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Differing responses of the two forms of photosystem II carbonic anhydrase to chloride, cations, and pH

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Abstract

The effects of Cl^-, Mn^{2+}, Ca^{2+}, and pH on extrinsic and intrinsic photosystem II carbonic anhydrase activity were compared. Under the conditions of our in vitro experiments, extrinsic CA activity, located on the OEC33 protein, was optimum at about 30 mM Cl^-, and strongly inhibited above this concentration. This enzyme is activated by Mn^{2+} and stimulated somewhat by Ca^{2+}. The OEC33 showed dehydration activity that is optimum at pH 6 or below. In contrast, intrinsic CA activity found in the PSII complex after removal of extrinsic proteins was stimulated by Cl^- up to 0.4 M. Ca^{2+} appears to be the required cofactor, which implies that the location of the intrinsic CA activity is in the immediate vicinity of the $CaMn_4$ complex. Up to now, intrinsic CA has shown only hydration activity that is nearly pH independent.
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Keywords: Bicarbonate; Carbonic anhydrase; Chloride; OEC33; Manganese Stabilizing protein; Photosystem II

1. Introduction

The function of bicarbonate on the donor side of photosystem II (PSII) remains an unsettled issue. It is known, for example, that HCO_3^- accelerates the photoassembly of the manganese cluster [1], implying a possible structural role. On the other hand, a role as an electron donor to PSII, possibly as an evolutionary step in the development of direct water splitting, has been suggested [2]. A more central role for bicarbonate in oxygen evolution was proposed by Helmut Metzner [3] and Werner Kreutz [4]. They suggested that bicarbonate acts as a “shuttle” to bring solvent water to the catalytic site. Thus, in this case, bicarbonate is the immediate precursor to O_2 while water is the ultimate source. Crucial to this proposal would be the necessary presence of a PSII-associated carbonic anhydrase (CA), to rapidly rehydrate catalytic amounts of CO_2 before it could be lost from the sample. Evidence for such PSII-associated CA has been documented [5,6].

At least two sources of carbonic anhydrase (CA) activity are closely associated with photosystem II (PSII) in photosynthetic membranes [5,6]. One source is located on the extrinsic 33 kDa protein [7], also called the oxygen-evolving complex 33 (OEC33), the manganese stabilizing protein (MSP), or the PsbO protein (reviewed in [8]). This protein, which we call extrinsic CA or CA_{ext}, is removable by washing PSII membrane fragments with 1 M $CaCl_2$ and has been overexpressed in *Escherichia coli* [7]. When the OEC33 is removed, another source of CA activity is revealed [5,9], this one associated with the core PSII complex [9]. Before the physiological role of each source of CA can be tested, it is necessary to thoroughly characterize them with respect to their responses to experimental and likely in vivo variables. This work represents a first effort toward that goal. We tested the response of each source of CA activity to Cl^-, Mn^{2+}, Ca^{2+}, and pH. We find very different responses of the two sources of PSII CA activity to these factors.

2. Materials and methods

Both maize (*Zea mays L.*) and pea (*Pisum sativum*) plants were grown in a greenhouse and mesophyll thylakoids were extracted as previously described [10]. PSII-enriched membranes were prepared by the procedure of Kuwabara et

Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(N-morpholinoethanesulfonic acid; OEC33, extrinsic photosystem II protein with apparent mol. wt. of 33 kDa; Pipes, Piperazine-1,4-bis(2-ethanesulfonic acid); prOEC33, precursor OEC33; PSII, photosystem II; Tricine, N-[Tris(hydroxymethyl)methyl]glycine

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al. [11] as modified in Xu and Bricker [12]. The PSII membranes were kept at − 80 °C until used.

The method used to obtain OEC33-depleted PSII membranes and purified OEC33 protein was modified from Hashimoto et al. [13] as detailed in [7]. Some experiments were done with precursor OEC33 (prOEC33) overexpressed in *Eschericia coli*. The procedure for obtaining this protein was also detailed in [7]. The method to extract calcium from stripped PSII membranes was followed by Kirk et al. [14]. The Ca-depleted PSII membranes were kept at − 80 °C until use.

