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Carbonic anhydrase associated with thylakoids and Photosystem II particles from maize

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Carbonic anhydrase activity was detected in osmotically shocked, washed thylakoids and in Photosystem II particles from maize. In thylakoids, light had a strong inhibitory effect on the carbonic anhydrase activity. The effect of light was partially reversed by the Photosystem II modifiers hydroxylamine and atrazine. The specific carbonic anhydrase inhibitor, acetazolamide, completely eliminated the thylakoid activity. Anionic Photosystem II inhibitors formate and chloride (at high concentration) reduced thylakoid carbonic anhydrase activity. In Photosystem II particles, carbonic anhydrase activity was frequently enriched, when compared, on the basis of chlorophyll content, to the thylakoids from which they were derived. Carbonic anhydrase activity in 'Cl^--depleted' thylakoids was stimulated by low (10 mM) concentrations of added Cl^- and Br^-, but not by F^- or SO_4^{2-}. Ca^{2+} also stimulated the activity. Thylakoid carbonic anhydrase is unusual in being sensitive to Zn^{2+}, which almost totally inhibits the activity. The effect of Zn^{2+} can be reversed by added Mn^{2+}, but not by Mg^{2+} or Ca^{2+}. The results suggest that one of the polypeptides in the Photosystem II complex has carbonic anhydrase activity. The interaction of anions at two sites on this polypeptide could explain many anion effects on Photosystem II, particularly those related to the 'HCO_3^- effect'.

Introduction

The activity of the Photosystem II (PS II) complex in thylakoid membranes and the activity of the enzyme carbonic anhydrase respond in parallel fashion to a number of chemical reagents (for detailed discussion, see Ref. 1). Examples are numerous. The specific carbonic anhydrase inhibitor, acetazolamide, is also effective against PS II [2,3], as is the basic form of imidazole [3]. Monovalent anions inhibit both carbonic anhydrase and PS II, with similar pH dependence [3]. Included among these anions is bicarbonate which, at high pH, inhibits both PS II [3] and carbonic anhydrase [4]. Those similarities lead us (Stemler and Jursinic, Ref. 3) to suggest that carbonic anhydrase could serve as a model to explain the bicarbonate effect associated with PS II. Since that time, other similarities between carbonic anhydrase and PS II have been uncovered. For example, it has been shown that two anion-binding sites exist on carbonic anhydrase [5]. Sulfonamide prevents anion binding to one site, but not to the other. Recent evidence indicates that there are also two binding sites for HCO_3^- on PS II [6,7]. High concentrations of atrazine or DCMU can eliminate

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Abbreviations: Chl, chlorophyll; DMBQ, 2,6-dimethylbenzoquinone; PS II, Photosystem II; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

one site on PS II, but not the other [6,8]. Bromoacetate has been shown to bind covalently to a specific histidine located near the anion-binding sites on carbonic anhydrase [9]. Bromoacetate-treated thylakoids no longer bind $H^{14}CO_3^-$ [1], suggesting that the anion binding sites on PS II are likewise permanently occupied after treatment with this reagent.

Considering the carbonic anhydrase-like properties of PS II, it seemed reasonable to ask whether or not a component of the PS II complex actually has carbonic anhydrase activity. This possibility was raised and discussed in a review by Reed and Graham [10]. Relevant to this question, it has been reported by Vaklinova et al. [11,12] that thylakoid membranes and PS II particles demonstrate such activity. To verify and extend these results, a mass spectrometer capable of measuring isotopic exchange between $C^{18}O_2$ and $H_2{}^{16}O$ was used. This method provided a sensitive and direct measurement of carbonic anhydrase activity. The results indicate that osmotically shocked and washed thylakoid membranes to demonstrate such activity. PS II particles likewise show carbonic anhydrase activity. The activity is inhibited by light, high concentrations of anions, Zn^{2+}, and by other PS II reagents.

Materials and Methods

Maize (*Zea mays* L.) plants were grown in potting soil in a greenhouse and harvested 2–3 weeks after planting. In the winter months, supplementary illumination was provided. To obtain thylakoids, leaves were ground in a Waring Blender, and the slurry was quickly filtered through eight layers of cheesecloth. The grinding medium normally contained 0.05 M sodium phosphate (pH 7.4)/0.2 M NaCl/5 mM $MgCl_2$. The filtrate was centrifuged at $1500 \times g$ for 5 min. The chloroplasts in the resulting pellet were given an osmotic shock by resuspending them in grinding medium diluted 10-fold with cold distilled water. The suspension was centrifuged for 1 min at $300 \times g$. The supernatant was poured into clean test tubes and centrifuged again at $1500 \times g$ for 5 min. The thylakoids in the resulting pellet were washed once by suspending them in 0.01 M sodium phosphate (pH 7.0)/0.01 M NaCl/5 mM MgCl/0.4 M sucrose. After a final centrifugation, the pellet was resuspended in a small amount of wash buffer, divided into aliquots, and kept frozen in liquid nitrogen until used.

