Oxidation–Reduction Potential Dependence of Photosystem II Carbonic Anhydrase in Maize Thylakoids

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ABSTRACT: In characterizing the carbonic anhydrase (CA) found in maize thylakoid membranes, it was observed that the enzyme's activity was inhibited somewhat when the Hill oxidant, ferricyanide, was given in the dark [Stemler, A. (1986) *Biochim. Biophys. Acta 850*, 97–107]. In the present work, a redox titration of this effect shows that the CA activity is mediated by a component that has a midpoint potential (E_m) of about 485 mV at pH 6.5 and a pH dependence of 60 mV/pH. These redox titration characteristics are identical to those of the redox mediator "D_{480}", which modulates formate and bicarbonate binding affinity to photosystem II (PS II). Bicarbonate binds to PS II more readily, and CA activity is higher, when D_{480} is reduced, whereas both bicarbonate binding and thylakoid-bound CA activity are low when D_{480} is oxidized in the dark by ferricyanide. Both the low bicarbonate binding affinity and the low CA activity induced by the presence of ferricyanide are reversed by a single saturating flash of light. In contrast, the activity of soluble CA, which is extracted from maize mesophyll cytosol, does not exhibit any redox dependence in the range 400–550 mV. Furthermore, thylakoid-bound CA activity is inhibited by 5 mM $ZnCl_2$ by as much as 75%, whereas the activity of soluble CA shows no significant decrease induced by $ZnCl_2$. Also, at a medium potential of 400 mV, ferricyanide (1 mM) inhibits soluble CA activity by 88% and thylakoid-bound CA activity by only 18%. It is concluded from these results that CA activity observed in thylakoids arises from CA inherent to PS II and is not some form of contamination by soluble CA. Possible roles of CA in PS II reaction mechanisms are discussed.

Photosynthesis begins with light-driven charge separations in PS II.[1] Subsequent electron transport through this multicomponent complex is inhibited upon binding of monovalent anions like formate, acetate, nitrate, and others (Good, 1963; Stemler & Murphy, 1985). The inhibitory effect of these anions can be reversed by their removal through the binding of bicarbonate, which is not inhibitory (Vermaas & van Rensen, 1981; Jursinic & Stemler, 1988) except at high pH (8.0) (Stemler, 1980a), and when silicomolybdate is used as the Hill oxidant (Jursinic & Stemler, 1986). The partial reactions within PS II that are affected by formate and bicarbonate occur both on the electron-acceptor side [for a review, see Blubaugh and Govindjee, (1988)] and on the electron-donor side (Stemler, 1981; Vermaas et al., 1984; Jursinic & Dennenberg, 1990).

It was shown previously (Stemler & Jursinic, 1993) that the binding affinity of PS II for formate is modulated by the oxidation state of a one-electron redox mediator with a midpoint potential (E_m) of about +480 mV at pH 7 (the E_m has a pH dependence of approximately 60 mV/pH). This PS II component was tentatively identified as the electron donor (E_m = +475 mV at pH 7.6) that was first described by Bearden and Malkin (1973) and later investigated by Babcock and Sauer (1975). For convenience, we refer to this yet uncharacterized component as "D_{480}". It was observed that anion binding (formate and bicarbonate) to PS II was low when D_{480} was oxidized in the dark by ferricyanide and high when D_{480} was reduced (Stemler & Jursinic, 1993). The low-affinity anion-binding state present when D_{480} is oxidized can be reversed by a single flash of light, from which it was concluded that D_{480} can be reduced in the light (Stemler & Jursinic, 1993).

It was shown in another study (Stemler, 1986) that a form of the enzyme carbonic anhydrase (CA) not only exists in washed maize thylakoids but is also concentrated in PS II-enriched membranes made from them. Membrane-bound CA has also been found in *Chlorella* cells (Pronina & Semenenko, 1984, 1988). There are a number of similarities between membrane-bound CA activity in maize and electron-transport activity. Thylakoid CA activity, like PS II electron transport, is inhibited by formate as well as other anions (Stemler, 1986). Inhibition by anions and imidazole shows the same pH dependence in both cases (Stemler & Jursinic, 1983). The specific CA inhibitor acetazolamide is also effective against PS II (Swader & Jacobson, 1972; Lonergan & Sargent, 1978; Stemler & Jursinic, 1983); an inhibitory response to this compound is usually considered diagnostic for the presence of CA.

Virtually all other inhibitors of CA that have been tested are also effective against PS II electron transport (Stemler & Jursinic, 1983). Thylakoid CA and PS II show the same qualitative dependence on Cl^-. This anion, at millimolar concentrations or less, stimulates PS II by activating the oxygen-evolving mechanism (Kelley & Izawa, 1978). Cl^- given to "Cl^--depleted" thylakoids also stimulates CA activity in thylakoids (Stemler, 1986). Ca^{2+} was also shown to have stimulatory effects on both PS II (Piccioni & Mauzerall, 1978; Brand, 1979) and thylakoid-bound CA activity (Stemler, 1986). These observations are all consistent with the hypothesis that there exists a form of CA tightly bound to thylakoid membranes and that the CA activity is associated with PS II electron transport. However, despite all the

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[1] Abbreviations: CA, carbonic anhydrase; chl, chlorophyll; D_{480}, uncharacterized photosystem II component with a midpoint potential of approximately +480 mV at pH 7.0; MES, 2-(*N*-morpholino)ethanesulfonic acid; PS II, photosystem II; rubisco, ribulose bisphosphate carboxylase/oxygenase; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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circumstantial evidence supporting this idea, it was not completely ruled out in the initial study (Stemler, 1986) that the CA activity was a form of contamination from extrathylakoid sources.