Carbonic anhydrase activity measurement was carried out by using the assay procedure developed by Stemler [15] with modifications as described by Moubarak-Milad and Stemler [10]. Hydration or dehydration of added $^{14}CO_2$ or $H^{14}CO_3^-$ (specific activity 96%) was measured in these experiments. The reaction mixtures are given in the figure legends. All samples with PS II membranes and isolated OEC33 were assayed in near-darkness. Each reaction tube that contained 0.3 mL of reaction mixture was injected with 50 µL of the diluted substrate to a final concentration of 12 µM $^{14}CO_2$ or $H^{14}CO_3^-$. The CA activity was determined with a scintillation counter. Buffered reaction media were routinely either boiled or ultra-filtrated to eliminate CA-containing microorganisms.

Oxygen evolution activity was measured with a Clark-type O_2 electrode at 25 °C under saturating white light. Reaction media are given in the figure legends. Chlorophyll concentration was determined spectrophotometrically by the method of Porra et al. [16], and the concentration of protein by the method of Bradford [17]. Bovine serum albumin (BSA) was used as a standard.

3. Results

3.1. CA activity in PSII as a function of Cl^- concentration

CA activity was measured in desalted purified OEC33 as a function of chloride concentration (Fig. 1). Addition of 5 mM Cl^- increased the activity approximately 3-fold. The activity appeared to plateau between 5 and 20 mM and then declined sharply above 20 mM. Cl^-. Other halides were tested for their stimulatory effect on the OEC33 CA activity (Fig. 2). By far the most effective halide was Cl^-, though Br^- also produced some stimulation. There may have been a small stimulation with I^-, but for this anion, and F^-, activity was statistically indistinguishable from the control. These results roughly match the effects of halides on oxygen evolution [18].

When intrinsic CA activity was measured as a function of Cl^- concentration (Fig. 3), the results contrasted greatly from

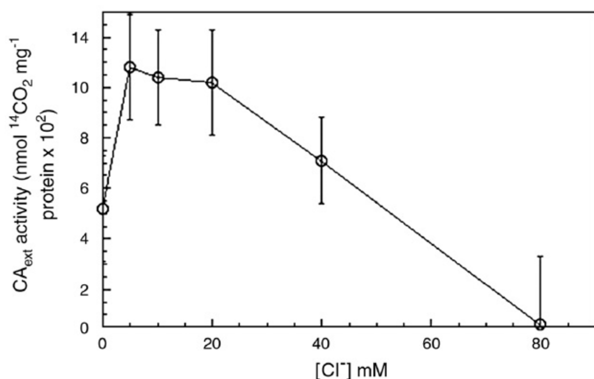

Fig. 1. CA activity in desalted OEC33 extracted from pea PSII-enriched membranes with 1 M $CaCl_2$ as a function of Cl^- concentration. The reaction mixture contained 0.05 M Na–Mes, pH 5.5, 5 mM $MnSO_4^-$, NaCl to the concentration indicated, and 3.7 µg protein mL^{-1}. Error bars represent 1 SE, $n=20$.

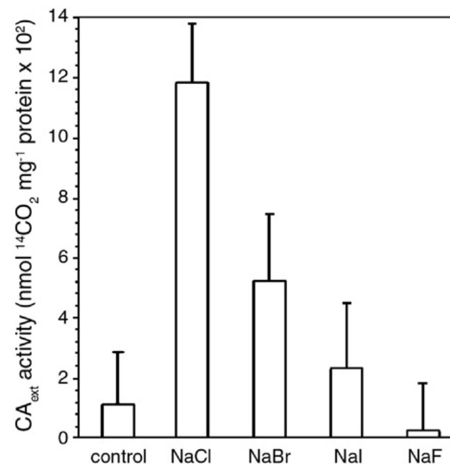

Fig. 2. The effect of the halide anions on manganese-stimulated CA activity of expressed prOEC33. Sodium halide salts (30 mM) were added in the reaction buffer (50 mM Na–Mes pH 5.5, 5 mM $MnSO_4$) with 3.4 µg of purified prOEC33 mL^{-1}, respectively. The protein was suspended in reaction buffer for a 10 min incubation time at room temperature. SE, $n=40$.

those with the OEC33. After PSII membranes were washed with 1 M $CaCl_2$, to remove extrinsic proteins i.e., "stripped", desalted, and then given Cl^-, CA activity increased up to 0.4 M, the highest concentration tested. As previously observed by Kuwabara et al. [11], oxygen evolution from these membranes during a Hill reaction responded in a related fashion to added Cl^-.