To obtain 'chloride-depleted' thylakoids, the same procedure was followed, except that the grinding medium contained 0.05 M Tricine (pH 7.8)/5 mM $MgSO_4$/0.4 M sucrose, while the wash buffer contained 0.01 M Tricine (pH 7.6)/5 mM $MgSO_4$/0.2 M sucrose.

Photosystem II particles were made by subjecting a portion of normally obtained thylakoids (see above) to the modified Kuwabara and Murata [13] procedure described by Bricker et al. [14].

The measurements of carbonic anhydrase activity were carried out with a MAT Atlas CH-4 mass spectrometer. A temperature-controlled glass reaction cell was connected to the spectrometer by means of a vacuum line. A polypropylene membrane at the bottom of the magnetically stirred cell allowed dissolved gases to be introduced into the line. Molecular masses 44, 46 and 48, representing $C^{16}O^{16}O$, $C^{18}O^{16}O$ and $C^{18}O^{18}O$, respectively, were monitored alternately; each sweep required approx. 30 s. The mass spectrometer output was fed into a Gespac, model 720, computer that recorded the areas under the peaks and performed the appropriate calculations. CO_2/HCO_3^-, labelled with ^{18}O, was made by dissolving a saturating amount of normal $NaHCO_3^-$ in $H_2{}^{18}O$ (more than 97% ^{18}O) and allowing the solution to equilibrate for several days. For each measurement, a small volume (8 μl) of this solution was then injected into the reaction cell which contained 8 ml of reaction mixture. The final CO_2/HCO_3^- concentration was 0.7 mM. It was necessary in performing these experiments to keep both the pH and the temperature of the reaction mixture constant from sample to sample. Small changes in these variables could alter the spontaneous exchange rate. Whenever a new substance or condition was introduced, controls were always performed in the absence of thylakoids but with an equivalent amount of thylakoid suspension medium. It was thus possible to measure any effect of the new condition on the spontaneous isotopic exchange. Reaction mixtures and other conditions are specified in the figure and table legends.

Hill reaction rates in thylakoids and PS II particles were measured with a Rank Brothers, Clark-type, oxygen monitor under saturating white light.

Results

Carbonic anhydrase activity associated with washed maize thylakoids

The rate of isotopic exchange between H_2O and dissolved CO_2 can be monitored with a mass spectrometer as a decrease in mass 48 ($C^{18}O^{18}O$) in solution. Fig. 1 shows the results of one such experiment. Immediately after injection of $HC^{18}O_3^-$, the mass 48 signal increases for about 1 min. This reflects the rate of chemical equilibration between HCO_3^- and CO_2 and also the response time of the instrument. After a peak is reached, the decay of mass 48 proceeds exponentially. The top redrawn trace in Fig. 1a shows the decay of mass 48 due to spontaneous isotopic exchange. In this case, thylakoids were not present, only an equivalent amount of suspension medium. When thylakoids were present, the bottom trace was obtained if the experiment was done in the dark. When the thylakoids were exposed to room light, the middle trace was observed. The faster decay of the mass-48 signal in the presence of thylakoids means that some carbonic anhydrase activity is present. The activity is inhibited by light.

For convenience in comparing the effects of various treatments on carbonic anhydrase activity, data such as that shown in Fig. 1a were plotted as a logarithm vs. time starting 3 min after injection of $HC^{18}O_3^-$ (Fig. 1b). These semilogarithmic plots were found to be straight lines. The slopes of experimental lines, minus the slope of the thylakoid-free, spontaneous exchange measurement, give relative carbonic anhydrase activity in arbitrary units. Corrections can be made for differences in chlorophyll concentration. Thus the data shown in Fig. 1a and b are also represented in Table I, lines 1 and 2. Comparing these values, we see that light inhibited the carbonic anhydrase activity by 71% in this experiment.

