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Low Bundle Sheath Carbonic Anhydrase Is Apparently Essential for Effective C_4 Pathway Operation

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ABSTRACT

Bundle sheath cells from leaves of a variety of C_4 species contained little or no carbonic anhydrase activity. The proportion of total leaf carbonic anhydrase in extracts of bundle sheath cells closely reflected the apparent mesophyll cell contamination of bundle sheath cell extracts as measured by the proportion of the mesophyll cell marker enzymes phosphoenolpyruvate carboxylase and pyruvate,Pi dikinase. Values of about 1% or less of the total leaf activity were obtained for all three enzymes. The recorded bundle sheath carbonic anhydrase activity was compared with a calculated upper limit of carbonic anhydrase activity that would still permit efficient functioning of the C_4 pathway; that is, a carbonic anhydrase level allowing a sufficiently high steady state [CO_2] to suppress photorespiration. Even before correcting for mesophyll cell contamination the activity in bundle sheath cell extracts was substantially less than the calculated upper limit of carbonic anhydrase activity consistent with effective C_4 function. The results accord with the notion that a deficiency of carbonic anhydrase in bundle sheath cells is vital for the efficient operation of the C_4 pathway.

The concentrating of inorganic carbon in bundle sheath cells is the key function of the unique reactions of the C_4 pathway (13). The prime purpose of this process is to favor the carboxylation reaction catalyzed by Rubisco[1] at the expense of oxygenase activity, thereby eliminating photorespiration. Since CO_2 as such is the substrate for Rubisco (5), and since it is the ratio of CO_2 to O_2 that determines the relative rates of carboxylase and oxygenase activities (1), it is the concentration of the CO_2 component of the inorganic carbon which is critical. Recently, we reported that CO_2 is the product of each of the three decarboxylase reactions operating in C_4 plants (15). Based on the assumption that CO_2 is both the product of decarboxylation in bundle sheath cells and the substrate for Rubisco a model was developed which predicted that the inorganic carbon pool generated during steady state photosynthesis would exist predominantly as CO_2 (8). That is, a steady state situation could exist where the relative concentrations of CO_2 and HCO_3^- remain far removed from thermodynamic equilibrium.

In this model another key assumption for such a steady state where CO_2 is the predominant inorganic carbon species was that bundle sheath cells contain little or no carbonic anhydrase. In fact, there is some evidence that the carbonic anhydrase activity in leaves of C_4 plants is at least largely associated with mesophyll cells (9, 16). Carbonic anhydrase would be essential in mesophyll cells to sustain rapid conversion of CO_2 entering the cell to bicarbonate, the substrate for PEP carboxylase (15). However, these studies did not exclude the possibility of bundle sheath cells containing in the region of 2 to 10% of the total leaf carbonic anhydrase which still represents relatively high activity in absolute terms. The purpose of the present paper was to determine the likely upper limit of carbonic anhydrase activity in bundle sheath cells. This activity is compared with an estimate of the upper level of carbonic anhydrase activity in bundle sheath cells that would still permit effective functioning of the C_4 pathway.

[1] Abbreviations: Rubisco, ribulose 1,5-bisphosphate carboxylase-oxygenase; PEP, phosphoenolpyruvate.

MATERIALS AND METHODS

Materials. Plants were grown in soil in a naturally illuminated glasshouse maintained between 20 and 30°C. The species examined were *Urochloa panicoides, Chloris gayana, Panicum miliaceum, Atriplex spongiosa, Sorghum vulgare*, and *Zea mays*. Biochemicals and reagent enzymes were obtained from either Sigma Chemical Co. or Boehringer-Mannheim (Australia). Solutions of carbon dioxide were prepared as previously described (15).

