the labelling of PGA, hexose phosphates and the end-products sucrose and starch only reached maximum rates after increasing time lags. The kinetics of labelling of alanine (Fig. 1a) was comparable to that of carbons 1, 2 and 3 of the C_4 acids malate and aspartate (results not shown) and the amount of ^{14}C incorporated was also similar. Since the radioactivity in carbons 1, 2 and 3 of C_4 acids is likely to rapidly exchange with alanine via pyruvate and PEP we concluded that the alanine pool was comparable in size to the combined pool of malate plus aspartate. From the data quoted below it can be deduced that this pool would be about 1.5 μmol·mg^{-1} Chl. The existence of such a large and dynamically labelled pool would be consistent with a role for alanine in C_4 acid metabolism.

The patterns of labelling of the C-4 carboxyl of malate and aspartate, provide information about the size and likely flux of carbon through these pools. For both these acids the C-4 carbon, which is derived directly from fixed $^{14}CO_2$, approached saturation at about 50–60 s (Fig. 1b). The amount of label in this carbon at saturation indicated that the pool of malate involved in photosynthesis was about 2.3 times that of aspartate. The initial rates of labelling of the C-4 carboxyl was consistent with the flux of carbon through the malate pool also being about two-fold higher. Based on the label in the C-4 carboxyl at saturation and the specific activity of the $^{14}CO_2$ supplied, the active pools of malate and aspartate were calculated to be 1.05 and 0.46 μmol·mg^{-1} Chl, respectively.

The kinetics for the loss of ^{14}C from the C-4 carboxyl of malate and aspartate during a chase in $^{12}CO_2$ (following a 30-s pulse in $^{14}CO_2$) provided more critical information about the flux of carbon through these pools (Fig. 2). In this experiment the ratio of ^{14}C in the C-4 carboxyl of malate and aspartate at the end of the 30-s pulse in $^{14}CO_2$ was about the same (approx. 2.2) as in the pulse experiment shown in Fig. 1. In the following chase ^{14}C was rapidly lost from the C-4 carboxyl of both malate and aspartate (Fig. 2a). Although more label was lost from the larger pool of malate the relative rate of loss from the C-4 of aspartate was higher. This can be more readily seen by plotting the loss of ^{14}C from C-4 as a percentage of that present at the start of the chase (Fig. 2b). This result is not in conflict with our proposal (see below and Fig. 3) that decarboxylation of aspartate occurs in the bundle sheath chloroplasts following conversion to malate; the malate pool in bundle sheath chloroplasts would represent only a small part of the total pool of malate involved in photosynthesis. The fact that ^{14}C is depleted more rapidly from aspartate clearly shows that it is not simply a side pool of the main malate pool that exchanges radiolabel through

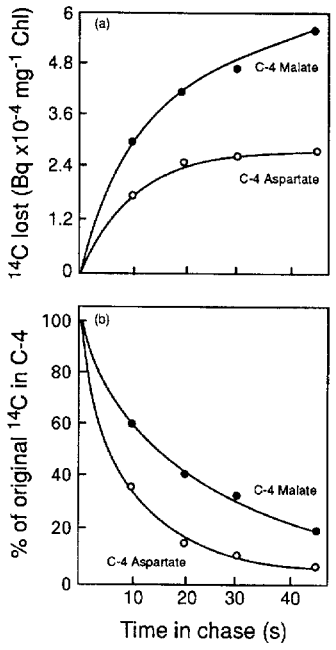

Fig. 2a, b. Kinetics of the loss of ^{14}C from the C-4 carboxyl of malate and aspartate in *F. bidentis* leaves during a chase in $^{12}CO_2$. **a** Total ^{14}C lost from the C-4 carboxyl. **b** Change in ^{14}C expressed as a percentage of that present at the end of the pulse in $^{14}CO_2$. Leaves were initially exposed to a 30-s pulse in $^{14}CO_2$ under steady-state conditions with 900 μmol quanta·m^{-2}·s^{-1} PAR. The figures show the change in radiolabelling of the C-4 carboxyl during a subsequent chase. Photosynthesis, measured as $^{14}CO_2$ fixed in the pulse, was 4.8 μmol·min^{-1}·mg^{-1} Chl