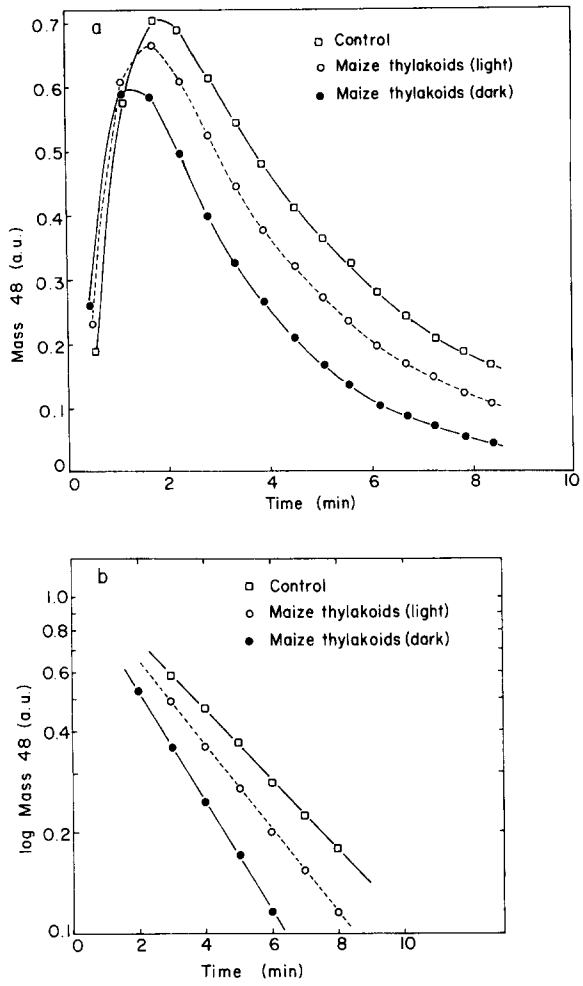

Fig. 1. Carbonic anhydrase activity in osmotically shocked, washed maize thylakoids. The reaction mixture contained 0.05 M Hepes (ph 7.0)/0.1 M NaCl/approx. 200 μg Chl per ml. The temperature was 25°C. Samples were routinely given a 5 min equilibration period before addition of labeled bicarbonate. $NaHC^{18}O_3$ was injected at time zero to a concentration of 0.7 mM. Mass 48 represents the amount of $C^{18}O^{18}O$ present in solution. The decay of the control signal (Fig. 1a, top curve) represents spontaneous isotopic exchange of oxygen between $C^{18}O^{18}O$ and $H_2{}^{16}O$. The bottom trace represents isotopic exchange when thylakoids were present in complete darkness. The middle trace was obtained when thylakoids were present and the experiment was done in room light. Other conditions are specified in Materials and Methods. Fig. 1b shows the same data converted to logarithmic values.

Effects of various treatments on thylakoid carbonic anhydrase activity

Thylakoids were subjected to a number of treatments in order to further characterize their carbonic anhydrase activity. When the membranes were suspended in reaction mixture, then centri-

TABLE I

THE EFFECTS OF VARIOUS TREATMENTS ON MAIZE THYLAKOID CARBONIC ANHYDRASE ACTIVITY

Membranes were suspended in reaction mixture that contained 0.05 M Hepes (pH 7.0), 0.1 M NaCl, 100–200 μg Chl·ml^{-1}, plus the additions indicated. The supernatant (line 3) was obtained by centrifugation of a normally constituted, thylakoid-containing sample. The thylakoid-containing pellet (line 4) was then resuspended in fresh reaction medium. Hydroxylamine stock solutions were freshly prepared and brought to pH 7.0 with NaOH before addition. Sulfate medium contained 0.05 M Hepes (pH 7.0)/0.01 M NaCl/0.05 M Na_2SO_4. 'Low-salt' medium contained 0.05 M Hepes (pH 7.0)/0.01 M NaCl. All experiments were done in complete darkness, unless otherwise noted. Other conditions were the same as mentioned in the legend of Fig. 1. The relative carbonic anhydrase activity was obtained as described in the text. Room light was from overhead incandescent lamps. The intensity near the sample holder was approx. 5 W·m^{-2} (about 2 μmole photons·m^{-2}·s^{-1}). The arbitrary units (a.u.) were normalized to the same chlorophyll concentration and time base.

Treatment or addition	Relative carbonic anhydrase activity in a.u. (per mg Chl per min)
1. Control (thylakoids in dark)	759
2. Room light	218
3. Supernatant	12
4. Thylakoid pellet (resuspended)	375
5. Silicomolybdate (0.1 mM)	57
6. Hydroxylamine (5 mM, dark)	813
7. Hydroxylamine (5 mM, light)	333
8. Acetazolamide (0.1 mM)	517
9. Acetazolamide (1.0 mM)	17
10. Formate (10 mM)	499
11. Formate (20 mM)	298
12. Formate (50 mM)	217
13. Formate (100 mM)	163
14. Sulfate medium	2100
15. 'Low-salt' medium	2974

fuged, little or no activity was observed in the supernatant (Table I, line 3). About half the initial activity was recovered in the pellet (line 4). This above that the carbonic anhydrase activity is definitely attached to the membranes, but is somewhat labile.

Silicomolybdate, a thylakoid membrane modifier, almost completely eliminated carbonic anhydrase activity (Table I, line 5). Hydroxylamine, an inhibitor of the PS II oxygen-evolving mechanism, increased slightly (7%) the carbonic anhydrase activity in the dark (line 6). It has a much greater stimulatory effect in the light (compare lines 2 and 7). In other words, hydroxylamine partially reversed the inhibitory effect of light on the carbonic anhydrase activity. Acetazolamide, the most commonly used inhibitor of carbonic anhydrase, inhibited activity 32% at 0.1 mM concentration (line 8) and 98% at 1.0 mM concentration (line 9). This is approximately the concentration range that inhibits PS II [3]. A related compound, ethoxyzolamide, was not obtainable commercially and was not tested. Since it is less effective against Photosystem II compared to acetazolamide, it would be of interest, in the future, to test its relative effectiveness against the carbonic anhydrase activity found here. Formate is a frequently used anionic inhibitor of PS II. It also inhibits carbonic anhydrase activity in thylakoids (lines 10–13). The amount of carbonic anhydrase inhibition is directly related to the formate concentration.