In the preliminary study on thylakoid-bound CA, it was observed that the activity was inhibited when samples were incubated in the dark in the presence of ferricyanide (Stemler, 1986). We therefore tested the possibility that D_{480}, the PS II redox component that modulates anion binding, also modulates thylakoid CA activity. We also compared membrane-bound CA to a soluble isoenzyme that was extracted from maize. We did this to further differentiate thylakoid-bound CA from soluble CA.

It is found that the binding of anions to PS II and the CA activity in thylakoid membranes show the same dependence upon oxidation–reduction potential of the medium. On the other hand, soluble and thylakoid-bound CA exhibit clear differences with respect to their responses to Zn^{2+} and ferricyanide treatments. We established that thylakoid-bound CA is not a tightly bound contaminant, but is indeed an inherent property of PS II.

MATERIALS AND METHODS

Thylakoid Source and Isolation. CA-containing thylakoids were isolated from maize (*Zea mays* L.) mesophyll tissue. Maize was chosen for this study, and for the previous study (Stemler, 1986), because of early reports that CA was difficult to isolate from maize and reports that soluble enzyme was confined to the cytoplasm of mesophyll cells (Everson & Slack, 1968). The amount of thylakoid CA varied substantially among preparations. We found that the highest activity was obtained if the maize leaves were harvested immediately after a 1-h or longer exposure to bright light. Thylakoids isolated on cloudy days were sometimes devoid of activity. The reason for this is not known.

Thylakoids were isolated from 2–3-week-old, greenhouse grown maize plants as previously described (Stemler, 1986). The grinding medium contained 0.025 M Tricine (pH 7.8), 0.2 M NaCl, and 5 mM $MgCl_2$. The suspension medium contained 0.025 M Tricine (pH 7.8), 0.3 M sucrose, and 5 mM $MgCl_2$. The chloroplast suspension was divided into aliquots and kept frozen at −80 °C until used for the determination of CA activity associated with thylakoids and for bicarbonate ($NaH^{14}CO_3$) binding experiments.

Soluble CA were extracted from 10-day-old leaf samples as described by Burnell et al. (1990) except that the extraction buffer consisted of 50 mM Tricine–NaOH (pH 8.2), 1 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM NaCl. One gram fresh weight of maize leaves was ground in 9 mL of extraction buffer.

Carbonic Anhydrase Activity Measurements. CA activity measurements were carried out by using the assay procedure previously developed (Stemler, 1993). The reaction vessels we now use were made from 60-mm-long glass cylinders (outside diameter, 12.5 mm; inside diameter, 9.5 mm). The $^{14}CO_2$-porous membrane fastened to the bottom of the cylinder was a Millipore poly(vinylidene difluoride) (PVDF) transfer membrane (Immobilon). The CO_2 permeability of this membrane is superior to that described by Stemler (1993), and it is more readily available. However, alcohols and wetting agents cannot be used in the reaction mixture with a PVDF membrane. CA activity measurements started when a solution of $NaH^{14}CO_3$ was injected into the stirred reaction medium consisting of maize thylakoids in 0.05 M Na-MES (pH as indicated in figure captions) and 0.01 M NaCl (to which a redox couple was added when needed). As $H^{14}CO_3^-$ was converted to $^{14}CO_2$, the radioactively labeled gas diffused through the membrane and was trapped by a NaOH-impregnated glass filter immediately below the reaction cylinder. After the reaction was stopped, the glass filter was immersed in scintillation fluid and radioactivity was determined. The duration of the assay was 10 s unless otherwise specified. For all experiments, CA-free control samples were included in order to assess the rate of spontaneous conversion of $H^{14}CO_3^-$ to $^{14}CO_2$. To make up these controls, an equivalent amount of chloroplast suspension medium, minus thylakoids, was added to the reaction mixture. The amount of radioactivity found in these controls was subtracted from that found in the experimental samples. In this report, this net CA activity in washed thylakoids is referred to as thylakoid-bound CA activity. CA activity is expressed in relative terms as counts/minute (cpm). Buffered solutions were routinely boiled for 5 min to kill any microorganisms and to reduce extraneous CO_2 and then cooled before use as reaction media. All experiments were carried out at 25 °C. Samples were kept in the dark until the assays were complete.

