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PSII carbonic anhydrase activity and the bicarbonate effect.

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Introduction

Among the many chemical models for oxygen evolution, several incorporate the idea that bicarbonate anions (HCO_3^-), rather than water molecules directly, are the immediate source of both electrons and oxygen (Metzner and Fischer, 1969; Kreutz, 1974; Stemler, 1980; Zeinalov, 1982). While water remains the ultimate source of oxygen, CO_2, in the form of bicarbonate, plays a necessary "catalytic" role. Although the several models differ in detail, they can all be summarized as follows:

$$2\,HCO_3^- + 2\,H^+ \xrightarrow{4\,h\nu} O_2 + 2CO_2 + 4\,H^+ + 4\,e^-$$

$$\uparrow \underline{\qquad\qquad\qquad\qquad} \downarrow$$

$$+\,2\,H_2O$$

In this scheme, bicarbonate is proposed to bind to the manganese cluster and be dehydrated to CO_2 in the course of oxygen evolution. The CO_2 is then rehydrated and the reaction proceeds in a cyclic manner. However, a potential problem with this model is that the spontaneous hydration rate of CO_2 to form HCO_3^- is slow, and very little of this anion is stable at the low pH environment of the oxygen-evolving mechanism. For the scheme to work, a specific prediction was made (Stemler, 1985) that the PSII complex must itself be able to quickly hydrate CO_2 to form the substrate HCO_3^-. That is, PSII must behave like carbonic anhydrase, the enzyme that normally catalyzes this reaction.

The discovery of PSII carbonic anhydrase:

The presence of CA activity in PSII has been verified in a number of labs (Stemler 1986; Karlsson et al 1995; Moskvin et al 1998, Dai et al 2001). Early evidence obtained by the first author is presented in Figure 1. From the results of mass spectrometry, it can be seen that maize PSII-enriched membranes catalyze a rapid rise in the mass 48 signal due to conversion of injected $H^{18}CO_3^-$ to $C^{18}O_2$, followed by a rapid isotopic exchange between added $HC^{18}O_3^-$ and $H_2{}^{16}O$, clear indication of CA activity.

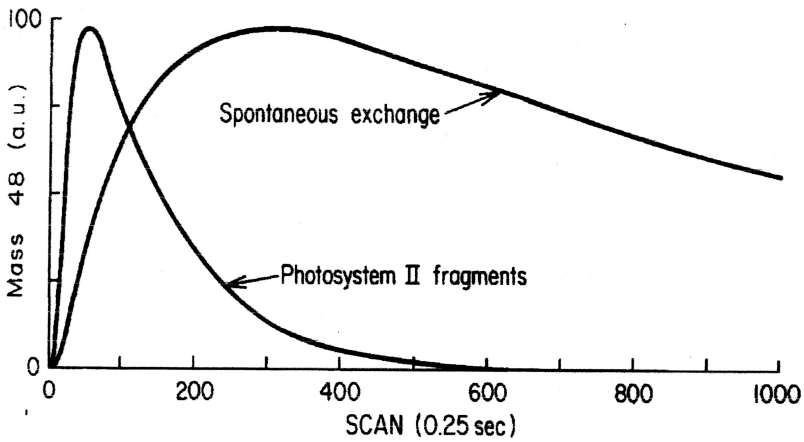

Figure 1. Carbonic anhydrase in PSII-enriched membranes catalyses isotopic exchange between $C^{18}O_2$ and $H_2{}^{16}O$. For details see Stemler (1986).

Some characteristics of PSIICA

While the CA associated with PSII is only partially characterized, some unique features are now in evidence. For example, maize thylakoid CA shows definite redox dependence (Figure 2). The Em of the PSII component that modulates the activity is about +480mV at pH 7.0. This is the same component that modulates bicarbonate and formate binding to PSII (Figure 2). We have proposed that the component is "D_{480}", an auxiliary electron donor to PSII discovered by Bearden and Malkin (1973).

