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Communication

Carbonic Anhydrase Activity in Leaves and Its Role in the First Step of C_4 Photosynthesis

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ABSTRACT

In C_4 plants carbonic anhydrase catalyzes the critical first step of C_4 photosynthesis, the hydration of CO_2 to bicarbonate. The maximum activity of this enzyme in C_4 leaf extracts, measured by H^+ production with saturating CO_2 and extrapolated to 25°C, was found to be 3,000 to 10,000 times the maximum photosynthesis rate for these leaves. Similar activities were found in C_3 leaf extracts. However, the calculated effective activity of this enzyme at *in vivo* CO_2 concentrations was apparently just sufficient to prevent the rate of conversion of CO_2 to HCO_3^- from limiting C_4 photosynthesis. This conclusion was supported by the mass spectrometric determination of leaf carbonic anhydrase activities.

The initial carboxylation reaction of C_4 photosynthesis, catalyzed by PEP[2] carboxylase, utilizes bicarbonate rather than CO_2 as the inorganic carbon substrate (9). To sustain this process, atmospheric CO_2 entering mesophyll cells must be rapidly converted to HCO_3^- and this reaction should rightly be regarded as the first step in C_4 photosynthesis. Consistent with this concept is the fact that the CA of C_4 leaves is very largely or exclusively confined to the cytosol of mesophyll cells (4, 6, 8), where PEP carboxylase is also located. In spite of this apparently critical role of CA in C_4 photosynthesis, the quantitative aspects of the operation of this enzyme have been largely neglected. The implicit assumption has been that the CA is present in a substantial excess in C_4 and also C_3 leaves. Hence, most studies have focused on the physical and kinetic properties of the enzyme (10).

In this paper we report on the maximum activity of CA in leaf extracts of C_4 and C_3 plants expressed in units which allow comparison with photosynthetic activity. An error in the leaf activities reported in an earlier study (4) is corrected. The V_{max} values were orders of magnitude higher than maximum photosynthesis rates, but the effective capacity for CA-catalyzed CO_2 hybridization *in vivo* was only just adequate to support photosynthesis.

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[2] Abbreviations: PEP, phospho*enol*pyruvate; CA, carbonic anhydrase.

MATERIALS AND METHODS

Materials

Plants were grown in soil in a naturally illuminated glasshouse maintained between 20 and 30°C. Biochemicals were obtained from either Sigma Chemical Co. or Boehringer-Mannheim (Australia).

Preparation of Leaf Extracts

About 0.5 g of leaf tissue was vigorously ground for about 20 s in a chilled mortar with 1 mL of either 40 mM barbitone-KOH or 40 mM Hepes-KOH (pH 8.0), each containing 10 mM dithiothreitol. After adding an additional 1 mL of the buffer mixture the homogenate was filtered through Miracloth. A sample of this filtrate was used to determine the Chl content of the extract (1), and the remainder was diluted in the blending buffer as required (usually 1 to 10 diluted) and stored at 0°C prior to being assayed.

Assay of Carbonic Anhydrase

CA was assayed by two different procedures. In the more conventional assay, the rate of CO_2 hydration was measured at 0°C by following the change of pH traced on chart recorder. Reactions contained 25 mM barbitone-KOH buffer (pH 8.2), in a final volume of 1 mL. The reaction was started by adding a CO_2 solution (distilled water saturated with CO_2 at 0°C, approximately 70 mM; see ref. 12) and stirring the mixture by up and down agitation of the precooled pH electrode probe. The nonenzymic reaction rate was measured by adding the CO_2 solution to the buffer without enzyme. Maximum CA activity was determined with a final concentration of 35 mM CO_2. With this concentration, the rate of change of pH remained essentially constant between 8.2 and 7.6. For determining K_m values, the activity with limiting CO_2 was determined from the initial slopes of the curves for changing pH. For these studies, more accurate initial slopes were obtained if the reaction was continuously stirred with a magnetic stirrer. The observed rate of change of pH was converted to equivalent μmol H^+ generated (and hence equivalent CO_2 hydrated assuming complete dissociation of H_2CO_3) by titrating the usual reaction mixture buffer through the range from pH 8.3 to 7.6 with a standard solution of H_2SO_4. Activity observed at 0°C was converted to equivalent activity at 25°C by multi-

