EVIDENCE FOR REASSIMILATION OF CO_2 RELEASED FROM C_4 ACIDS BY PEP CARBOXYLASE IN LEAVES OF C_4 PLANTS

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1. Introduction

In recent years, mesophyll and bundle sheath cells isolated from leaves of C_4 plants have been extensively used in elucidating the photosynthetic control mechanisms operating in these plants for maintaining high rates of photosynthesis [1, 2–5]. Since the currently proposed formulations for the C_4 pathway of photosynthesis require a strong coordinated function of the two cell types [1,6], any predictions made from studies with the isolated cell types or organelles may not reflect the true in vivo situation. We believe that the use of thin leaf slices should certainly bridge this gap between whole leaf and isolated cell or organelle studies and provide further insight into the mechanism and function of C_4 photosynthesis [7].

A relatively strict stoichiometry between C_4 acid synthesis, C_4 acid decarboxylation and refixation of the released CO_2 is essential for efficient photosynthesis [1]. In spite of greater stomatal resistance for gaseous diffusion [8,9], the observed activities of PEP carboxylase with very high affinity for HCO_3^- constitues a strong sink for atmospheric CO_2 [1]. The CO_2 concentration at the mesophyll cell surface was estimated to be about 1 μM [1,7]. The minimal estimate of CO_2 present in the bundle sheath cells during active C_4 acid decarboxylation was approx. 25 μM [1,7]. Currently no significant

Abbreviations: PEP, phosphoenolpyruvate; RuDP, ribulose 1,5-diphosphate

back-flux of CO_2 from bundle sheath to mesophyll cells has been warranted in spite of the existence of a large difference in CO_2 concentrations and a high frequency of plasmodesmata between the two cell types [1,7].

It is further perplexing to note that the measured K_m (CO_2) (45 μM) of the high-affinity RuDP carboxylase from C_4 plants was twice that of the C_3 plants, and the C_4 RuDP carboxylase was further characterized by a V_{max} which is only half that of C_3 RuDP carboxylase [10]. The refixation of released CO_2 through RuDP carboxylase thus becomes a key strategic step during the C_4 pathway of photosynthesis. The present paper reports the occurrence of a back-flux into mesophyll cells of CO_2 released during C_4 acid decarboxylation in bundle sheath cells, but an ultimate outward diffusion of the CO_2 into the atmosphere was restricted due to an efficient reassimilation by PEP carboxylase.

Materials and methods

Bundle sheath strands were enzymatically isolated from leaves of the C_4 plants (2–3 week old) *Digitaria sanguinalis* (NADP-malic enzyme type), *Panicum miliaceum* (NAD-malic enzyme type) and *Eriochloa borumensis* (PEP carboxykinase type) as previously described [3,11]. Leaf slices, 0.5 mm width, were obtained by cutting the leaves in the leaf-cutter (described by Huber and Edwards, [12]) fitted with a sharp razor blade. The strands and leaf slices were suspended in 50 mM Hepes (pH 7.6) containing 0.3 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM KH_2PO_4. Decar-

boxylation experiments were run in sealed ampules as previously described [3–5]. The assays were run with a reaction mixture containing, 0.3 M sorbitol, 50 mM Tricine–KOH (pH 8.0), 1 mM $MgCl_2$, 2 mM KH_2PO_4 and 3 mM sodium isoascorbate. The reactions were initiated unless otherwise mentioned, by adding L-[4-^{14}C]aspartate + α-ketoglutarate or L-[4-^{14}C]malate + phospho-3-glyceric acid (for details see ref. [4]) The released CO_2 was trapped into 0.2 ml 1.0 M hyamine hydroxide placed in a centrally suspended one half of a gelatin capsule (No. O, Eli Lilly Co., Indianapolis, USA) [3–5]. Other additions and conditions are described in the text. The conditions used for studies with leaf slices were at their optimum; and complete details of the characteristics of photosynthesis by C_4 leaf slices will be reported elsewhere (in preparation). The activities are expressed on the basis of chlorophyll present in the bundle sheath strands or leaf slices. The rates with the strands are generally double those of leaf slices, as the decarboxylations, being exclusively confined to the bundle sheath cells [1,2–5] are expected to be about two-fold higher in activity on a Chl basis than in whole leaves.