We also observed CA activity and oxygen evolution in PSII membranes that were washed in 1 M NaCl to remove only the extrinsic 24 and 17 kDa extrinsic proteins, leaving the OEC33 in place [19]. As such, the membranes retained both sources of CA activity. Assays were then conducted

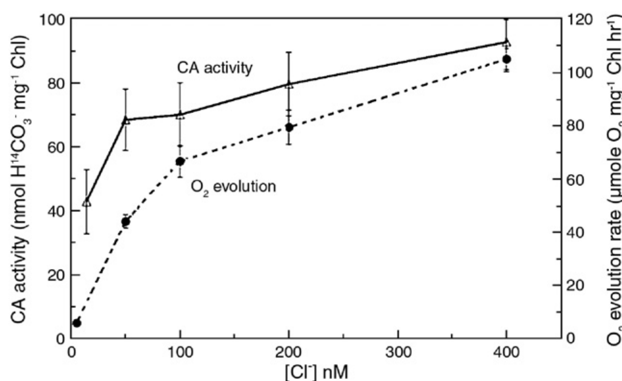

Fig. 3. CA and oxygen-evolving activities as a function of chloride concentration in maize PS II-enriched membranes washed in 1 M $CaCl_2$ to remove the extrinsic 33, 24, and 17 kDa proteins. The membranes were suspended in 0.05 M Na–HEPES, pH 7.2, 2 mM $CaCl_2$ and the indicated concentration of Cl^- (as NaCl). For the CA assay, the chlorophyll concentration was initially 184 µg chl ml^{-1}. Samples were incubated in the dark for at least 10 min then assayed in the dark. SE, $n=30$. Oxygen evolution was measured in the same reaction mixture as the CA assay, but at a chlorophyll concentration of 20 µg chl ml^{-1}. Just before saturating white light was applied, DCBQ (2,6-dichloro-p-benzoquinone), was injected to a concentration of 200 µM. Data represent initial rates, averaging 5 measurements at each concentration.

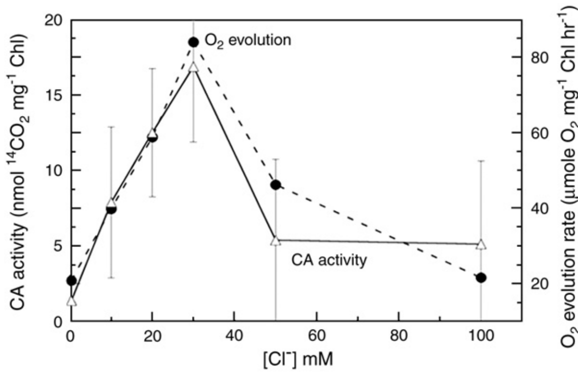

Fig. 4. CA and oxygen-evolving activities as a function of chloride concentration in maize PS II-enriched membranes washed in 2 M NaCl to remove the 24 and 17 kDa extrinsic proteins. The reaction mixtures and conditions were similar to those described in the Fig. 2 legend, except that much lower chlorophyll concentrations were required (on the order of 20–30 μg chl ml^{-1}) for the CA assay.

varying the Cl^- concentration (Fig. 4). With the addition of Cl^-, both CA activity and oxygen evolution increased to the same optimum at 30 mM Cl^-. Concentrations greater than 30 mM inhibited both. Thus the response of the CA activity to Cl^- more closely resembles that of the OEC33 (Fig. 1) and not that of intrinsic CA (Fig. 3). It should also be noted that the quantitative responses of CA activity and oxygen evolution to Cl^- were not the same. Addition of Cl^- produced at most a 2-fold increase in CA activity but a 4–5 fold increase in oxygen evolution.