Table I. *Carbonic Anhydrase Activity in Leaves and K_m for CO_2*

Photosynthetic Type and Species	Maximum Activity at 25°C[a]	CO_2 to HCO_3^- Rate *In Vivo*		K_m CO_2[b]
		Deduced from V_{max}[c]	From mass spectroscopy assay[d]	
	μmol CO_2 hydrated min^{-1} (mg Chl)$^{-1}$			*mM*
C_4 species				
Zea mays	34,000	68	51	2.8
Sorghum vulgare	17,500	35	36	2.5
Echinochloa crusgalli	32,000	64	110	1.8
Panicum miliaceum	45,000	89	130	1.5
Chloris gayana	14,000	28	37	0.8
Atriplex spongiosa	40,000	79	63	2.5
C_3 species				
Triticum aestivum	31,000			
Hordeum vulgare	19,500			
Spinacia oleracea	70,000			
Pisum sativum	65,000			

[a] An average of two determinations using the pH assay system at 0°C with 35 mM CO_2. For *Z. mays* only, the activity of 34,000 is the average of seven separate determinations ± SD 7000. [b] Estimated from the $[CO_2]$ response curves generated with the pH assay procedure at 0°C. [c] Deduced from the recorded V_{max} values at 25°C and assuming an average of K_m CO_2 value of 2 mM and a mesophyll cell $[CO_2]$ of 4 μM (see text for details). [d] Calculated from the mass spectrometer assay (1000 μM total inorganic carbon, 13.7 μM CO_2, see "Materials and Methods") and adjusted to give the rate for 4 μM CO_2 *in vivo* (see text for details).

plying by a factor of 4.8 determined from the Q_{10} for maize CA (4).

CA activity was also measured by a mass spectrometer procedure based on the method developed by Silverman (11) and described in detail by Badger and Price (2). The reaction mixture contained 25 mM barbitone-KOH in an final volume of 2 mL (pH 7.98) and was started by adding 2 μL of 1 M $NaHC^{18}O_3$ to give a final concentration of 1 mM. After measuring the rate of ^{18}O loss due to the nonenzymic reaction, the enzyme-catalyzed rate was determined by adding a sample of extract containing CA. CA activity in the CO_2 hybridration direction was deduced from the increase in rate of ^{18}O loss over that observed without enzyme by applying this factor to the nonenzymic first order rate constant for the CO_2 hydration reaction (25°C and an ionic strength of 0.1 [7]). With 1 mM total inorganic carbon at pH 7.98 the CO_2 concentration was taken as 13.7 μM at thermodynamic equilibrium.

RESULTS AND DISCUSSION

Quantitation of CA Activity

CA activities in leaves and other tissues have generally been expressed in arbitrary units which cannot be related to the rates of processes such as photosynthesis (10). In the present study enzyme activity, measured as the rate of change of pH as CO_2 is hydrated to give HCO_3^- plus H^+, was converted to equivalent μmol of H^+ generated so that comparisons could be made with photosynthetic rates. The conversion factor was determined by titrating a normal buffered reaction mixture with a standard acid solution at 0°C. With a 1 mL reaction mixture containing 25 mM barbitone buffer, 0.105 μmol H^+ was required to give a pH change of 0.1 unit.

CA Activity in Leaves

Maximum CA activities measured in the direction of CO_2 hydration were very high in extracts of both C_3 and C_4 leaves and fell within a similar range (Table I). The values recorded here are much higher than those reported previously (4), due primarily to a consistent calculation error made in the earlier study. This did not affect the major conclusion of the earlier investigation, which was to show that bundle sheath cells contained a very low or negligible proportion of the CA activity in C_4 leaves.

The maximum activities of leaf CA recorded with 35 mM CO_2 and extrapolated to 25°C (Table I) are about four orders of magnitude greater than the maximum photosynthesis rates for C_4 (4–8 μmol min^{-1} [mg Chl]$^{-1}$)] or C_3 (approximately 3 μmol min^{-1} [mg Chl]$^{-1}$) leaves (5). However, the rates *in vivo* will be much lower because of the very low CO_2 concentrations prevailing in the cells during photosynthesis. For instance, the $[CO_2]$ in C_4 mesophyll cells during maximum steady-state photosynthesis will be, at the most, about 4 μM (13). Taking this value for $[CO_2]$ and assuming an average K_m for CO_2 of 2 mM (the average of K_m CO_2 values shown in Table I for C_4 species) the potential rate of CO_2 hydration can be calculated from the equation $v = V[S]/(K_m + [S])$ where $[S]$ is the CO_2 concentration. The values obtained, recorded in Table I, ranged between 28 and 89 μmol min^{-1} (mg Chl)$^{-1}$.