3. Results and discussion

During the C_4 pathway of photosynthesis, the decarboxylation of C_4 acids in the leaf bundle sheath cells is facilitated, depending upon the species, by specific decarboxylases: NADP-malic enzyme, NAD-malic enzyme and PEP carboxykinase; and consequently the C_4 plants are subclassified on the basis of their major decarboxylase. We have presented evidence that malate decarboxylation in leaves of *D. sanguinalis,* a malate-former, proceeds through NADP-malic enzyme in the light with no decarboxylation in the dark [2,4,5]. Aspartate decarboxylation through NAD-malic enzyme in *P. miliaceum,* an aspartate-former, has been shown to be light-independent as there was no net energy requirement due to a stoichiometric balancing of pyridine nucleotide oxidation–reduction [2,4,5]. Recent evidence indicates that light-independent decarboxylation of aspartate (20–25% of total) in the leaves of *E. borumensis,* an aspartate former, is mediated through its residual NAD-malic enzyme system, while 75–80% of aspartate decarboxylation is catalyzed by its ATP-dependent PEP carboxykinase in a light-dependent manner [2–5].

The results presented in table 1 demonstrate the capacity of isolated bundle sheath strands and narrow leaf slices of *D. sanguinalis* for L-[4-^{14}C]malate decarboxylation during preferential inhibition of mesophyll and bundle sheath carboxylations. In studies with isolated bundle sheath strands, the site of both C_4 acid decarboxylation and RuDP carboxylation [1,2–5], it was found necessary to inhibit the refixation of the CO_2 released from C_4 acids for estimating the true in vivo potential for C_4 acid decarboxylation (for complete details see ref. [3,4]). It is also clear from table 1 that

Table 1
Effect of inhibitors of mesophyll and bundle sheath metabolism on
L-[4-^{14}C]malate decarboxylation by *D. sanguinalis* in light

Conditions	Rate of decarboxylations (μmol/mg Chl/h)[a]	
	Bundle sheath strands	Leaf slices
Malate	100	0
Malate + glyceraldehyde	406	0
Malate + oxalate	0	0
Malate + maleate	94	0
Malate + glyceraldehyde + maleate	319	224

[a]See ref. [5] for assay details

Substrate concentrations used were 10 mM L-[4-^{14}C]malate, 50 mM D,L-glyceraldehyde; 0.4 mM oxalate and 4 mM maleate. There was no significant malate decarboxylation in the dark

the apparent rate of malate decarboxylation by bundle sheath strands of *D. sanguinalis* was very low. However, there was a four-fold stimulation in the light-dependent malate decarboxylation activity, as measured by the release of $^{14}CO_2$, when D, L-glyceraldehyde, a specific inhibitor of ribulose-5-phosphate kinase [13], was included in the reaction mixtures. This apparent stimulation was due to the prevention of the refixation of the $^{14}CO_2$ liberated from malate into the Calvin cycle. Oxalate, a specific inhibitor of NADP-malic enzyme [4,5] completely blocked malate decarboxylation, indicated the involvement of NADP-malic enzyme. The addition of maleate, a potent inhibitor of PEP carboxylase [7,15], had no effect. Under similar conditions, leaf slices fed with malate did not release $^{14}CO_2$ even in the presence of D,L-glyceraldehyde. In the absence of a functional Calvin cycle the $^{14}CO_2$ released should ultimately diffuse into the atmosphere unless it is reassimilated by the PEP carboxylase localized in the mesophyll cells. It was found to be the case indeed since the $^{14}CO_2$ released during malate decarboxylation was readily detected when glyceraldehyde was added together with maleate, i.e., $^{14}CO_2$ liberated in the bundle sheath cells during C_4 acid decarboxylation eventually escapes out of the leaf.