The reaction medium for these experiments initially contained a high concentration (100 mM) of NaCl, added to maintain ionic and osmotic strength. This condition was also chosen because much of the past work on the 'HCO_3^--effect', which prompted the present effort, was done under similar high-salt conditions. Chloride at high concentration, however, inhibits both PS II [15] and carbonic anhydrase (for a review , see Ref. 16). Indeed, when SO_4^{2-} was substituted for Cl^-, nearly a 3-fold increase in carbonic anhydrase activity was observed (line 14). An even greater stimulation was observed (line 15) when sulfate was omitted and the reaction medium contained only buffer plus 0.01 M NaCl. This showed that chloride, at high concentrations, was a powerful inhibitor of the carbonic anhydrase activity. The remainder of the experiments reported here were therefore done under low-salt conditions. The information in Table I, however, is more directly comparable to previous work [38].

Atrazine given to thylakoids in the dark had little or no effect on carbonic anhydrase activity (Table II, line 3). In strong light, however, atrazine significantly increased the carbonic anhydrase activity (lines 2 and 4). While light alone produced a 68% decrease in carbonic anhydrase activity, it

TABLE II

THE EFFECTS OF VARIOUS TREATMENTS ON CARBONIC ANHYDRASE ACTIVITY ASSOCIATED WITH MAIZE THYLAKOIDS IN 'LOW-SALT' MEDIUM

Conditions were the same as described in the Table I legend, except that the reaction mixture here contained 0.05 M Hepes (pH 7.0), 0.01 M NaCl plus the additions specified. 'Strong light' was provided by an unfocused 150 W flood lamp spaced 8 cm from the reaction cell.

Treatment or addition	Relative carbonic anhydrase activity in a.u. (per mg Chl per min)	Change (%)
1. Control (thylakoids in dark)	2974	–
2. Strong light throughout	942	−68
3. Atrazine (10 μM), dark	2829	−5
4. Atrazine (20 μM), strong light throughout	1701	−43
5. Ferricyanide (1 mM), dark	1244	−58
6. DMBQ (100 μM), dark	1814	−39
7. Ferricyanide (1 mM) plus DMBQ (100 μM), dark	803	−73
8. $ZnCl_2$ (1 mM), dark	208	−93

affected only a 43% decrease in the presence of atrazine. Thus, atrazine, like hydroxylamine, partially reversed the inhibitory effects of light. The Hill oxidant, ferricyanide, given to thylakoids in the dark affected a 58% reduction in carbonic anhydrase activity (line 5). Likewise, the membrane-soluble electron acceptor, DMBQ, also produced a 39% inhibition of activity (line 6). Together, ferricyanide and DMBQ had an additive effect and reduced carbonic anhydrase activity 73% (line 7).

As was shown by Tripathy and Mohanty [17], Zn^{2+} inhibits PS II at a site near the oxygen-evolving mechanism. On the other hand, Zn^{2+} is known to be the required cofactor for nearly all naturally occurring carbonic anhydrase, and should not inhibit this enzyme. It was therefore surprising to find that 1 mM $ZnCl_2$ all but eliminated the carbonic anhydrase activity associated with thylakoids (line 8). Co^{2+} and Mn^{2+}, at concentrations of 5 mM, had little or no effect (data omitted). This effect of Zn^{2+} will be described in more detail in the subsection Inhibition of carbonic anhydrase by Zn^{2+}.

Inhibition of carbonic anhydrase activity by light

The nature of inhibition of thylakoid carbonic anhydrase activity by light was investigated further. Light could conceivably induce a slow irreversible inactivation of the carbonic anhydrase activity, or could produce a rapid, reversible effect in consequence to even limited electron transport. To test these possibilities, control chloroplasts were incubated in the reaction cell for 12 min in the

TABLE III

EFFECTS OF LIGHT ON THYLAKOID CARBONIC ANHYDRASE ACTIVITY IN 'LOW-SALT' MEDIUM

Conditions were as described in the Table II legend.