$H^{14}CO_3^-$ Binding to Thylakoid Membranes. The steady-state binding of $H^{14}CO_3^-$ to thylakoid membranes was measured with techniques already described in detail (Stemler & Murphy, 1983). Maize chloroplasts, containing a total of 40 μg of chl, were suspended in 0.05 M Na-MES, pH 6.5, 100 mM NaCl, 1 mM potassium ferricyanide, and ferrocyanide to the desired E_h. The total volume was 0.25 mL. To start binding, $NaH^{14}CO_3$ (1.4 μCi; final concentration, 96 μM) was injected into the samples. After incubation at room temperature in the dark for 30 min, binding was stopped by the addition of 1 mL of ice-cold buffered solution that contained 0.01 mM sodium phosphate, pH 7.6, and 0.01 M NaCl. The sample tubes were placed in a cold rotor, and the thylakoids were collected by centrifugation. After centrifugation, samples were processed and radioactivity determined as previously described (Stemler & Murphy, 1983).

To determine the effects of flashes on $H^{14}CO_3^-$ binding, we used the methods of Stemler et al. (1984). Thylakoids (40 μg of chl) were suspended in the reaction mixture described in the preceding paragraph. They were incubated in the dark for 10 min; then 0–3 saturating flashes were given, $NaH^{14}CO_3$ was injected immediately after the last flash, and binding was stopped 20 s later. The thylakoids were then processed, and the radioactivity was determined as described above. For all experiments, flashes were administered by placing the samples atop, and in direct contact with, an EG&G Model Fx200 xenon lamp operated at 4.9 J (1400 V with a 5.0-μF capacitance input energy). Flashes were spaced 1 s apart for all experiments.

Statistical analysis of data involved one-factor analysis of variance (ANOVA) with CA activity or bicarbonate binding being the factors investigated.

Measurements of Redox Potentials. Solution redox potential, E_h, was set by varying the potassium ferricyanide/ferrocyanide ratio. In all experiments, the concentration of potassium ferricyanide was kept constant. Ferrocyanide was added to obtain the desired E_h level. Solution E_h values were measured with a Cole-Parmer Model J-5994-21 combination platinum/reference electrode as described previously (Stemler & Jursinic, 1993).

More details are given in the figure captions and table footnotes.

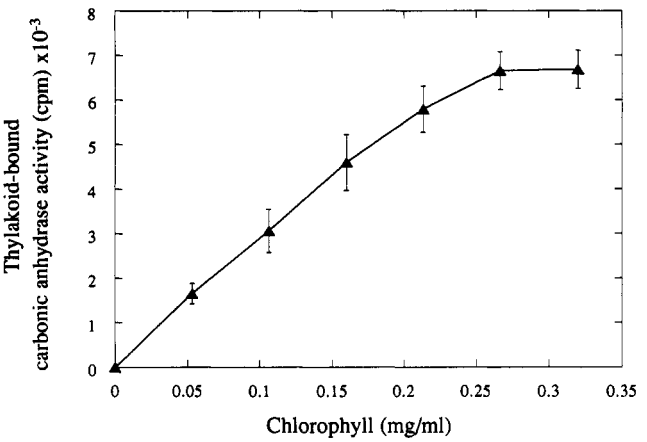


FIGURE 1: Thylakoid-bound carbonic anhydrase activity as a function of chlorophyll concentration. Chloroplasts (chlorophyll concentration as indicated) were suspended in the reaction mixture, pH 6.5. After a 50-s incubation the reaction was started by injection of 100 μL of bicarbonate solution that contained 0.5 μCi of $NaH^{14}CO_3$. The final reaction volume was 300 μL. The final concentration of $NaHCO_3$ was 28.7 μM. The measurement was stopped 10 s later by removing the reaction vessel from the glass filter trap (Stemler, 1993). Carbonic anhydrase activity is expressed in counts/min (cpm). Each point on the graph represents an average of at least six observations. Bars represent 1 SE from the mean.

RESULTS

Thylakoid-Bound Carbonic Anhydrase Activity as a Function of Chlorophyll Concentration. It has been shown previously (Stemler, 1986) with mass spectrometry that some form of CA is indeed associated with washed maize thylakoids and PS II-enriched membrane fragments. In this study, we used a new CA assay (Stemler, 1993). Figure 1 shows that thylakoid-bound CA activity is directly proportional to chlorophyll concentration. With these particular chloroplasts and conditions, the enzyme reached saturation at a concentration around 0.26 mg of chl/mL, a value that differed among preparations. In the experiments to be reported, we always used enzyme concentrations that were below saturation, that is, on the linear portion of the curve.

Redox Titration of ^{14}C-Bicarbonate Binding to Photosystem II and Thylakoid-Bound Carbonic Anhydrase Activity. It has been shown (Stemler & Jursinic, 1993) that the binding affinity of PS II for formate and bicarbonate depends on the oxidation state of an unidentified component, D_{480}. In these previous studies, binding affinity was deduced from the effects of these anions on the Hill reaction. Here, we repeated the redox titration of D_{480}, this time measuring radiolabeled bicarbonate binding directly. This method has the advantage that it can be done in complete darkness, eliminating any possible ambiguity introduced by light. The results are shown in Figure 2. The binding of $H^{14}CO_3^-$ is maximal at E_h = 400 mV and declines as E_h is raised to about 550 mV. The point at 400 mV was the same for controls given no redox couple, or those given only ferrocyanide (data omitted). The E_m for this redox effect of $H^{14}CO_3^-$ binding was about +485 mV. These results confirm that a photosystem II component with an E_m of about + 485 mV, D_{480}, modulates anion binding.