3.2. Cation effects on PSII CA activities

The CA activity of PSII is sensitive to cations as well as anions. We have already documented that the OEC33 CA activity is stimulated by Mn^{2+} and to a lesser extent by Ca^{2+} [7]. The cations Zn^{2+}, Co^{2+}, Mg^{2+} and Na^+ had no effect. What we have found since this earlier work is that the response of the OEC33 to Mn varies with source and method of preparation. At times, activity is high and no response is elicited from added Mn, presumably because the protein still retains the Mn it had in vivo. In other preps, the response to Mn^{2+} is dramatic. The reason for this variability will require extensive study now in progress.

Calcium was found to have a major effect on the intrinsic CA activity of stripped PSII membranes. The cation was specifically removed by treating the membranes with citric acid [20] leaving the Mn in place. After treatment, both oxygen evolution and intrinsic CA activity was eliminated (Fig. 5). Thus Mn, or Mn alone, is not sufficient for intrinsic CA

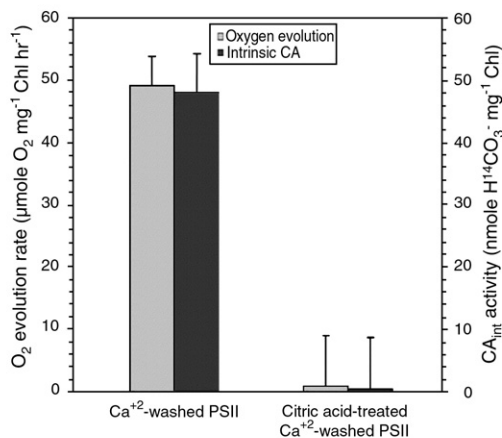

Fig. 5. Oxygen evolution and intrinsic CA activity in 1 M $CaCl_2$-washed pea PSII membranes subsequently either washed in citric acid or left unwashed. For oxygen evolution, the reaction medium contained 50 mM Na–Mes, pH 6.1, 5 mM $CaCl_2$, and 0.4 M NaCl. Intrinsic CA activity was measured in reaction mixture that contained 50 mM Na–HEPES, pH 7.5, 5 mM $CaCl_2$, and 0.4 M NaCl. Other conditions for both assays were as described in the Fig. 3 legend. CA activity was measured as $^{14}CO_2$ hydration after a 10 min incubation time at room temperature. Error bars represent 1 SE, $n=40$.