Preparation of Bundle Sheath Cell Strands. The basic procedure for preparing bundle sheath cell strands was very similar to that described previously (2). Except where otherwise specified, 15 g of deribbed leaf tissue was thinly sliced with a razor blade and then blended in a Sorvall Omnimixer in 100 ml of an ice-cold solution containing 0.3 M sorbitol, 50 mM Hepes-KOH (pH 7.5), 5 mM $MgCl_2$, 2 mM Pi, 5 mM dithiothreitol, 0.5% (w/v) BSA, and 0.2% (w/v) sodium isoascorbate. The tissue was blended in bursts of about 10 s at 40% of line voltage until microscopic examination of the homogenate showed that most of the bundle sheath cells occurred in strands free of mesophyll cells. Filtration of this homogenate through 1 mm and then 0.65 mm nylon nets further purified the preparation with respect to tissue containing contaminating mesophyll cells. For the studies described in Table II the strands recovered through the 0.65 mm net were collected by filtration on Miracloth and then subjected to a further blending for bursts of 10 s at 30% of line voltage followed by microscopic examination. The bundle sheath cell preparations, free of mesophyll cells, were collected and extracted as described below.

Preparation of Leaf and Cell Extracts. Deribbed leaf tissue or bundle sheath cell strands were extracted by grinding the tissue in a cold mortar with a solution containing 50 mM Hepes-KOH (pH 7.5), 2 mM Pi, 10 mM $MgCl_2$, and 5 mM dithiothreitol. The homogenate was filtered through Miracloth, sampled for determination of Chl (20), and then centrifuged at 10,000*g* for 2 min to remove particulate material.

Assay of Enzymes. Pyruvate,Pi dikinase (4), PEP carboxylase (12), NADP-malic enzyme (11), NAD malic enzyme (14), and PEP carboxykinase (10) were activated, where required, and then assayed as previously described.

Carbonic anhydrase was assayed at 0°C in a 1 ml reaction containing 20 mM barbitone buffer pH 8.0 and 50 mM CO_2 by following the change of pH with an electrode attached to a chart recorder. The rate of CO_2 hydration was determined from the

slope of the trace in the region of pH 7.9 to 7.6 following the addition of a CO_2 solution. A correction was applied for the nonenzymic rate of CO_2 hydration determined in a control reaction run in the absence of added enzyme.

Carbonic Anhydrase Units. Carbonic anhydrase activity, determined as described above, was transformed to the units of μmol CO_2 hydrated min^{-1} at 25°C as follows. The observed change of pH was transformed to equivalent μmol of H^+ by titration of a sample of the standard reaction mixture with HCl. Rates were then converted to equivalent activity at 25°C using the temperature coefficient (Q_{10}) of 1.9 determined in the present study for maize leaf carbonic anhydrase (see Fig. 3).

RESULTS

Leaf extracts of six C_4 species (listed in Table II) contained carbonic anhydrase activities comparable to those found in the leaves of C_3 plants (Table I). Bundle sheath cell strands prepared from leaves of various C_4 plants by a routine procedure involving a single extensive blending (see "Materials and Methods," also Ref. 2) contained between 1.5 and 5% of the total leaf carbonic anhydrase activity (results not shown). Notably, these extracts contained about the same proportion of the total leaf PEP carboxylase and pyruvate,Pi dikinase activities, enzymes generally taken as quantitative markers for mesophyll cells (6, 13).

To further assess the significance of the low levels of carbonic anhydrase detected in bundle sheath cell preparations we followed the activity of this enzyme during successive blending treatments (Fig. 1). Leaf tissue from *U. panicoides* was subjected to a brief blending treatment (two 10 s periods in a Sorvall blender at 40% of line voltage) and the homogenate was filtered on a fine nylon net (see legend, Fig. 1) to remove most of the larger pieces of tissue. The filtrate contained material enriched in single strands of bundle sheath cells. A sample of this tissue was extracted by grinding in a mortar. PEP carboxylase and carbonic anhydrase activities in this extract were between 7 and 8% of the whole leaf activity. The partially purified strands were then reprocessed through a series of identical blending and filtering steps. The activity of both enzymes declined in parallel through to the fourth repetition. At this point carbonic anhydrase and PEP carboxylase activities were less than 1% of the activity in whole leaf extracts (Fig. 1).

Table I. *Carbonic Anhydrase Activities of Leaf Extracts of C_3 and C_4 Plants*

Species	Carbonic anhydrase[a]
	µmol min^{-1} mg^{-1} Chl
C_3 plants	
Spinach	3500
Wheat	1100
Barley	700
C_4 plants	
Six species[b]	830–1300

[a] Corrected to give activity at 25°C, see "Materials and Methods." [b] Range of activities for the six C_4 species listed in Table II.