A four-fold stimulation by glyceraldehyde of L-[4-^{14}C]aspartate decarboxylation in isolated bundle sheath strands of *P. miliaceum* was observed in the light, the rates being comparable to the rates in dark (table 2). As there is no net energy requirement for aspartate decarboxylation through the NAD-malic enzyme system, it was not surprising that the rate of decarboxylation was similar in light and dark. As indicated earlier, the observed apparently low rates of aspartate decarboxylation in the absence of glyceraldehyde in light was presumably due to the immediate refixation of the released $^{14}CO_2$ by the Calvin cycle.

In experiments with leaf slices, no significant release of $^{14}CO_2$ was detected in the light (in the presence of either glyceraldehyde or maleate) unless both glyceraldehyde and maleate were added together (table 2). However, $^{14}CO_2$ was readily released from leaf slices fed with aspartate in the dark as there was no functional Calvin cycle or PEP carboxylation, due to a lack of photochemical production of ATP, and NADPH, required to refix the $^{14}CO_2$.

The rate of L-[4-^{14}C]aspartate decarboxylation by bundle sheath strands of *E. borumensis* was stimulated six-fold in light by glyceraldehyde with no effect on the dark rates (table 3). 3-Mercaptopicolinic acid, a specific inhibitor of PEP carboxykinase [3,4,16], abolished the light-dependent rates with no effect on the dark rates, thus indicating that the decarboxylation in light was catalyzed by the ATP-dependent PEP carboxykinase. Maleate and/or glyceraldehyde had no inhibitory effect on the decarboxylation activities of the isolated strands. Evidence was presented earlier that the light-independent decarboxylation of aspartate is mediated by their NAD-malic enzyme system [3].

Unless both glyceraldehyde and maleate were added together, the leaf slices of *E. borumensis* fed with

Table 2
Effect of inhibitors of mesophyll and bundle sheath metabolism on L-[4-^{14}C]aspartate decarboxylation by *P. miliaceum*

Conditions	Rate of decarboxylation (μmol/mg Chl/h)[a]			
	Bundle sheath strands		Leaf slices	
	Light	Dark	Light	Dark
Aspartate	82	287	0	163
Aspartate + glyceraldehyde	327	294	0	181
Aspartate + maleate	90	281	12	141
Aspartate + glyceraldehyde + maleate	284	323	143	149

[a]Assay conditions were as described in Materials and methods

Substrate concentrations used were as those of table 1: L-[4-^{14}C]aspartate, 10 mM

Table 3

Effect of inhibitors of mesophyll and bundle sheath metabolism on L-[4-^{14}C]aspartate decarboxylation by *E. borumensis*

Conditions	Rate of decarboxylation (μmol/mg Chl/h)[a]			
	Bundle sheath strands		Leaf slices	
	Light	Dark	Light	Dark
Aspartate	58	77	0	39
Aspartate + glyceraldehyde	369	94	0	48
Aspartate + 3-MPA	96	72	0	37
Aspartate + maleate	50	79	0	56
Aspartate + glyceraldehyde + maleate	352	96	140	54

[a]Experimental conditions were as described in Materials and methods

Substrate concentrations used were as those of table 1; 3-mercaptopicolinic acid (3-MPA), 0.4 mM. 3-MPA was a generous gift from Dr H. L. Saunders, Smith Kline and French Labs, Philadelphia, PA

aspartate did not release any $^{14}CO_2$ in the presence of either of the two compounds in light (table 3).