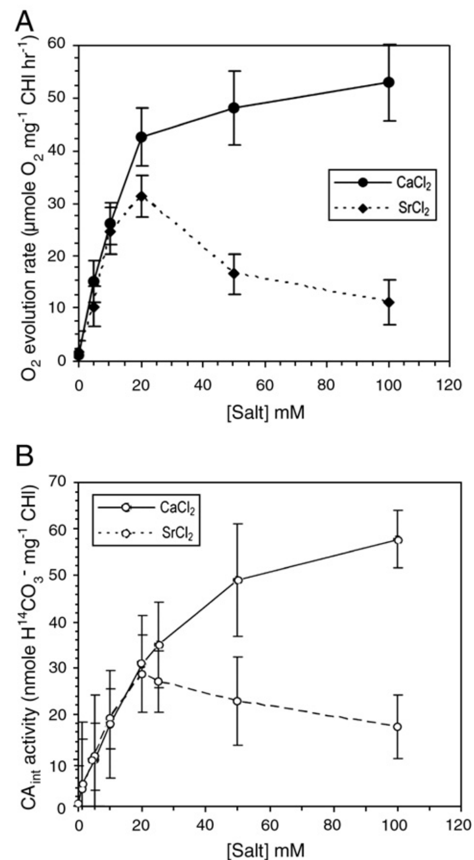

Fig. 6. Oxygen evolution and intrinsic CA activity as a function of Ca^{2+} or Sr^{2+} concentrations in stripped pea PSII membranes washed in citric acid. (A) Oxygen evolution measured with Ca-washed and citric acid treated PSII membranes in the reaction medium that contained 0.4 M NaCl, and 50 mM Na–Mes, pH 6.1, 25 μg chl mL^{-1}. (Error bars represent 1 SE, $n=5$. (B) Intrinsic CA activity was measured as $^{14}CO_2$ hydration at room temperature. The reaction mixture contained 0.4 M NaCl, and 50 mM Na–HEPES pH 7.5, 100 μg chl mL^{-1}. Error bars represent 1 SE, $n=40$. Activity was restored before both measurements by suspending the citric acid- treated membranes in 0.4 M sucrose and 50 mM Mes, pH 6.5 with various concentrations of either $CaCl_2$ or $SrCl_2$. All samples were incubated in the dark on ice for 1 hr and then centrifuged. The pellets were washed and centrifuged twice with 10 mM Na–Mes pH 6.5, 10 mM NaCl and then resuspended in a small amount of 0.4 M sucrose and 50 mM Na–Mes, pH 6.5. The x-axes, therefore, indicate the concentrations of cations given during the pretreatment.

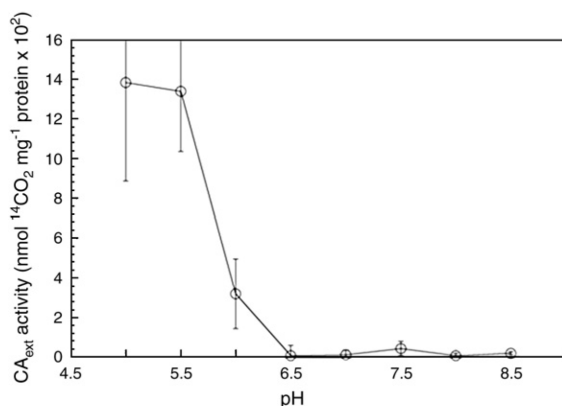

Fig. 7. Extrinsic CA activity as a function of pH in OEC33 extracted from pea PSII-enriched membranes with 1 M $CaCl_2$. The reaction mixture contained 50 mM buffer-Na (Mes pH 5–6.5, Pipes pH 6.5–7.5, Tricine pH 7.5–8.8) 2.5 mM $CaCl_2$ and 4 μg protein mL^{-1}.

activity. Both activities were restored by adding back either Ca^{2+} or Sr^{2+} (Fig. 6). Calcium saturated oxygen evolution at about 20 mM, while this amount half-saturated CA activity. The difference is not surprising. The rate-limiting step for oxygen evolution during Hill reaction shifts from that controlled by Ca^{2+} to other steps, usually considered to be on the electron-acceptor side of PSII. Hence, a hyperbolic response curve is expected. Measurement of CA activity involves only the one reaction directly controlled by Ca^{2+} and may require a higher concentration to saturate. Hence a more linear response is expected.

Strontium is known to substitute for Ca^{2+} as part of the $CaMn_4$ complex, but only to a degree [21]. We show in Fig. 6, that up to 20 mM Sr^{2+} increases intrinsic CA activity. Higher concentrations inhibit the CA activity. A parallel effect of Sr^{2+} is seen on oxygen evolution. Again, activity increased up to 20 mM, and inhibition was seen at higher concentrations.

3.3. CA activities as a function of pH

Carbonic anhydrase activity is normally strongly influenced by pH [22]. We measured dehydration activity of the OEC33 in vitro (Fig. 7). Activity was highest at pH below 6, and practically immeasurable above 6.5. The in vitro hydration activity of this enzyme was much more difficult to determine reproducibly and we continue to study the controlling factors involved. The hydration activity of the intrinsic CA, in contrast, was easily observable (Fig. 8). Curiously, the activity was practically insensitive to pH. Despite numerous attempts, we have so far been unable to observe dehydration activity in Ca-washed PSII membranes.

4. Discussion

The dependence of the PSII carbonic anhydrases on Cl^- is one indication of their unique character relative to typical CA enzymes. Normally, Cl^- and other monovalent anions are strong inhibitors of CA [23]. Intrinsic CA increases in activity at least to 0.4 M Cl^-, whereas the OEC33 activity is stimulated to about 30 mM Cl^- and only inhibited at higher concentrations. Likewise, the cofactor requirements of the PSII CAs set them apart. The metal cation required for activity of nearly all CAs is Zn^{2+}, although a Cd^{2+}-requiring enzyme has been documented [24]. The reason for activation of the OEC33 by Mn^{2+} and to some extent by Ca^{2+} is not readily apparent. However, that the OEC33 can bind these two cations has been reported by Shutova et al. [25]. These authors suggested that the high flexibility shown by the OEC33 as a function of pH might play an important role in reversible dissociation reactions of the $CaMn_4$ cluster under physiological condition.