On the basis of the study described in Figure 1 bundle sheath cell strands were prepared from leaves of several C_4 species by a modified procedure incorporating a repetition of the blending and filtering steps (see "Materials and Methods"). For NAD-malic enzyme-type and PEP carboxykinase-type species the carbonic anhydrase activities in extracts of these bundle sheath cells ranged between 0.5 and 0.8% of the activities recorded in leaf extracts (Table II). It should be noted that the bundle sheath cells contain only part of the leaf Chl (varies between about 40 and 70%; [17]). The data in Table II has not been corrected to compensate for this. The bundle sheath cell extracts contained very similar low proportions of the mesophyll marker enzymes pyruvate,Pi dikinase and PEP carboxylase. For NADP-malic enzyme-type species slightly higher values were obtained for both carbonic anhydrase and the mesophyll marker enzymes. Notably, the activity of the C_4 acid decarboxylating enzymes typical of each subgroup of species was between 1.5- and 2.4-fold higher in bundle sheath cell extracts compared with leaf extracts. These enzymes are presumed to be very largely or totally confined to bundle sheath cells (13).

Arguments in the following discussion depend on an assessment of the maximum potential carbonic anhydrase activity in bundle sheath cells. This calculation requires information about the K_m for CO_2 and the effect of varying temperature on the C_4 leaf enzyme. The K_m CO_2 for the maize leaf enzyme, determined from a Lineweaver-Burk plot, was about 8.7 mM (Fig. 2). Figure

Table II. *Carbonic Anhydrase and Cell Marker Enzyme Activities in Leaves and Bundle Sheath Cells of C_4 Plants*

Bundle sheath cell (BSC) strands were prepared by a modified procedure including repeated blending described in "Materials and Methods."

Species and C_4 Type	Enzyme Activity[a]							
	Carbonic anhydrase[b]		PEP carboxylase		Pyruvate,Pi dikinase		C_4 acid decarboxylase	
	Leaf	BSC	Leaf	BSC	Leaf	BSC	Leaf	BSC
	µmol min^{-1} mg^{-1} Chl							
PCK-Type							PEP carboxykinase	
U. panicoides	1280	7.7 (0.6%)	25	0.16 (0.6%)	2.7	0.02 (0.7%)	6.3	10.2
C. gayana	1060	5.9 (0.5%)	27	0.17 (0.6%)	2.1	0.02 (1%)	9.2	14.4
NAD-ME-Type							NAD malic enzyme	
P. miliaceum	1090	7.4 (0.7%)	23	0.16 (0.7%)	3.7	0.03 (0.8%)	5.8	12.3
A. spongiosa	1070	8.3 (0.8%)	21	0.17 (0.8%)	4.4	0.04 (0.9%)	9.4	15.8
NADP-ME-Type							NADP malic enzyme	
S. vulgare	860	15 (1.8%)	23	0.38 (1.6%)	4.1	0.06 (1.5%)	4.8	11.8
Z. mays	830	13 (1.6%)	20	0.30 (1.5%)	6.5	0.09 (1.4%)	5.9	13.7

[a] Bundle sheath cell activity as a percentage of the whole leaf activity is shown in parentheses. [b] Carbonic anhydrase activities are calculated values for 25°C (4.8 times the activity recorded at 0°C; see "Materials and Methods").