A similar range of potential *in vivo* CA activities was obtained using the mass spectrometric-based assay system (Table I). With this procedure the activity due to CA is initially measured as a multiple of the nonenzyme-catalyzed rate (see "Materials and Methods"). These measurements are made with a highly limiting concentration of substrates (total inorganic carbon 1 mM or 13.7 μM CO_2 at pH 7.98) so that the

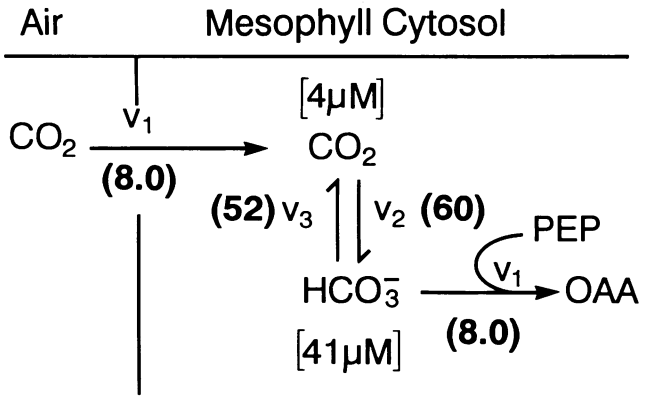

Figure 1. A model of CO_2 incorporation via PEP carboxylase in mesophyll cells. The values quoted on the scheme for CO_2 and HCO_3^- concentration and for v_1, v_2 and v_3 (bold values in round brackets) are based on the following assumed values: a net photosynthesis rate of 6.4 μmol min^{-1} (mg Chl)$^{-1}$ and 25% overcycling of the C_4 acid cycle giving a PEP carboxylation rate (v_1) of 8.0 μmol min^{-1} (mg Chl)$^{-1}$ (7), a mesophyll cell CO_2 concentration of 4 μM under conditions of maximum photosynthesis (7, 13), a CA activity in the CO_2 to HCO_3^- direction (v_2) of 60 μmol min^{-1} (mg Chl)$^{-1}$ (average of values for C_4 species recorded in Table I) and a mesophyll cytosolic pH of 7.2. Rate constants for the interconversion of CO_2 and HCO_3^- were calculated for particular pH values and for 25°C and 0.1 ionic strength as described previously (7). With these rate constants (k_f = 3.9 × $10^{-2}s^{-1}$ for the forward reaction and k_r = 0.33 × $10^{-2}s^{-1}$ for the reverse reaction) and the above assumed values, the HCO_3^- concentration was calculated from the relationship $v_3/v_2 = [HCO_3]k_r/[CO_2]k_f$.

activity due to added CA can be deduced from the known first order rate constant for CO_2 hydration at 25°C. Likewise, the potential *in vivo* rate corresponding to a CO_2 concentration in mesophyll cells of 4 μM can then be calculated from the above values (see above and legend of Fig. 1) assuming again a pseudo first order response to varying CO_2. It should be noted that the calculated rates for CA-catalyzed CO_2 hydration under physiological conditions (shown as *in vivo* rates in Table I) are about 10^4 times the calculated nonenzymatic rate of CO_2 hydration that would occur in mesophyll cells under the same conditions (*i.e.* 25°C, pH 7.2, 4 μM CO_2 and other conditions described in the legend to Fig. 1).

Table I also lists K_m values for CO_2 determined for several C_4 leaf CA. These values, determined from $[CO_2]$ response curves with the pH assay, ranged between 0.8 and 2.8 mM. Values are only approximate because, with this method, accurate initial rates could only be obtained for CO_2 concentrations of about 2 to 3 mM or above. Apparent K_m CO_2 values for the wheat and spinach enzymes were about 1 mM and 5 mM, respectively.

In vivo Carbonic Anhydrase Activity in Relation to Requirements

The estimated *in vivo* rates of CO_2 hydration with 4 μM listed in Table I are between 5 and 15 times the maximum photosynthetic rates for C_4 leaves. However, the considerations below will show that this apparent excess of carbonic anhydrase activity would be essential to maintain a steady-state where both net CO_2 hydration rates and the HCO_3^- concentrations for PEP carboxylase are adequate to support maximum rates of photosynthesis. Using the model system outlined in Figure 1, it is possible to calculate the concentration of HCO_3^- that would develop during steady-state photosynthesis (when $v_1 = v_2 - v_3$) with 4 μM CO_2 and any given values for cytosolic pH, the rate of PEP carboxylation to oxalacetic acid, and the gross rate of CO_2 hydration in meosphyll cells (see legend to Fig. 1). For instance with 4 μM CO_2, a pH of 7.2, a PEP carboxylation rate of 8 μmol min^{-1} (mg Chl)$^{-1}$, and a potential CA activity in the direction of CO_2 hydration of 60 μmol min^{-1} (mg Chl)$^{-1}$ (average for C_4 species in Table I), the steady-state HCO_3^- concentration would be 41 μM (Fig. 1). This value is within the range of K_m HCO_3^- values (25–100 μM) reported for the C_4 PEP carboxylase for pH in the range of pH 7 to 8 (3, 9).