Conditions	Relative carbonic anhydrase activity in a.u. (per mg Chl per min)	Change %
1. 12 min dark, assay in dark (control)	2000	–
2. 12 min dark, assay in strong light	1765	−12
3. 10 min strong light, 2 min dark assay in dark	762	−62
4. 10 min room light, 2 min dark assay in dark	1760	−12

dark before injection of $NaHC^{18}O_3$. These thylakoids showed high rates of carbonic anhydrase activity (Table III, line 1). Another sample was incubated for 12 min in the dark, then continuously illuminated with strong light beginning just as the $NaHC^{18}O_3$ was injected. Membranes so treated showed a 12% reduction in carbonic anhydrase activity (line 2). This value represents an 'average' difference in carbonic anhydrase activity measured over the time period 2–8 min after injection of $HC^{18}O_3^-$ and application of light. A closer look at the data, however, showed that there was change in activity with time. There was almost no measurable difference in the carbonic anhydrase activity in the illuminated sample compared to the dark control in the time period 2–4 min after $HC^{18}O_3^-$ injection. In the time period 6–8 min, in contrast, a 38% decrease in carbonic anhydrase activity was recorded for the illuminated sample. This means that the sample slowly lost activity during the period of measurement in the light. When thylakoids were first given 10 min of strong light, then dark adapted 2 min before assay (also in the dark) the carbonic anhydrase activity was reduced 62% (line 3). A closer look at these data showed that this decrease in activity was the same 2–4 min compared to 6–8 min after injection. This means there was no apparent increase in carbonic anhydrase activity as the measurement proceeded in the dark. That is, inactivation by light appeared permanent, at least within a 10 min dark period following illumination. This last experiment, when done with much weaker 'room light', produced only a 12% decline in activity (line 4). Clearly, under these conditions, light produces a slow, irreversible inactivation of the carbonic anhydrase activity, rather than a rapid reversible effect. High light intensity is much more effective than low intensity. These results, obtained in 'low-salt' reaction mixture differ from

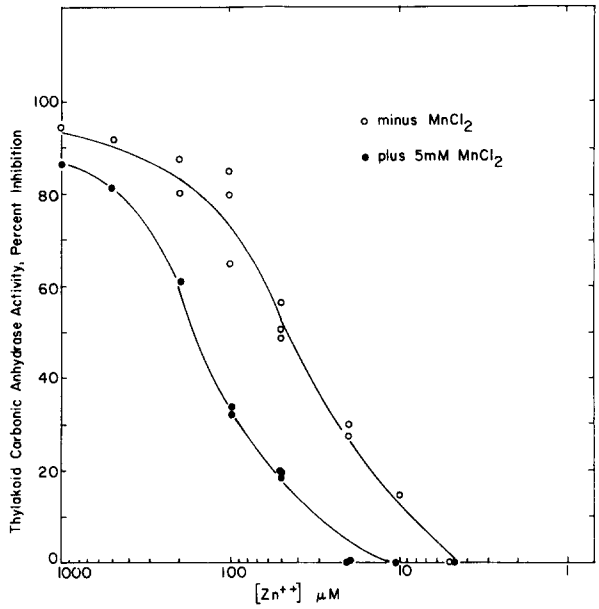

Fig. 2. Inhibition of thylakoid carbonic anhydrase activity as a function of Zn^{2+} concentration in the presence or absence of Mn^{2+}. The reaction mixture contained 0.05 M Hepes, (pH 7.0), $MnCl_2$ and $ZnCl_2$ to the concentration indicated. Chloride concentrations were kept comparable by the addition of 10 mM NaCl to the $MnCl_2$-free reaction mixtures. Other conditions were as described in the legend at Table I.

TABLE IV

THE ABILITY OF VARIOUS DIVALENT CATIONS TO REVERSE THE INHIBITION OF THYLAKOID CARBONIC ANHYDRASE ACTIVITY BY Zn^{2+}

The reaction mixture contained 0.05 M Hepes (pH 7.0), and either 10 mM NaCl or 5 mm of the salt indicated. Other conditions were as described in the Table I legend.

Additions	Relative carbonic anhydrase activity in a.u. (per mg Chl per min)	Change (%)
1. None (control)	2701	–
2. $ZnCl_2$ (50 μM)	1161	−57
3. $ZnCl_2$ (50 μM), $MgCl_2$ (5 mM)	1269	−53
4. $ZnCl_2$ (50 μM), $CaCl_2$ (5 mM)	1215	−55
5. $ZnCl_2$ (50 μM), $CoCl_2$ (5 mM)	1539	−43
6. $ZnCl_2$ (50 μM), $MnCl_2$ (5 mM)	1998	−26

those observed in high-salt medium (Table I, line 2). In high-salt medium, room light throughout an experiment was sufficient to produce a greater than 70% inhibition of carbonic anhydrase activity. Evidently, either the presence of high anion concentration makes the carbonic anhydrase activity more susceptible to photoinactivation by weak light, or low light can increase the affinity of the enzyme for inhibitory anions such as Cl^-. Light is known to affect the relative affinity of PS II for various inhibitory anions [7].

Inhibition of carbonic anhydrase by Zn^{2+}

The unusual inhibition of thylakoid carbonic anhydrase by Zn^{2+}, the metal cofactor associated with this enzyme in both animals and plants (for reviews, see Refs. 16 and 18) was investigated in more detail. A concentration study showed that as little as 50 μM Zn^{2+} was sufficient to reduce the carbonic anhydrase activity by half (Fig. 2, upper curve). The effect of Zn^{2+} could be countered, however. When the experiment was repeated in the presence of 5 mM added Mn^{2+}, the inhibitory effect of Zn^{2+} declined such that a 3- to 4-fold greater Zn^{2+} concentration (approx. 170 μM) was required to reduce activity by half (Fig. 2, lower curve).