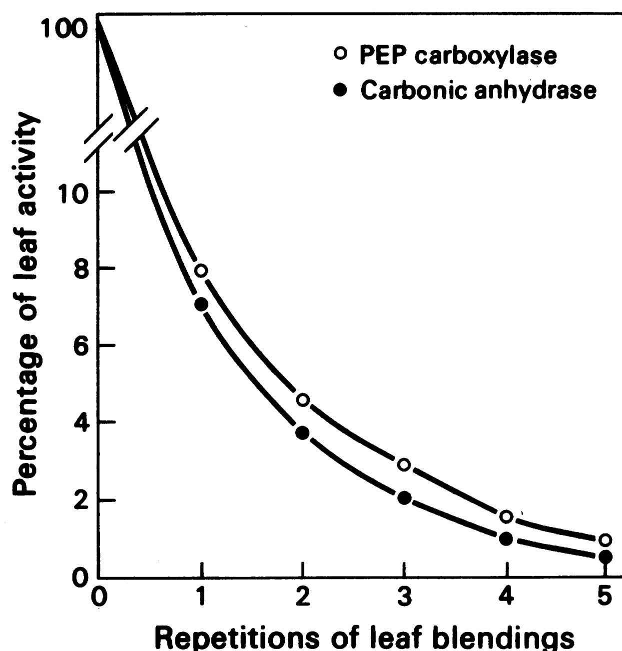

FIG. 1. Effect of repetitive blending on the carbonic anhydrase activity associated with isolated bundle sheath cell strands of *U. panicoides*. The tissue blending and filtering procedure was modified from that described in "Materials and Methods." Tissue was initially blended for two periods of 10 s at 40% of line voltage and then filtered on a 0.65 mm net. A sample of the recovered bundle sheath cell strands was extracted and then the remaining tissue reblended and filtered as described above. This procedure was repeated three more times.

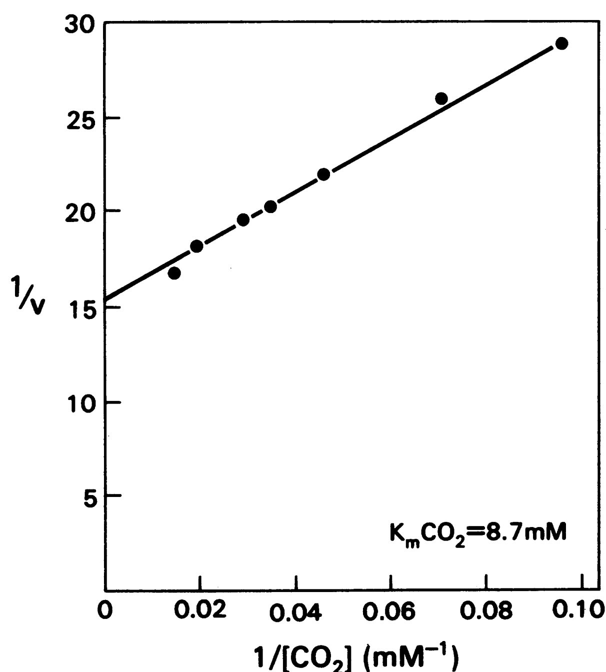

FIG. 2. Lineweaver-Burk plot of the effect of varying [CO_2] on maize leaf carbonic anhydrase activity. The temperature was 0°C. See "Materials and Methods" for other details.

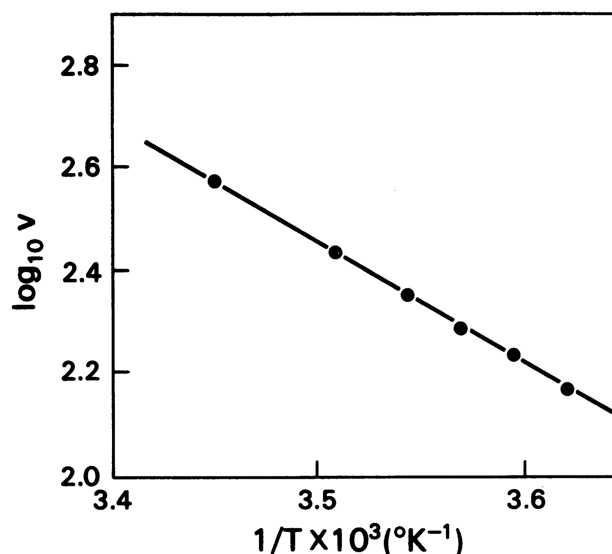

FIG. 3. Effect of temperature on maize leaf carbonic anhydrase activity. Data for temperatures varying between 0 and 17°C are presented as an Arrhenius plot.

3 shows an Arrhenius plot for the effect of varying temperature in the range from 0 to 17°C on maize leaf carbonic anhydrase activity. The Q_{10} derived from this plot was about 1.9.