Since ferricyanide treatment in the dark also inhibited thylakoid CA activity (Stemler, 1986), we tested to see whether this effect was dependent on the E_h of the medium. The results are also shown in Figure 2 to allow direct comparison with the $H^{14}CO_3^-$ binding data. In fact, both bicarbonate binding affinity and thylakoid CA activity show the same redox dependence. The curves are superimposable. The E_m for inhibition of CA is likewise about +480 mV. It should be

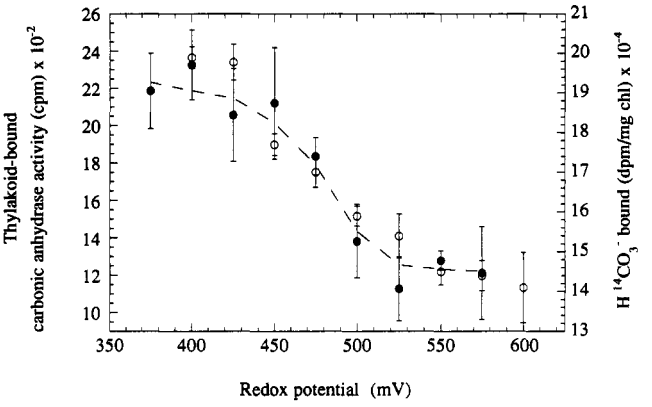


FIGURE 2: Bicarbonate binding to photosystem II and thylakoid-bound carbonic anhydrase activity as a function of redox potential of the medium. The procedure for measuring $H^{14}CO_3^-$ binding (○) to thylakoid membranes is given in Materials and Methods. For carbonic anhydrase measurements (●), chloroplasts (60 μg of chl) were suspended in 100 μL of redox-poised reaction medium (pH 6.5). Solution redox potentials were set as described in Materials and Methods. The ferricyanide concentration was kept at 1 mM. After 12 min of incubation, the reaction was started by injection of 50 μL of bicarbonate solution that contained 0.5 μCi of $NaH^{14}CO_3$. The final reaction volume was 170 μL. The final concentration of $NaHCO_3$ was 50.7 μM. Measurements were stopped 10 s later as described in the Figure 1 caption. Each point on the graph represents an average of at least six observations. Bars represent 1 SE from the mean.

Table 1: Comparison of the Effects of Varying Redox Potentials on Thylakoid-Bound and Soluble Carbonic Anhydrase Activities[a]

	soluble CA activity (cpm × 10^{-3})	% activity	thylakoid-bound CA activity (cpm × 10^{-3})	% activity
control	7.96 ± 0.74	100	7.96 ± 0.31	100
400 mV	0.93 ± 0.08	12	6.52 ± 0.63	82
575 mV	0.96 ± 0.05	12	3.39 ± 0.60	43
5 mM potassium ferrocyanide (E_h = 264 mV)	7.61 ± 0.54	96	7.91 ± 0.12	99

[a] Chloroplasts (60 μg of chl) and soluble carbonic anhydrase were incubated in 100 μL of reaction medium (pH 6.5). The solution redox potentials of samples redox poised at 425 or 550 mV were set as described in Materials and Methods. The concentration of ferricyanide in these samples was kept at 1 mM. The concentration of ferrocyanide in samples redox poised at 400 mV was about 5 mM. Controls were incubated without a redox couple. Potassium ferrocyanide reaction mixtures did not contain any ferricyanide. After 12 min of incubation, 100 μL of bicarbonate solution that contained 0.5 μCi of $NaH^{14}CO_3$ was injected. The final reaction volume was 240 μL. The final $NaHCO_3$ concentration was 35.9 μM. After 10 s, measurements were stopped as described in the caption of Figure 1. Carbonic anhydrase activity is expressed in counts/minute (cpm). Data are normalized to the same control values. Each value is the mean and SE of at least six observations.

mentioned that the highest CA activity, seen at 400 mV, was 82% of the control rate in samples given no redox couple or only ferrocyanide (Table 1). We attribute this decrease in activity to a slight inhibition of thylakoid-bound CA by 1 mM ferricyanide that is not a function of E_h. Increasing E_h from 400 to 575 mV causes a 48% (p = 0.0002) decrease in thylakoid CA activity and a 30% (p = 0.0001) decrease in bicarbonate binding affinity.