Several divalent cations were tested to see if they had a similar effect as Mn^{2+}. The results are shown in Table IV. Both Mg^{2+} and Ca^{2+} were found to be ineffective at reversing Zn^{2+} inhibition of Ca activity. Co^{2+}, in contrast, was able to counter the effect of Zn^{2+} to a small extent. While 50 μM Zn^{2+} produced a 57% inhibition of carbonic anhydrase activity when given alone, it produced only a 43% inhibition when given in the presence of 5 mM Co^{2+}. This metal was tested because of its ability (along with Mn^{2+}) to substitute for Zn^{2+} in in vitro studies of carbonic anhydrase. Both Co^{2+} and Mn^{2+} yield partially active carbonic anhydrase, whereas other metals give only inactive enzyme. The effect of Co^{2+} at reversing Zn^{2+} inhibition was not, however, as great as that of Mn^{2+}, which reduced inhibition of 50 μM Zn^{2+} 26%.

In doing these experiments, it was found that the injection of 5 mM $MnCl_2$, and other salts, could raise the pH of a buffered solution slightly. One effect of increasing the pH above 7 is to reduce the solubility to Zn^{2+}, which precipitates, apparently as Zn $(OH)_2$ or ZnO. This could explain, quite trivially, the ability of Mn^{2+} to reduce Zn^{2+} inhibition of carbonic anhydrase activity. To avoid this problem, $MnCl_2$ was injected into a reaction mixture buffered at a pH below the controls. The final pH was then identical in Mn^{2+} containing solutions and in the controls. The pH was rechecked in each sample after the experiments were finished. Thus, the effect of Mn^{2+} in countering Zn^{2+} inhibition was not an indirect effect of a change in pH.

Carbonic anhydrase activity in PS II particles

If thylakoid carbonic anhydrase activity is asso-

TABLE V

A COMPARISON OF HILL REACTION AND CARBONIC ANHYDRASE ACTIVITY IN MAIZE THYLAKOIDS AND PS II PARTICLES DERIVED FROM THEM

A representative experiment is presented, see text for ranges. The reaction mixture for carbonic anhydrase measurements contained 0.05 M Hepes (ph 7.0), 0.01 NaCl and 100–200 μg Chl·ml^{-1}. The reaction mixture for the Hill reaction measurements was the same, but contained only 20–30 μg Chl·ml^{-1}/plus 0.5 mM $K_3Fe(CN)_6$/0.1 mM 2.6-dimethylbenzoquinone/1 μM gramicidin. Other conditions for carbonic anhydrase measurements were as described in the legend to Table I. Strong light was used throughout the experiments where indicated. It was provided by an unfocused 150 W flood lamp spaced 8 cm from the reaction cell.

Treatment	Chl *a*/*b*	Hill activity (μmol O_2 per mg Chl per h)	Carbonic anhydrase activity (dark) in a.u. (per mg Chl per min)	Carbonic anhydrase activity (light) in a.u. (per mg Chl per min)	Carbonic anhydrase activity (dark) plus 1 mM acetazolamide in a.u. (per mg Chl per min)
Thylakoids	3.24	100	980	539	0.0
PS II particles	2.48	53	1462	1419	0.0

ciated with PS II, it should be observable in PS II particles. With this in mind, particles from maize were prepared, and their carbonic anhydrase activity was compared to that of the thylakoids from which they were derived. In doing this, a number of difficulties were experienced. The yield of particles was quite low, only 12–15% of the initial chlorophyll was recovered in the particles. In addition, there was variability in the amount of carbonic anhydrase activity in the thylakoids and in the amount recovered in the particles. In all, valid data were obtained for five separate particle preparations. The results from a representative experiment are found in Table V. Because the results were not easily averaged, the range of data will be presented in the following paragraph.

Chlorophyll *a/b* ratios ranged from 3.24 to 4.15 in the thylakoids and 1.57 to 2.48 in the PS II particles (Table V). Hill reaction rates in the thylakoids ranged from 86 to 100 μmol O_2 per mg Chl per h. About half this activity was present in the particles. In the thylakoids, carbonic anhydrase activity ranged from 310 to 980 units. In every case, light had a strong inhibitory effect, ranging from 45 to 73%, on the carbonic anhydrase activity. With equal consistency, PS II particles were found to have significant amounts of carbonic anhydrase activity. Often the PS II particles were strongly enriched in carbonic anhydrase activity when compared on a chlorophyll basis to the thylakoids from which they were derived. Enrichment in carbonic anhydrase activity was observed in three out of five PS II particle preparations. Considering that the carbonic anhydrase activity appears to be labile (Table I, line 4 vs. 1) and that the preparation of particles requires several additional steps rigorous enough to remove 85% of the chlorophyll from the membranes, these results strongly argue that the carbonic anhydrase activity is closely associated with the PS II complex.