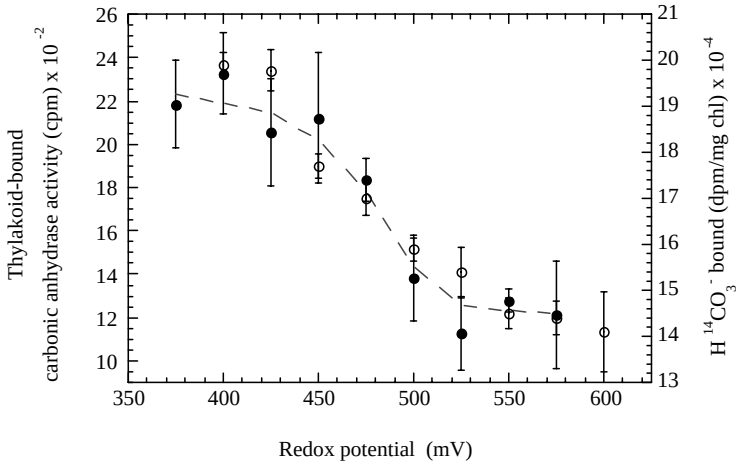

Figure 2. Redox dependence of PSII carbonic anhydrase activity (closed circles) and binding affinity of $H^{14}CO_3^-$ (open circles) in maize thylakoid membranes. For details see Moubarak-Milad and Stemler (1994).

Flashing light also modulates the CA activity in maize thylakoids (Figure 3). After incubating the membranes in the dark, the first flash of a series increases CA activity, while a second flash decreases it again. Oscillations, however, are not pronounced and damping occurs rapidly. While it is clear that the CA activity is somehow linked to PSII turnover, a detailed model awaits further study.

The CA activity associated with PSII-enriched membranes that were washed in 1 M $CaCl_2$ to remove extrinsic proteins is shown in Figure 4. Both oxygen evolution and CA activity are highly dependent on added Cl^-, with rates increasing up to and beyond

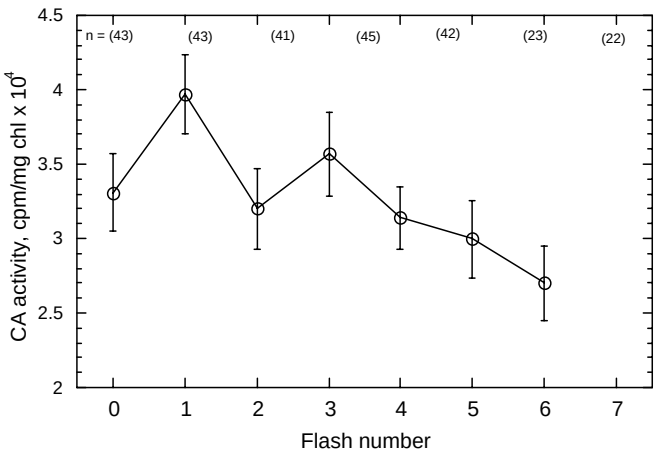

Figure 3. CA activity (hydration) in maize thylakoids as a function of flash number given 5s before the 10 s assay. The reaction mixture contained 0.05 M Na-HEPES, pH 7.2, 0.01 M NaCl, 114µg chl/mL

0.4 M. This is very unusual behavior for a CA, all others described in the literature are inhibited by high concentrations of monovalent anions such as chloride, formate, acetate, etc.

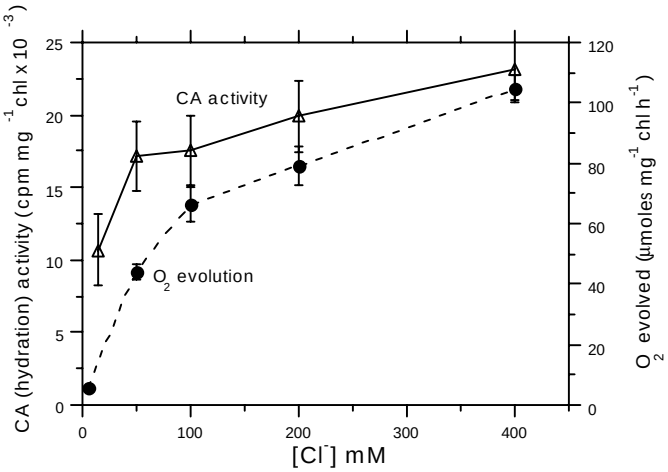

Figure 4. Carbonic anhydrase activity and oxygen evolution as a function of Cl^- concentration in maize PSII-enriched membranes washed with 1 M $CaCl_2$. For details see Stemler 1998.