We repeated the CA redox titration at a higher pH (7.6) than the one shown in Figure 2 (pH 6.5). Results are shown in Figure 3. The midpoint potential clearly shifted to a lower potential (from around 480 to 410 mV, or 64 mV/pH unit) at the higher pH value. It appears, therefore, that the E_m of the component determining CA activity shows the same pH dependence as the E_m of the redox mediator that determines

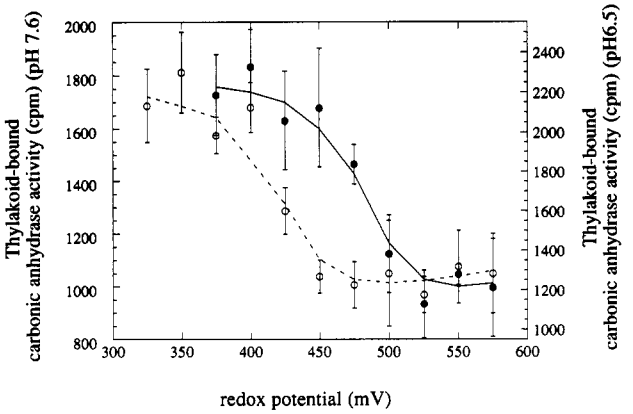


FIGURE 3: Redox titrations for maize thylakoid carbonic anhydrase activity at two different pH values. Protocol and conditions were the same as described in Figure 2 [carbonic anhydrase measurements at pH 6.5 (●)] except that in the redox titration at pH 7.6 (○), MES was substituted by Tricine buffer and only 30 μg of chl was used. To start the reaction, 0.85 μCi of $NaH^{14}CO_3$ was injected; the final concentration of $NaHCO_3$ was 86.2 μM.

formate/bicarbonate affinity to PS II (Stemler & Jursinic, 1993).

Flash Dependence of ^{14}C-Bicarbonate Binding to Photosystem II and Thylakoid-Bound Carbonic Anhydrase Activity. Previous studies (Stemler & Jursinic, 1993) showed that the effect of ferricyanide in decreasing formate affinity could be reversed by a single flash. Hill activity was used as an indicator of formate binding. In the present study we measured $H^{14}CO_3^-$ binding as a function of flashes given to dark-adapted chloroplasts. The thylakoids were first incubated for 10 min in reaction medium set at either 50 or 425 mV or without a redox couple. The samples were given 0–3 saturating flashes, $NaH^{14}CO_3$ was injected, and binding was stopped 20 s later by raising the pH (Stemler et al., 1984). The results are shown in Figure 4A. In the dark, bicarbonate binding was higher in samples poised at 425 mV or in non-redox-poised controls than in samples poised at 550 mV. This shows, consistently with the data in Figure 2 and with previous results (Stemler & Jursinic, 1993), that the affinity of PS II for bicarbonate is greater when the PS II component, D_{480}, is reduced. After the first flash, bicarbonate binding significantly increased (p = 0.0001) in samples redox poised at 550 mV and reached the same values as samples poised at 425 mV or not redox poised. Bicarbonate binding remained constant after subsequent flashes. There was no change in bicarbonate binding in response to flashes in samples poised at 425 mV or in non-redox-poised controls.

We were also interested to see whether depression of CA activity by ferricyanide (see Figure 2) is reversed by a single flash. Thylakoids were incubated in the dark for 6 min, either as controls (no redox couple) or in samples poised at 425 or 550 mV. Zero to three saturating flashes were administered just before CA activity was assayed. Results are shown in Figure 4B. In the non-redox-poised controls, flashes have no effect on the thylakoid-bound CA activity. The same lack of response to flashes was observed in samples poised at 425 mV. The apparent decrease in activity after the third flash in both treatments was not significant (p = 0.3155 and 0.52, respectively). In contrast, CA activity was initially lower in samples set at 550 mV, consistent with the results shown in Figures 2 and 3. The CA activity significantly increased (p = 0.0021) after the first flash and remained constant on subsequent flashes. After the first flash, CA activity, although significantly higher (p = 0.0021) than before flashes were