An additional, rather puzzling result was obtained. While PS II particles showed high carbonic anhydrase activity, and responded normally to acetazolamide, there were no effect of light on their activity. Somehow, in the preparation of PS II particles, the ability of the carbonic anhydrase to respond to light is lost. There is no obvious explanation for this consistent effect at present.

Carbonic anhydrase activity in 'Cl$^-$-depeleted' and reconstituted thylakoids

PS II is inhibited by high concentrations of Cl^- [14]. At the same time Cl^-, at low concentrations, stimulates PS II by activating the oxygen-evolving mechanism [19]. Since high concentrations of Cl^- inhibit thylakoid carbonic anhydrase activity (Table I), one might expect also low concentrations to have a stimulatory effect. Thylakoids were isolated

TABLE VI

A COMPARISON OF HILL REACTION AND CARBONIC ANHYDRASE ACTIVITIES IN 'Cl$^-$-DEPLETED' MAIZE THYLAKOIDS GIVEN VARIOUS ANIONS

The reaction mixture contained 0.05 M Hepes (pH 7.0), 0.1 M sucrose, plus the anion indicated. Other additions and conditions were as described in the legend to Table IV. The 'Cl$^-$-depleted' thylakoids were obtained as described in Materials and Methods. The same preparation of thylakoids was used for both measurements. The numbers in parentheses represent reaction rates in the presence of the anion divided by the control rate.

Additions	Hill activity (μmol O_2 per mg Chl per h)	Relative carbonic anhydrase activity in a.u. (per mg Chl per min)
1. Control (no addition)	21	666
2. NaCl (10 mM)	143 (6.81)	1260 (1.89)
3. $CaCl_2$ (5 mM)	150 (7.14)	1486 (2.23)
4. NaBr (10 mM)	129 (6.14)	894 (1.34)
5. $NaNO_3$ (10 mM)	94 (4.48)	284 (0.42)
6. $NaNO_3$ (1 mM)	– –	746 (1.12)
7. NaF (10 mM)	28 (1.33)	674 (1.01)
8. Na_2SO_4 (10 mM)	20 (0.95)	634 (0.95)

in the absence of Cl^- and tested for the effects of added anions on both Hill reaction and carbonic anhydrase activity. The results are shown in Table VI. With respect to the Hill reaction, the results of Kelley and Izawa [19] were reproduced. Addition of 10 mM NaCl increased Hill activity nearly 7-fold (line 2). If the Cl^- was given as 5 mM $CaCl_2$ (line 3), little or no additional stimulation resulted. Br^- (line 4) also had a strong stimulating effect (6.14-fold increase) as had NO_3^- (line 5). In contrast, F^- and SO_4^{2-} had little or no stimulating effect on the Hill reaction (lines 7 and 8).

When carbonic anhydrase activity was measured, the effects of anions were similar in several respects, but different in others. The carbonic anhydrase activity was easily measurable even in the absence of added anion (Table VI, line 1). Cl^-, however, did increase the activity by a factor of about 1.9 (line 2). When the Cl^- was added as $CaCl_2$, an even greater stimulation, 2.23-fold, was noted (line 3). This effects of Ca^{2+} was modest but occurred in all of 4 thylakoid preparations. As with the Hill reaction, Br^- also stimulated carbonic anhydrase activity (line 4). On the other hand, 10 mM NO_3^- not only failed to stimulate the carbonic anhydrase activity, it produced a clear inhibition of more than 50% (line 5). A lower NO_3^- concentration, 1 mM, did produce a slight (1.12-fold) stimulation (line 6). As with the Hill reaction, F^- and SO_4^{2+} had little or no stimulating effect on carbonic anhydrase activity (lines 7 and 8).

The results show that Cl^- stimulates carbonic anhydrase activity associated with thylakoids. However, the results are not quantitatively similar to those obtained for the Hill reaction. It should be noted that the two measurements were done under different conditions and may not be directly comparable. The Hill reaction was measured in the light and in the presence of added electron acceptors. Carbonic anhydrase activity was measured in the dark without added acceptors. Thus, the significance of the quantitative differences is not clear. It should also be noted that NO_3^-, shown here to stimulate Hill activity, can also, under other conditions, have an inhibitory effect [7,20].

Discussion

The results presented here confirm the findings of Vaklinova et al. [11,12]: chloroplast thylakoids and PS II particles possess carbonic anhydrase activity. Additional evidence supports the notion that the activity is closely associated with the PS II complex. The following PS II modifying treatments or reagents have pronounced effects on the thylakoid carbonic anhydrase activity; light (photoinactivation), hydroxylamine and atrazine (both active in the light), acetazolamide, silicomolybdate, ferricyanide, DMBQ, Zn^{2+} and Mn^{2+}. Both PS II and the thylakoid carbonic anhydrase activity are stimulated by low concentrations of Cl^- and Br^-, but are inhibited by high concentrations of anions (formate, Cl^-, NO_3^-). Ca^{2+} also appears to have a stimulatory effect on both PS II [21,22] and on carbonic anhydrase activity. In addition, PS II particles are frequently enriched in carbonic anhydrase activity compared to the thylakoids from which they were derived.