The dependence of intrinsic CA activity on Ca^{2+} (or Sr^{2+}) is equally unusual. While the reason for activation by Ca^{2+} is also not clear, we can conclude that the source of the intrinsic CA activity must be the $CaMn_4$ complex and its immediate surroundings. Considerable information is now available concerning the structure of PSII, particularly, the catalytic site for O_2 production that consists of an inorganic core of four oxobridged manganese ions and one calcium ion, a special redox-active tyrosine residue (termed Y_Z), and the associated amino acids of the surrounding proteins [26]. As such, it may not be possible to isolate a single protein bearing intrinsic CA activity as can be done with the OEC33. Rather, intrinsic CA activity appears to be a function of the oxygen-evolving mechanism, which itself is not extractable as a single protein.

The correlation between PSII CA activity and oxygen evolution with respect to Cl^- and Ca^{2+} is remarkable. So closely do these functions often track, that it could be inferred that CA activity may be necessary for oxygen evolution. A further speculation is that the function of Cl^- and Ca^{2+} may be to activate the PSII CAs which, in term, allows oxygen to be evolved. Dismukes et al. [2] pointed out that using bicarbonate instead of water to generate hydroxide for oxygen evolution would require no additional energy input in PSII. This is consistent with our conclusion that the intact water-oxidizing complex, with Ca^{2+}, Mn^{2+}, and Cl^-, can hydrate CO_2 along with its other functions. The bicarbonate could be

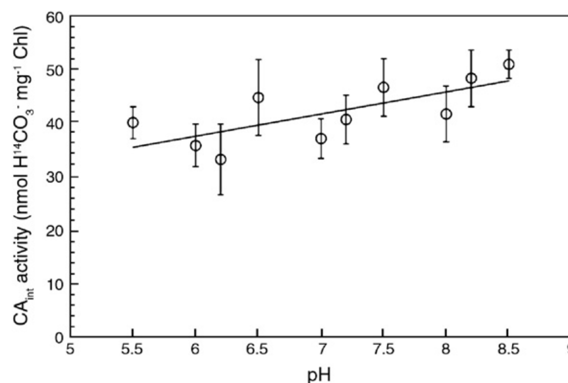

Fig. 8. Intrinsic CA activity in stripped maize PSII membranes as a function of pH. The reaction mixture contained 0.4 M NaCl and 10 mM $CaCl_2$, the buffers indicated in Fig. 7 legend, and 80 μg chl mL^{-1}.

a source of hydroxide, or take part in some other mechanism of oxygen evolution. However, we have observed and reported conditions under which CA activity and oxygen evolution appear “uncoupled”. For example, when oxygen evolution and CA activity is measured as a function of Cl^- concentration in maize *thylakoids*, as opposed to PSII membranes shown here (Fig. 4), CA activity is inhibited much more at high concentrations than is oxygen evolution [27]. This could mean that CA activity is not necessary for oxygen evolution, or that it is simply not rate-limiting under the experimental conditions. An added complication is that there are at least two forms of CA in thylakoids and very possibly more. The CA that is inhibited by high Cl^- may not be the one active in oxygen evolution. Therefore, whether CA activity in PSII is necessary for oxygen evolution remains an open question.

4.1. Remark

Our experience in trying to characterize the two PSII carbonic anhydrases prompts a note of caution. Besides the factors discussed here, there are other variables that dramatically change the activity of both CAs. One of these, light, has already been reported [28–30] and will be further described in future publications. Others may include the source and methods for obtaining the separated enzymatic activity as well as experimental conditions and protocol. Moreover, it does not appear that these variables are all independent. Therefore we emphasize that a great deal of additional work is necessary before the PSII CAs are fully characterized individually and in tandem in the membrane.

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