DISCUSSION

The prime advantages offered by the C_4 pathway would almost certainly be negated by the presence of high levels of carbonic anhydrase in bundle sheath cells (see Ref. 8 and "Introduction" above). Such activity would lead to HCO_3^- rather than CO_2 being the predominant inorganic carbon species. For instance, at pH 8 (a likely stromal pH in the light) the HCO_3^- concentration would be 50 times the CO_2 concentration at equilibrium (19). In the following discussion we make a calculation of the upper limit of bundle sheath carbonic anhydrase activity that would be compatible with maintaining effective functioning of the C_4 pathway. In essence, we wish to determine the level of carbonic anhydrase that would still allow development of a sufficient concentration of CO_2 to effectively suppress Rubisco-mediated oxygenase activity and accompanying photorespiration. These calculations will show that this limit is in excess of the uncorrected upper limits of carbonic anhydrase assayed in bundle sheath cell extracts.

An estimate of the minimum bundle sheath CO_2 concentration compatible with the efficient operation of the C_4 pathway can be calculated from the equations developed by Laing *et al.* (18) and discussed by Andrews and Lorimer (1). For this calculation we will assume the worst case of a large accumulation of O_2 in bundle sheath cells during photosynthesis (7). With limited gas diffusion between mesophyll and bundle sheath cells the steady state concentration of O_2 during photosynthesis could be up to four times the atmospheric concentration (calculation based on a diffusion coefficient in water of 2.9×10^{-5} cm^{-2} s^{-1} and assumptions used in the model developed by Furbank and Hatch [8]).Thus the oxygen concentration may be as high as 1000 μM in solution at 25°C. Of course, NADP-malic enzyme-type C_4 species would not accumulate O_2 since there is little photosynthetic O_2 evolution in bundle sheath cells (Chapman *et al.* [3]). We will take as a base for effective C_4 pathway operation (suppression of photorespiration) a ratio of photosynthetic CO_2 assimilation to photorespiratory CO_2 production of 30 or greater. This is equivalent to a ratio of photosynthetic CO_2 assimilation to oxygenase mediated O_2 uptake of 15. The CO_2 concentration

needed to meet this requirement can be calculated as follows. In the equation

$$\frac{v_c}{v_o} = S_{rel}\frac{[CO_2]}{[O_2]}$$

v_c and v_o are the carboxylation velocity and oxygenation velocity, respectively and S_{rel} can be taken as the proportionality constant specifying the ratio of carboxylation to oxygenation activities (1). This equation was developed by Laing *et al.* (18) and the term S_{rel} is equivalent to their term V_cK_o/V_oK_c. Assuming an S_{rel} value for higher plant Rubisco of about 100 (1), then it can be calculated that with the above assumption $[CO_2]$ would have to be 15% of $[O_2]$ or more. Hence, with an $[O_2]$ of 1000 μM, a $[CO_2]$ of 150 μM or more would be required.

We now proceed to calculate the upper permissible level of carbonic anhydrase in bundle sheath cells giving a steady state CO_2 concentration of at least 150 μM. For this we take a bundle sheath cell inorganic carbon pool of 55 nmol mg^{-1} Chl together with the other assumptions used in an earlier study (8) to develop the model for predicting the bundle sheath cell inorganic carbon status. The only exception is that a pH of 8.0 instead of 7.0 is assumed and, accordingly, different rate constants for the reaction $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$ are taken (4.5×10^{-2} s^{-1} for $CO_2 \rightarrow HCO_3^-$ and 1.2×10^{-3} s^{-1} for $HCO_3^- \rightarrow CO_2$; [7]).

Our model (8) predicts that CO_2 would be the predominant inorganic carbon species if bundle sheath cells contain no carbonic anhydrase ($[CO_2]$ is 550 μM with an inorganic carbon pool of 55 nmol mg^{-1} Chl) but that the proportion of CO_2 would decline as carbonic anhydrase increases. Using the empirical procedure described earlier (8) we can calculate the increase in the rate constants of the reaction $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$ that would reduce the steady state $[CO_2]$ to 150 μM. The value obtained is an increase of about 43 times over the rate constants for the noncatalysed reaction. With this rate constant and 150 μM CO_2 the rate of hydration of CO_2 to HCO_3^- in bundle sheath cells would be about 0.78 μmol min^{-1} mg^{-1} Chl at 25°C. Assuming a K_m CO_2 of 8.7 mM (from Fig. 2) this is equivalent to a maximum velocity with saturating CO_2 of 45 μmol min^{-1} mg^{-1} Chl calculated from the equation $v = V[S]/(K_m + [S])$.