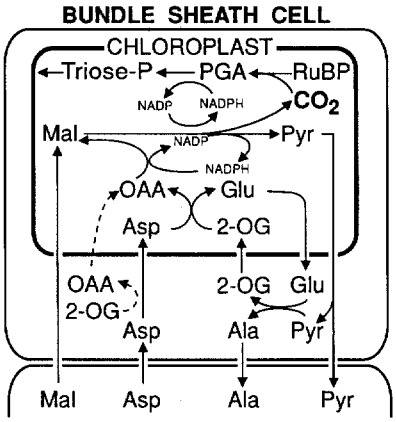

Fig. 3. Proposed paths of malate and aspartate metabolism during photosynthesis in *F. bidentis*. Not shown is the substantial capacity for PSII-mediated NADP reduction in these cells. *Mal*, malate; *Asp*, aspartate, *Pyr*, pyruvate; *Glu*, glutamate; *Ala*, alanine; *RuBP*, ribulose 1,5-bisphosphate

oxaloacetate. If it were, then the percentage loss of ^{14}C from the C-4 of aspartate would always be less than that observed for the C-4 of malate. These C-4 labelling kinetics contrast with those observed for *Z. mays* where ^{14}C in the C-4 of malate declined rapidly from the start of the chase whereas the ^{14}C in the C-4 of aspartate declined more slowly and only after a significant lag (Hatch 1971).

Concluding comments

Our studies with *F. bidentis* show that, compared with grasses from the NADP-ME-type C_4 group, aspartate has a much more important direct role in photosynthetic metabolism. Based on these data we propose the scheme in Fig. 3 to account for C_4 acid metabolism and decarboxylation in *F. bidentis* bundle sheath cells. The scheme proposes that malate derived from mesophyll cells can be directly metabolized in bundle sheath chloroplasts in the same way that it is in the NADP-ME-type C_4 grasses (Hatch 1987). Malate is metabolized via NADP-ME, generating CO_2 and NADPH. In addition, we propose that aspartate may be metabolized through OAA and then malate in a sequence of reactions involving aspartate aminotransferase, NADP-MDH and then NADP-ME (Fig. 3). We showed that all these enzymes are present in *F. bidentis* bundle sheath cells at levels greater than the maximum photosynthesis rates recorded for this species of about 5 μmol CO_2·min^{-1}·mg^{-1} Chl. Significantly, only about half the aspartate aminotransferase was located in chloroplasts so that if the remaining component contributes then OAA as well as aspartate must be transported into chloroplasts (see Fig. 3). The high rates of aspartate plus 2-OG-dependent O_2 evolution observed with isolated bundle sheath cells and chloroplasts provided additional support for the view that aspartate could contribute to carbon flux in those cells. These rates of O_2 evolution were similar to those observed with PGA plus HCO_3^-.

In other groups of C_4 species that actively metabolize aspartate, the leaves invariably contain high activities of both aspartate aminotransferase and alanine aminotransferase in both mesophyll and bundle sheath cells (Hatch 1987). The alanine aminotransferase functions to regenerate 2-OG from glutamate through the coupled conversion of pyruvate to alanine. We showed high alanine aminotransferase activities in both mesophyll and bundle sheath cells in *F. bidentis* and a similar role for this enzyme is proposed (Fig. 3). Notably, the kinetics of labelling from $^{14}CO_2$ indicated that the alanine pool was large with a rapid turnover.

The analysis of the kinetics of labelling of C_4 acids and other compounds from $^{14}CO_2$ provided additional support for the scheme outlined in Fig. 3. Particularly critical was the evidence that the relative rate of loss of ^{14}C from the C-4 carboxyl during a chase in $^{12}CO_2$ was faster from aspartate than from malate (Fig. 2b). This clearly shows that carbon fluxes independently through the aspartate pool. However, the malate pool was about twice the size of that of aspartate so that more carbon (measured as ^{14}C) was transferred from the C-4 of malate in the chase (Fig. 2a). Based on the initial rates of loss of ^{14}C from the C-4 carboxyl, aspartate apparently contributed between 35 and 40% of the carbon fluxing through the C-4 carboxyl of C_4 acids. With a net photosynthesis rate in this particular experiment of 4.8 μmol·min^{-1}·mg^{-1} Chl this would be equivalent to a turnover through the aspartate pool of about 1.8 μmol·min^{-1}·mg^{-1} Chl.

As indicated in the *Introduction*, to the extent that aspartate replaces malate as the source of of CO_2 in bundle sheath cells of NADP-ME-type species there will be a decrease in the reducing equivalents transferred to

these cells. Our results support the view proposed earlier (Repo and Hatch 1976; Chapman and Hatch 1981) that as the role of malate in CO_2 transfer declines the PSII activity of bundle sheath cells may be increased to compensate.

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