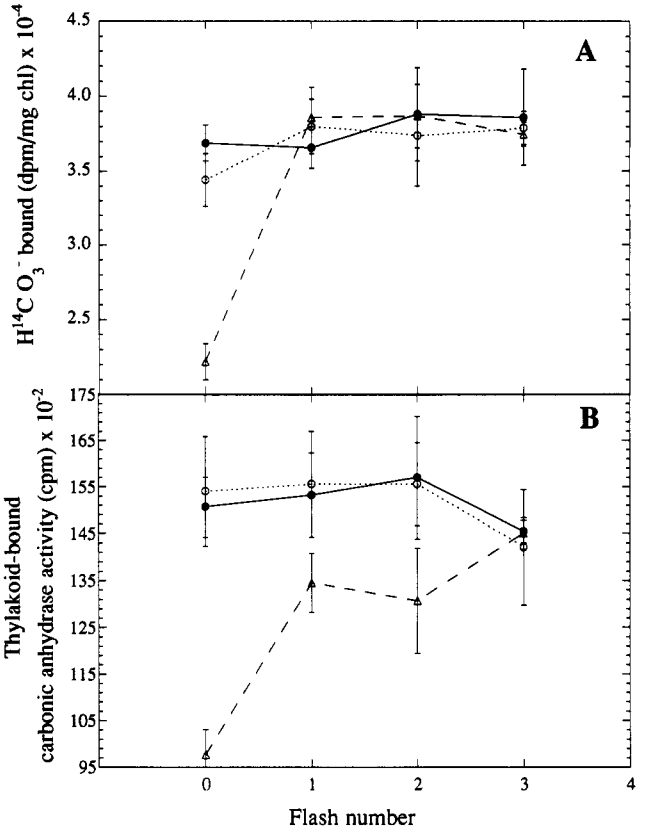


FIGURE 4: Bicarbonate binding and thylakoid-bound carbonic anhydrase activity at two different E_h values as a function of flash number. The binding of $H^{14}CO_3^-$ after flashes (A) was determined as described in Materials and Methods. For carbonic anhydrase activity measurements (B), chloroplasts (58 μg of chl) were suspended in 100 μL of reaction medium (pH 6.4). The concentration of ferricyanide in these samples was kept at 0.2 mM. After 6 min of incubation, 0–3 saturating flashes were given right before injection of 50 μL of bicarbonate solution that contained 0.5 μCi of $NaH^{14}CO_3$. The final reaction volume and the final $NaHCO_3$ concentration were the same as in Figure 2. After 10 s, measurements were stopped as described in Figure 1. For both (A) and (B), the solution redox potentials for samples poised at 425 (○) or 550 (△) mV were set as described in Materials and Methods. Controls (●) were incubated without the redox couple. Each point on the graph represents an average of at least 6 observations. Bars represent 1 SE from the mean.

administered, was still significantly lower (p = 0.0623) than the activity of those samples with no redox couple or poised at 425 mV. The reason for the lack of full recovery of CA activity after flashes is not known.

Comparison between Soluble and Thylakoid-Bound Carbonic Anhydrase. To eliminate further the possibility that the observed CA activity associated with thylakoids is a result of a tightly bound contaminant, we extracted CA from the cytosol (as described in Materials and Methods) and conducted a series of tests comparing soluble and membrane-bound enzymes. As shown in both Figure 2 and Table 1, CA activity associated with thylakoids is strongly dependent on the redox potential of the medium; its activity decreases by as much as 48% between 400 and 575 mV. On the other hand, the enzyme extracted from the cytosol (soluble CA) was strongly inhibited (88%) at 400 mV and did not respond further when the E_h was raised to 550 mV (Table 1). Thylakoid-bound CA activity decreased only by 18% at 400 mV. As mentioned earlier, this 18% reduction probably represents chemical inhibition of thylakoid CA by 1 mM ferricyanide not related to E_h. Samples which contained only ferrocyanide did not show any significant

Table 2: Comparison of the Effects of $ZnCl_2$ on Thylakoid-Bound and Soluble Carbonic Anhydrase Activities[a]

		carbonic anhydrase activity (cpm × 10^{-3})			
		$-ZnCl_2$	$+ZnCl_2$	% activity	p^b
±1 mM $ZnCl_2$	soluble CA	42.5 ± 1.7	43.0 ± 1.3	100	0.83
	thylakoid-bound CA	42.5 ± 3.2	26.2 ± 2.0	62	0.0003
±5 mM $ZnCl_2$	soluble CA	35.5 ± 4.8	31.5 ± 1.6	89	0.42
	thylakoid-bound CA	35.5 ± 3.7	8.8 ± 1.3	25	0.002

[a] Chloroplasts (63 μg of chl) and soluble carbonic anhydrase were incubated in 100 μL of reaction buffer (pH 6.3) with or without $ZnCl_2$. After 6 min, 100 μL of bicarbonate solution that contained 0.5 μCi of $NaH^{14}CO_3$ was injected. The final reaction volume was 220 μL. The final $NaHCO_3$ concentration was 39.1 μM. After 20 s, measurements were stopped as described in the caption of Figure 1. Carbonic anhydrase activity is expressed in counts/minute (cpm). For each $ZnCl_2$ concentration, data are normalized to the same control ($-ZnCl_2$) values. Each value is the mean and SE of at least six observations. [b] Probability from one-factor ANOVA.

decrease in activity in either soluble or thylakoid-bound CA (Table 1).