Figure 2 illustrates the effect of varying the *in vivo* CA activity on the steady-state levels of HCO_3^- that would develop in mesophyll cells during steady-state photosynthesis. These calculations are based on the assumptions for the model described in Figure 1 and curves are presented for three values of cytosolic pH. The calculations show that potential CA activities of about 100 μmol min^{-1} (mg Chl)$^{-1}$ in the CO_2

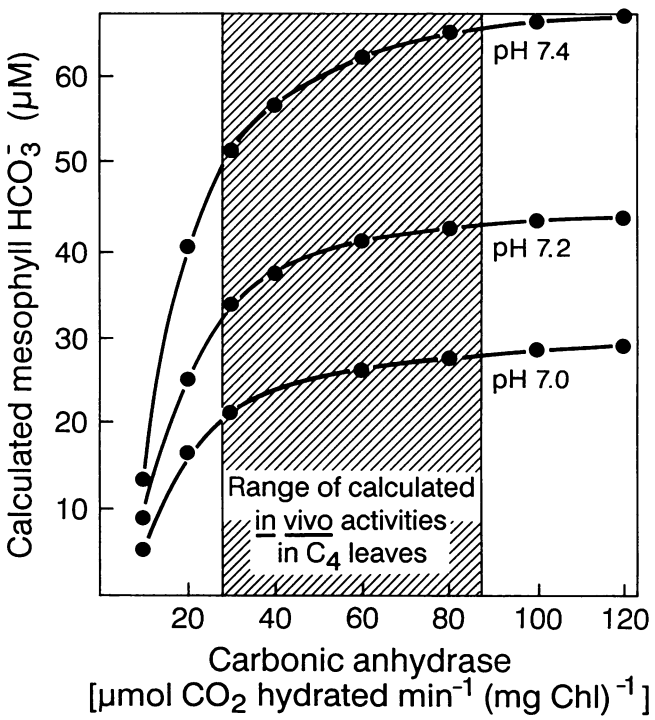

Figure 2. Effect of varying mesophyll cell CA activity on the estimated steady-state level of HCO_3^- and the comparison of this relationship with the estimated activity of CA *in vivo*. Calculations were made on the basis of the assumptions described in Figure 1 and for three pH values covering the range of likely cytosolic pH. Other details are provided in the legend to Figure 1. For comparison, the range of estimated *in vivo* CA activities for C_4 leaves (taken from Table I) is included.

hydration direction would be required to give [HCO_3^-] approaching the thermodynamic equilibrium concentration. As shown in Figure 2, with the CA activities estimated to occur *in vivo* in C_4 plants the corresponding HCO_3^- concentrations would be significantly below the thermodynamic equilibrium values. It should be noted that even a reduction by a factor of as little as two in this range of CA activities could result in a large decrease in the steady-state HCO_3^- concentration possibly to values well below the K_m HCO_3^- for PEP carboxylase (see above). CA in the leaves of C_4 plants is apparently poised on the threshold of limiting photosynthesis.

CONCLUDING COMMENTS

The maximum potential activity of CA in both C_3 and C_4 leaf extracts is orders of magnitude higher than the maximum photosynthesis rates. Although the function of CA in C_3 leaves remains uncertain (10) it apparently does not have a stoichiometric role in the photosynthetic process. On the other hand, CA in C_4 leaves almost certainly functions specifically and solely to convert CO_2 appearing in mesophyll cells to HCO_3^- which is then assimilated via PEP carboxylase (see Introduction). In view of this, the fact that C_3 and C_4 leaves contain similar CA activities would appear to be coincidental.

In spite of the very high potential CA in C_4 leaves, our analysis indicates that the effective activity *in vivo* may be just sufficient to ensure that the conversion of CO_2 to HCO_3^- in mesophyll cells does not limit photosynthesis. This is largely attributable to the very low CO_2 concentration prevailing in meosphyll cells during photosynthesis relative to the apparent K_m CO_2 values for the C_4 leaf CA. In addition, to establish a steady-state where concentrations of HCO_3^- are in the same order as the K_m HCO_3^- for PEP carboxylase, potential activities of CA for the CO_2 hydration direction need to be several fold higher than the maximum rate of photosynthesis.

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