The simplest interpretation of these results (tables 1–3) is that a back-flux of some of the CO_2 liberated in the bundle sheath cells during C_4 acid decarboxylation indeed occurs into the adjacent mesophyll cells. The CO_2, in spite of a steep concentration gradient, possibly could not escape out of the leaf due to an apparent reassimilation by PEP carboxylase, aided further by high stomatal resistance for gaseous exchange together with low cytoplasmic resistance for CO_2 diffusion and a high conduction of internal CO_2 diffusion [8,9]. The presence of higher internally exposed cell surface per unit cell volume in C_4 plants [8,9] may also expedite this reassimilation process.

The back-flux of endogenous CO_2 could be due to a lack of requisite stoichiometry between C_4 acid decarboxylation and subsequent refixation of the released CO_2. Even if strict stoichiometry exists, any photorespiratory release of CO_2 [17,18] might result in an imbalance of this ratio. The C_4 RuDP carboxylase, characterized with a low V_{max} and a high K_m (CO_2) [10], may in part, allow a backward diffusion of excess CO_2 to the adjacent mesophyll cells for fast reassimilation. The apparent lack of photorespiration in C_4 plants is, therefore, not only due to the maintenance of a high CO_2 concentration at the site of RuDP

carboxylase, but also to the fast reassimilation of the endogenously produced CO_2. Any absolutely quantitative measurement of such internal recycling in vivo is not yet possible. However, our assumptions of internal recycling of CO_2 may not be far from correct. To our knowledge, this is the first evidence for the fast reassimilation of endogenously liberated CO_2 by the PEP carboxylase localized in the mesophyll cells.

References

[1] Hatch, M. D. and Osmond, C. B. (1976) in: Encyclopedia of Plant Physiology (New Series), (Herber, U. and Stocking, C. R. eds.) Vol. 3, pp. 144–184, Springer-Verlag, Berlin.
[2] Rathnam, C. K. M. and Edwards, G. E. (1975) Arch. Biochem. Biophys. 171, 214–225.
[3] Rathnam, C. K. M. and Edwards, G. E. (1977) Planta 133, 135–144.
[4] Rathnam, C. K. M. and Edwards, G. E. (1977) Plant Cell Physiol. 182, 1–13.
[5] Rathnam, C. K. M. and Edwards, G. E. (1977) Arch. Biochem. Biophys. in press.
[6] Osmond, C. B. and Smith, F. A. (1976) in: Intercellular Communication in Plants: Studies on Plasmodesmata. (Gunning, B. E. S. and Robards, A. W. eds) pp. 229–241, Springer-Verlag, Berlin.
[7] Rathnam, C. K. M. (1977) Plant Physiol. 59 sup., Abs. 366b.
[8] El-Sharkawy, M. A. and Hesketh, J. (1965) Crop Sci. 5, 517–521.

[9] El-Sharkawy, M. A., Loomis, R. S. and Williams, W. A. (1976) Physiol. Plant. 20, 171−186.

[10] Badger, J. R., Andrews, T. J. and Osmond, C. B. (1975) in: Proc. 3rd Int. Cong. Photosynthesis.(Avron, M. ed.) pp. 1421−1429, Elsevier, Amsterdam.

[11] Kanai, R. and Edwards, G. E. (1973) Plant Physiol. 51, 1133−1137.

[12] Huber, S. C. and Edwards, G. E. (1975) Physiol. Planta. 35, 203−209.

[13] Bamberger, E. S. and Avron, M. (1975) Plant Physiol. 56, 481−485.

[14] Sarawek, S. and Davies, D. D. (1976) Phytochem. 15, 479−481.

[15] Bhagvat, A. S. and Sane, P. V. (1976) Ind. J. Exp. Biol. 14, 155−158.

[16] Ray, T. B. and Black, C. C. (1976) J. Biol. Chem. 251, 5824−5826.

[17] Chollet, R. (1974) Arch. Biochem. Biophys. 163, 521−529.

[18] Rathnam, C. K. M. and Das, V. S. R. (1974) Ind. J. Biochem. Biophys. 11, 303−306.