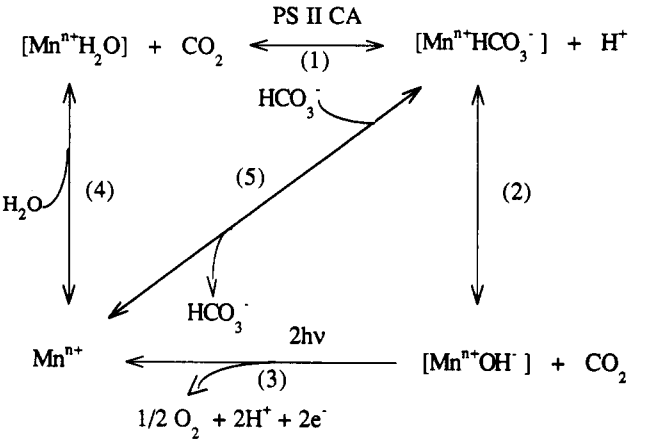
It was shown previously (Stemler, 1986) that $ZnCl_2$ inhibited CA associated with thylakoids, an unexpected result since Zn^{2+} is a required cofactor for all known CA. We compared the effects of $ZnCl_2$ on thylakoid-bound and soluble CA activities. As expected, soluble and thylakoid-bound CA responded differently to incubation with Zn^{2+} (Table 2). At 1 mM $ZnCl_2$, soluble CA did not decrease in activity. In contrast, thylakoid-bound CA activity decreased significantly by 38% ($p = 0.0003$). At 5 mM $ZnCl_2$, thylakoid-bound CA activity significantly decreased by 75% ($p = 0.002$), whereas the slight apparent decrease in soluble CA activity was not significant ($p = 0.42$). Furthermore, similarly to soluble maize CA, bovine CA activity did not decrease after incubation with 5 mM $ZnCl_2$ (data not shown). It is clear from these results that maize leaves contain at least two distinct pools of CA. One pool is present in the cytosol of mesophyll cells as determined by Everson and Slack (1968); a second is inherent to thylakoids and cannot be attributed to contamination.

DISCUSSION

On the basis of our results, we conclude the following: (1) CA activity in thylakoids [and PS II membranes; see Stemler (1986)] is an intrinsic function of PS II. It is not a contamination from other cellular sources. (2) The intrinsic CA is a target for inhibitory anions and certain other PS II inhibitors such as acetazolamide, imidazole (Stemler & Jursinic, 1983), and zinc (Tripathy & Mohanty, 1980). (3) The PS II CA activity is modulated by the oxidation state of a component with a midpoint potential of about 480 mV at pH 7. This appears to be the same component, D_{480}, that modulates formate/bicarbonate binding to PS II (Stemler & Jursinic, 1993).

Given the presence of CA activity in PS II, inferences can be made regarding the bicarbonate "requirement" for PS II electron transport. All carbonic anhydrases studied have a site where inhibitory monovalent anions bind. This site is near the zinc cofactor (Håkansson et al., 1992). Formate and other anions bind to this site and displace the substrate water molecule. For human CA, the three-dimensional structure of the inhibitory anion binding site has been described (Lindahl et al., 1993; Xue et al., 1993). It has been shown that bicarbonate will also bind to the inhibitory anion binding

Scheme 1



site on CA (Yeagle et al., 1975). However, the presence of bicarbonate at the inhibitory anion binding site is not necessary for activity, since the enzyme works optimally when this site is empty. We now feel justified in equating the inhibitory anion binding site common to all carbonic anhydrases with the formate/bicarbonate binding site on PS II. By inference, bicarbonate is not required *at this particular site*. In other words, "membrane-bound" bicarbonate, as measured here with $H^{14}CO_3^-$ (Figure 2), should not be necessary for PS II functions. This has already been concluded on the basis of direct measurements (Stemler, 1989; Jursinic & Stemler, 1992). However, the question of a CO_2/HCO_3^- requirement in PS II is still valid, if given a different focus. We can now ask, is CO_2/HCO_3^- necessary at the *catalytic* site (as opposed to the inhibitory anion binding site) on the PS II CA in order for electron transport to occur? In considering a bicarbonate requirement it is essential to distinguish between the two different sites for bicarbonate, and to view the question in this new context.

At the present time, the location and function of CA in PS II is not apparent. There is circumstantial evidence that the CA may be involved in reactions on the electron-donor side of PS II or in oxygen evolution. The component that modulates CA activity, D_{480}, can donate electrons directly or indirectly to the oxidized reaction center (Bearden & Malkin, 1973; Babcock & Sauer, 1975). The CA activity is stimulated by Ca^{2+} and by low concentrations of Cl^- (Stemler, 1986). Both of these are cofactors in oxygen evolution. Formate, among its other effects, slows down the release rate of oxygen (Stemler, 1981; Jursinic & Dennenberg, 1990) and prevents electron donation from Y_D to the higher s-states of the oxygen-evolving mechanism (Vermaas et al., 1984).

In interpreting the "bicarbonate effect" in PS II, several authors (Kreutz, 1974; Metzner, 1978; Stemler, 1980b) suggested that bicarbonate might actually be a chemical intermediate in the chemistry of oxygen evolution. There are many such schemes possible. For example, one might assume that oxygen evolution begins with a hydroxylated manganese, $[Mn^{n+}OH^-]$. The formation of this starting complex may be problematic at the low pH believed to be the environment of the oxygen-evolving mechanism. The concentration of OH^- would be low and the deprotonation of a manganese-bound water, $[Mn^{n+}H_2O]$, may be improbable in a reasonable time period. In such a scenario, the formation of the starting complex $[Mn^{n+}OH^-]$ could require CO_2 for deprotonation of a water adduct as shown in Scheme 1. In this simple scheme, CO_2 works catalytically to deprotonate a water molecule at the active site of oxygen evolution. Bicarbonate is also shown

binding directly and reversibly to Mn^{n+} (reaction 5). Reactions 1, 2, and 5, taken together, constitute CA activity, but their sole purpose is to hydroxylate Mn^{n+}.