Two sources of PSII CA activity

In *Chlamydomonas reinhardtii*, a CA located in the thylakoid lumen can be removed from PSII-enriched membranes by washing with 0.2 M KCl (Karlsson et al 1995). We applied various wash treatments to maize mesophyll PSII membranes in an attempt to likewise remove some of the CA activity. We found that even 1 M KCl was almost completely ineffective at removing CA activity (Figure 5). At the same 1 M concentration, NaCl was only slightly more effective. Total removal of the CA activity required 1 M $CaCl_2$. The activity was recoverable in the wash solutions, in particular, the $CaCl_2$ wash. It is important to note that under these assay conditions, pH 5.5, 10 mM NaCl, and measuring *dehydration*, the $CaCl_2$-washed PSII membranes showed no CA

activity. However, under different assay conditions, pH 7.2, 0.4 M NaCl, added Ca^{2+}, and measuring *hydration*, abundant CA activity is observed in these same $CaCl_2$-washed

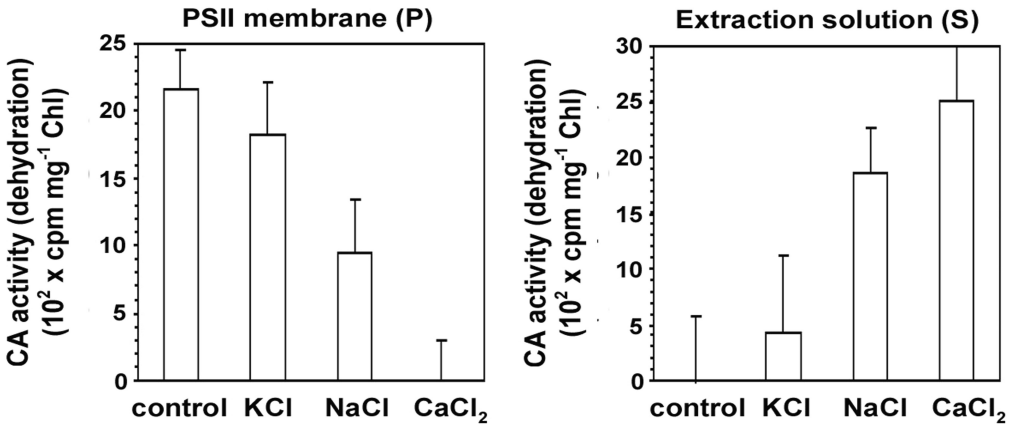

Figure 5. Removal of CA activity from PSII-enriched membranes by various 1 M salt solutions and recovery of the activity in the supernatant.

PSII membranes (Figure 4). We interpret this to mean that there are two distinct sources of CA activity associated with PSII. One, an extrinsic source, that we denote CA_{ext}, is removable by $CaCl_2$ wash. The other, an intrinsic source, remains after $CaCl_2$ wash and is denoted CA_{int}.

Extrinsic PSII CA

CA_{ext} found in the crude $CaCl_2$ wash solution can be characterized in vitro after desalting or dilution of the $CaCl_2$. CA activity as a function of pH is shown in Figure 6. The ability of CA_{ext} to dehydrate HCO_3^- is apparent only at or below pH 6.0. It is important to emphasize that dehydration is measured here. Despite our best efforts, the enzyme has not yet demonstrated hydration activity in vitro.

We have attempted to assess the molecular weight of the CA_{ext} with SDS-PAGE

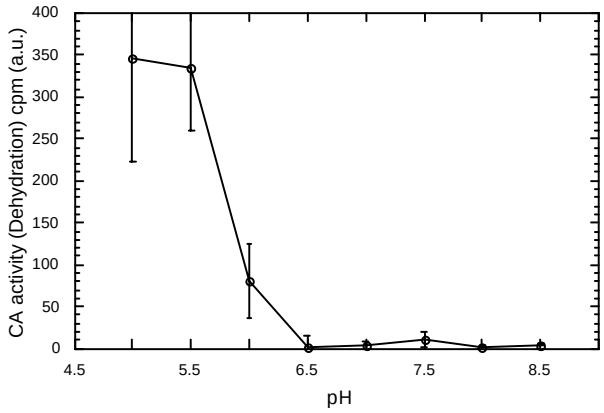

Figure 6. CA activity as a function of pH in enzyme extracted from PSII-enriched membranes with 1 M $CaCl_2$. The extract was diluted by a factor of 400 in the reaction mixture. Only dehydration activity was detected in the extract.

and antibodies against the *C. reinhardtii* enzyme and against the extrinsic 33 kDa PSII protein (OEC 33). Figure 7 shows that the CA_{ext} and the OEC33 protein appear to co-migrate when subjected to electrophoresis. There are several ways of interpreting these results. One possibility is that the CA_{ext} has a molecular weight of 33 kDa and is normally hidden in the OEC33 band. Another hypothesis is that the CA antibody cross-reacts with the OEC33. In this case, either there may be so little CA present that it is not detected in a separate band, or that the OEC33 *is* CA_{ext}, an interesting possibility. Our ongoing studies are testing these alternatives.