If the recorded values for carbonic anhydrase activity in bundle sheath cell extracts are corrected on the basis of the activity observed in the same extracts for mesophyll cell marker enzymes (Table II) then it would appear that bundle sheath cells contain insignificant levels of carbonic anhydrase. Of course it is possible that bundle sheath cells may contain low levels in the order of 1% of the leaf activity of these mesophyll marker enzymes. In any case, the maximum possible levels of carbonic anhydrase would be those recorded making no correction for mesophyll contamination. Using this 'worst case' assumption we could take an average value of about 8 μmol min^{-1} mg^{-1} Chl at 25°C (Table II) as an upper limit for bundle sheath carbonic anhydrase activity. This value is less than one-fifth of the calculated upper limit of carbonic anhydrase consistent with effective functioning of the C_4 pathway (45 μmol min^{-1} mg^{-1} Chl at CO_2 saturation and 25°C, see above). The key assumptions and results relating to this comparison are summarized in Table III.

Our model for predicting the inorganic carbon status of bundle sheath cells (8) can be used to calculate the steady state level of CO_2 that would prevail if the bundle sheath cell carbonic anhydrase activity was 8 μmol min^{-1} mg^{-1} Chl, the level taken here as the upper limit. The computed value is a CO_2 pool of 16.7 nmol mg^{-1} Chl in the nonvacuolar space giving a concentration of 375 μM. In this model a CO_2 pool of 16.7 nmol mg^{-1} Chl is equivalent to 44% of the total cytosolic inorganic carbon. This compares with a value of about 2% for the proportion of CO_2 if CO_2 and HCO_3^- reach thermodynamic equilibrium at pH 8.0. As reported previously (8), the model predicts that CO_2 would represent 81% of the cytosolic inorganic carbon at pH 8.0 with only uncatalyzed rates of CO_2/HCO_3^- interconversion operating.

Table III. *Maximum Observed Carbonic Anhydrase Activity in Bundle Sheath Cells and Computed Permissible Activity Consistent with Effective C_4 Function*

Observed bundle sheath cell carbonic anhydrase activity:	
Uncorrected range of activities (V_{max}, 25°C) recorded in Table II: 6–15 μmol min^{-1} mg^{-1} Chl	
Computed maximum permissible activity consistent with effective C_4 function[a]:	
Assumptions: $[O_2]$ 1000 μM, total inorganic carbon pool 55 nmol mg^{-1} Chl, ratio of CO_2 assimilation to photorespiratory CO_2 production of 30 or >	
Calculated $[CO_2]/[O_2]$	0.15
Corresponding $[CO_2]$ (with 1000 μM O_2)	150 μM
Computed carbonic anhydrase activity with 150 μM CO_2, 25°C	0.78 μmol min^{-1} mg^{-1} Chl
Equivalent V_{max} (with saturating CO_2)	45 μmol min^{-1} mg^{-1} Chl

[a] Further details about assumptions and the basis for calculations are provided in the text.

The level of carbonic anhydrase we calculated as an upper limit permitting the effective functioning of the C_4 pathway (0.78 μmol min^{-1} mg^{-1} Chl with $[CO_2]$ = 150 μM at 25°C or 45 μmol min^{-1} mg^{-1} Chl at saturating CO_2) assumed a total inorganic carbon pool in bundle sheath cells of 55 nmol mg^{-1} Chl. This value was the average observed for six C_4 species during steady state photosynthesis (8). Of course higher carbonic anhydrase activities could be tolerated but only at the expense of elevating the total inorganic carbon pool in bundle sheath cells even further to maintain an effective CO_2 concentration. Among the consequences of this solution would be increases in inorganic carbon leakage from bundle cells which may prove unacceptable in terms of efficiency (see "Discussion" by Furbank and Hatch [8]).

From the above discussion it is apparent that bundle sheath cells contain either no carbonic anhydrase or else so little as not to affect the efficiency of the C_4 pathway. The data and calculations presented here add further weight to the implied view (8) that the development of bundle sheath cells with little or no carbonic anhydrase was a vital step in the evolution of the C_4 pathway.

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