The observations discussed above support the proposal that one of the polypeptide components of the PS II complex is, in fact, a form of carbonic anhydrase. This would seem to be the simplest, most compelling explanation of the data. On the other hand, the evidence is circumstantial. It is not possible to eliminate completely the possibility that the observed carbonic anhydrase activity represents some form of tightly bound contamination. The contaminating enzyme must persist in the production of PS II particles and, by coincidence, be influenced by all of the PS II modifiers used here. This ambiguity can, however, be resolved with further testing. In this regard, the literature on carbonic anhydrase contains numerous suggestions. For example, it has already been shown that a number of reagents can covalently bind to specific sites on carbonic anhydrase (for a review, see Ref. 18). One of these, bromoacetate, appears to bind to the PS II complex at the HCO_3^--binding sites [1]. With radioactively labelled bromoacetate, it may be possible to identify to PS II component that contains these sites. This protein should have carbonic anhydrase activity. Such work is now in progress in our laboratory.

The finding of carbonic anhydrase activity in

PS II would seem fortuitous in light of the extensive information available on this enzyme (for reviews, see Refs. 16 and 18). This knowledge can provide insight into aspects of PS II chemistry. For example, much can be deduced concerning anion, and particularly HCO_3^-, effects on the photosystem. The anion binding sites on carbonic anhydrase are well characterized. There are at least two; a relatively low-affinity inhibitory anion binding site and a higher-affinity 'noninhibitory' site. Acetate has been shown to bind to both sites on carbonic anhydrase [5]. Likewise, on PS II, there appears to be at least two non-substrate binding sites for HCO_3^- [6,7]. There also appears to be at least two Cl^--binding sites. These sites for HCO_3^- and Cl^- appear, in fact, to be identical. Kelley and Izawa [19] showed that a low (10 mM) concentration of Cl^- stimulates electron transport through PS II. HCO_3^- can substitute, though only weakly. Thus, one site on PS II, the 'Cl^--binding site' also binds HCO_3^- to some extent. In contrast, Good [15] showed that high (more than 100 mM) Cl^- concentrations inhibited PS II and that the effect was reversed by added HCO_3^-. This suggests a competitive interaction between Cl^- and HCO_3^- at a second site on PS II. Thus, both carbonic anhydrase and PS II have two distinct anion-binding sites. It is therefore possible that the 'HCO_3^--binding site' on PS II is, in fact, the inhibitory anion binding site on a carbonic anhydrase associated with PS II; while the 'Cl^--binding site' on PS II is the 'noninhibitory' anion binding site on the enzyme. Again, this suggestion is testable if the protein can be identified.

One of the most surprising observations reported here is the Mn^{2+}-reversible inhibitory effects of Zn^{2+} on the thylakoid carbonic anhydrase activity. In all animal sources of carbonic anhydrase, a tightly-bound Zn^{2+} is invariably the metal cofactor. Not surprisingly, a commercially available bovine carbonic anhydrase was tested here and found to be insensitive to added Zn^{2+} (data omitted). Enzymes from parsley [24] and pea [25] also contain Zn^{2+}. However, there is at least one report by Chiba et al. [26] of a Zn^{2+}-inhibited carbonic anhydrase from spinach leaves. These workers found that 1 mM $ZnCl_2$ inhibited their enzyme preparation by only 28% whereas that concentration of Zn^{2+} almost totally eliminated the activity reported here with maize thylakoids (Fig. 2). These findings suggest that plants may contain at least two forms of carbonic anhydrase, a soluble Zn^{2+}-containing enzyme and a thylakoid-bound Zn^{2+}-inhibited protein. During isolation, either pool could serve as a contaminant in the other. From numerous in-vitro studies, it is known that the Zn^{2+} in carbonic anhydrase can, with some difficulty, be replaced by other metals. Among these, only Co^{2+} and Mn^{2+} yield a still-active enzyme. The findings reported here would suggest that thylakoid carbonic anhydrase may be a naturally-occurring Mn^{2+}-containing enzyme. This does not, however, explain inhibition by Zn^{2+}, which one might expect to substitute for Mn^{2+} without loss of activity. For a discussion of possible roles for carbonic anhydrase in Photosystem II, the reader is encouraged to consult Refs. 1 and 27.

Many results presented here are not easily explicable at this time. It is not clear, for example, why light inhibits carbonic anhydrase activity in thylakoids, but has no effect on PS II particles. Likewise, it is not obvious why ferricyanide and DMBQ, in the dark, inhibits carbonic anhydrase activity or why Ca^{2+} stimulates it. Clearly, a number of questions are posed by the results. At the same time, the large body of literature on carbonic anhydrase provides numerous suggestions for future work to resolve these problems.

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