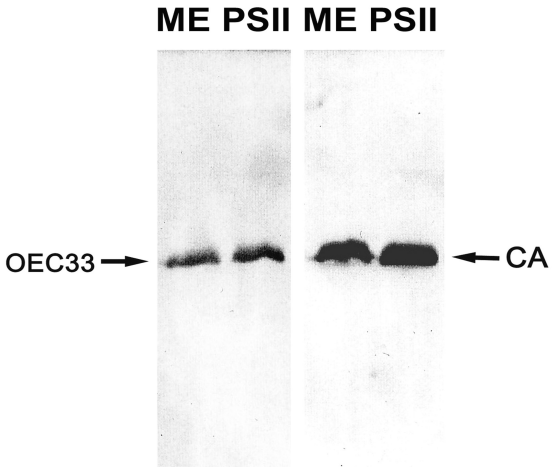

Figure 7. Western blots developed from maize mesophyll thylakoids (ME) and PSII membranes. The two tracks on the right show antibody to C. reinhardtii CA reacting at the same position as pea antibody against the 33kDa extrinsic PSII protein. (left two tracks).

Intrinsic PSII CA

The PSII-enriched membranes that remain after $CaCl_2$ wash likewise show CA activity (Figure 4). However, it is a distinctly different kind of activity (hydration vs. dehydration in the CA_{ext}) and requires a different set of experimental conditions. In Figure 8 is shown the hydration activity of washed PSII membranes as a function of pH.

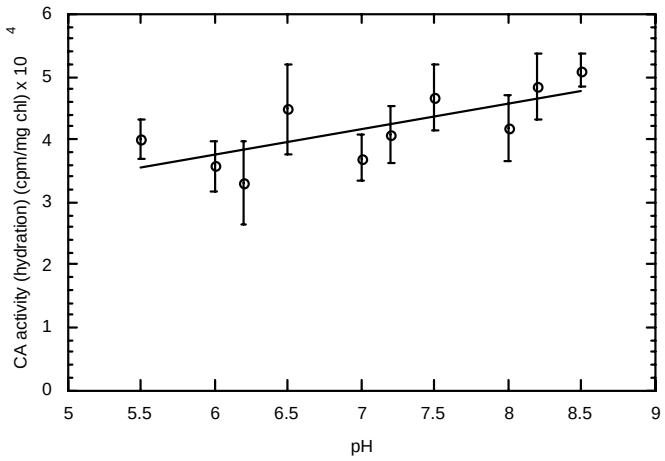

Figure 8. Intrinsic PSIICA activity as a function of pH. The reaction mixture contained 0.4 M NaCl and 0.01 M $CaCl_2$.

The CA_{int} demonstrates hydration activity that is practically pH independent. This means that the intrinsic PSIICA can manufacture bicarbonate even at the low pH that normally surrounds the oxygen-evolving mechanism. This is an unusual and

significant property of this particular CA. Typically, CA does not hydrate CO_2 at pH much below 7 (Coleman 1980). However, it is an absolute requirement if bicarbonate is an intermediate in oxygen evolution, as discussed in the introduction. We have not yet been able to observe dehydration activity in CA_{int}.

Conclusions and discussion

There are two distinct sources of carbonic anhydrase associated with PSII. They appear to have different catalytic functions with respect to the hydration of CO_2 vs. the dehydration of HCO_3^-. The intrinsic CA is probably associated with the oxygen-evolving mechanism, judging from the parallel stimulatory effects of chloride and calcium on oxygen evolution and CA activity. It may function to supply necessary bicarbonate for the operation of photosystem II. The extrinsic CA does not appear to be absolutely essential for oxygen evolution, since $CaCl_2$-washed membranes still evolve oxygen, albeit at a lower rate. We guess that the extrinsic CA may have some indirect role such as helping to supply CO_2 to the intrinsic CA. While the evidence presented here is consistent with bicarbonate acting as a chemical intermediate in oxygen evolution, direct evidence is still lacking.

Acknowledgements

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