All models that postulate a direct role for bicarbonate in the chemistry of oxygen evolution have been tested with isotope experiments [e.g., Stemler and Radmer (1975) and Radmer and Ollinger (1980)]. In experiments in which oxygen is evolved in the presence of either $HC^{18}O_3^-$ or $H_2{}^{18}O$, the evolved oxygen always approaches water in its isotopic content. However, these experiments have been interpreted without knowledge that PS II itself has CA activity. Such activity could quickly exchange ^{18}O between water and HCO_3^- in the vicinity of the oxygen-evolving mechanism, thus leading to ambiguous results.

Other experiments have been done in systems that have reached isotopic exchange equilibrium [e.g., Holt and French (1948) and Metzner et al. (1979)]. These experiments take advantage of the fact that, at equilibrium, CO_2/HCO_3^- in solution will be slightly enriched in ^{18}O compared to water. That is, a slight but easily measurable partitioning of ^{18}O takes place that favors CO_2/HCO_3^-. Yet even in these systems, the oxygen evolved by chloroplasts or algae is not enriched in ^{18}O to the extent predicted if bicarbonate were the source of the evolved oxygen. It would seem that the results rule out all of the hypotheses that include a role for bicarbonate in oxygen evolution. However, this interpretation contains at least one assumption. It is assumed that the oxygen-evolving process does not itself discriminate against heavier isotopes. Such discrimination is fairly common in biological systems, for example, catalysis by ribulose bisphosphate carboxylase/oxygenase (rubisco) (Christeller et al., 1976). A small isotopic discrimination factor in oxygen evolution could compensate for the partitioning effect of ^{18}O into CO_2/HCO_3^- and account for the observed results. We therefore question whether isotope experiments have so far yielded conclusive evidence against a possible catalytic role of CO_2/HCO_3^- in oxygen evolution.

While the PS II CA may be acting on the electron-donor side of PS II, other possibilities exist. There is also circumstantial evidence that CA may be involved on the electron-acceptor side as well. It is well known that formate, an inhibitor of PS II CA (Stemler, 1986), slows electron transfer between the primary stable acceptor Q_A and plastoquinone (Jursinic & Stemler, 1986; Eaton-Rye & Govindjee, 1988; van Rensen et al., 1988). The EPR signal associated with non-heme iron (Q_{400}) is dramatically altered by formate and other anions [Vermaas & Rutherford, 1984; for a discussion, see Diner et al. (1991)]. Another explanation of the bicarbonate effect to account for "acceptor-side" effects was that bicarbonate complexed with Q_B (plastoquinone) and, as a proton donor/acceptor, could facilitate "proton pumping" (Stemler, 1980). This idea has been further developed in recent times [van Rensen et al., 1988; for a discussion, see Govindjee (1993)]. We could now suggest a similar role for PS II CA in the protonation of plastoquinone. Since a proton is liberated in the hydration of CO_2, we can imagine it transferred to plastoquinone, PQ, as follows:

$$2CO_2 + 2H_2O + PQ^{2-} \xrightarrow{\text{PS II CA}} 2HCO_3^- + PQH_2$$

In order for the proton pump to operate in creating a $\Delta\mu_H$, it would be necessary, according to this acceptor-side scheme, for bicarbonate to be ejected into the chloroplast stroma. There, it could take up a proton, possibly utilizing a soluble CA, and re-form CO_2. The cycle could then continue, resulting in a net transfer of protons from the stroma to PQ^{2-}. While such a scheme could explain the effects of formate on the electron-acceptor reactions of PS II, there is still no direct evidence that bicarbonate or CA activity is necessary for the protonation of plastoquinone. Finally, it was proposed by other researchers (Pronina & Semenenko, 1988) that membrane-bound CA in *Chlorella* chloroplasts could be involved in converting bicarbonate in the stroma to CO_2, thereby concentrating CO_2 in the zone of carboxylation by rubisco. Our work, however, is done with maize mesophyll chloroplasts where rubisco is not present. Nevertheless, we will not exclude the possibility that PS II CA activity was retained in maize during the evolution of C_4 photosynthesis. Until additional studies are done, this scheme, and the others discussed here and elsewhere, to explain the function of PS II CA will remain speculative.

In conclusion, we consider several findings of this study to be particularly valuable. (1) The formate and bicarbonate effects on PS II can now be placed in a reasonable biochemical context. These anions bind to the inhibitory anion binding site on an intrinsic PS II CA. (2) A unique CA has been described. To the authors' knowledge this is the first CA in which activity and anion binding affinity have been redox titrated. It also seems to be the only CA inhibited by zinc, normally the metal cofactor at the catalytic site. It is not clear at present why this inhibition occurs. We can only repeat earlier speculation (Stemler & Jursinic, 1983; Stemler, 1986) that the PS II CA may be a manganese-containing instead of a zinc-containing enzyme. Zinc may inhibit by replacing the native manganese. (3) The study provides additional incentive to determine the chemical identity of D_{480}.

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