

# A quantitative assessment of the carbonic anhydrase activity in photosystem II

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## Abstract

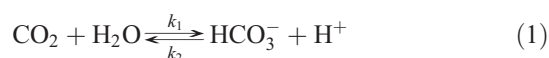
Using a carbonic anhydrase assay based on membrane inlet mass spectrometry (MIMS), we have extended our earlier investigations of Photosystem II (PSII)-associated carbonic anhydrase activity in spinach PSII preparations (W. Hillier, I. McConnell, M. R. Badger, A. Boussac, V. V. Klimov G. C. Dismukes, T. Wydrzynski *Biochemistry* 2006, 45:2094). The relationship between the carbonic anhydrase activity and O<sub>2</sub> evolution has been evaluated in terms of the effects of metal ion addition, preparation type, light, and response to specific inhibitors. The results indicate that the PSII-associated carbonic anhydrase activity is variable and appears not to be associated specifically with the oxygen evolving activity nor the 33 kDa extrinsic manganese stabilising protein.

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**Keywords:** Bicarbonate; Carbonic anhydrase; Manganese stabilising protein; Oxygen evolving complex; Photosystem II

## 1. Introduction

Carbonic anhydrase (CA) enzymes (EC 4.2.1.1) are ubiquitous in the biosphere. They catalyse the reversible hydration of carbon dioxide to the bicarbonate ion, i.e.:



There are four main classes of CA:  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ; recently the  $\epsilon$  class was also introduced [1]. In terms of the distribution of CAs within the biosphere, animals only have the  $\alpha$  CA; plants contain examples of each class except  $\delta$ ; algae and cyanobacteria contain a range of CAs but often not all classes in the same cell [2–4]; and the marine diatoms contain the

structurally novel  $\delta$  class [5]. All of these groups of CA, while varying structurally and in catalytic efficiency, use a Zn ion in the catalytic site which binds a hydroxide molecule and present this to the CO<sub>2</sub> for hydration to bicarbonate. It is of some interest then that a further classification has been advanced: the  $\zeta$  class, which natively utilises Cd ions as the catalytic metal centre [6]. The catalytic Zn ions have also been biochemically substituted with Co [7]. This diversity in catalytic metals and structures means that novel CAs can only be identified by isolation and subsequent assay [4].

Photosystem II (PSII) of plants, algae and cyanobacteria is a large pigment–protein complex found spanning the photosynthetic thylakoid membranes [8,9]. It functions as a light-dependent water-plastoquinone oxidoreductase, feeding electrons to the photosynthetic electron transport pathway [10]. The PSII photochemistry is uniquely important as it is the only biological reaction that catalyses the oxidation of H<sub>2</sub>O and produces molecular the O<sub>2</sub>. The O<sub>2</sub> by-product is critical for much of the life on Earth.

The catalytic site of water oxidation in PSII is a metal cluster containing four Mn and one calcium ion (i.e. Mn<sub>4</sub>Ca). The Mn<sub>4</sub>Ca cluster is ligated by the transmembrane intrinsic proteins D1 and CP43. In addition, several membrane extrinsic com-

*Abbreviations:* CCM, carbon concentrating mechanism; DCMU, 3-(3,4-dichlorophenyl)-1,1-methylurea; EZ, ethoxyzolamide; MSP, manganese stabilising protein; C<sub>i</sub>, inorganic carbon; MIMS, membrane inlet mass spectrometry; PSII, photosystem II; WOC, water oxidising complex

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ponents bind to PSII on the luminal side of the PSII complex in close proximity to the  $\text{Mn}_4\text{Ca}$  cluster. In higher plants the extrinsic proteins are PsbP, PsbQ and PsbO of approximately 16, 23 and 33 kDa molecular weight, respectively. In prokaryotes the PsbU, PsbV and PsbO proteins are present in PSII. (PsbP and PsbQ have been found in prokaryotic genomes, but not in the crystal structures of the complex [11].) None of the extrinsic proteins directly ligate the  $\text{Mn}_4\text{Ca}$  complex [12]. Instead they are suggested to act as an accessibility barrier to the  $\text{Mn}_4\text{Ca}$  cluster [13]. The PsbO protein in particular is universal and is needed to stabilise the Mn ions [14]; thus it is also termed the manganese stabilising protein (MSP). Interestingly, CA activity has been found to be associated with thylakoid membranes and PSII preparations from a range of species [15]. Two main hypotheses have been advanced to account for the CA activity: (1) the CA activity is associated with a protein that is separate to the PSII complex; and (2) the CA activity is expressed by a subunit or subunits of the PSII complex itself.

In *Chlamydomonas reinhardtii* a CA named Cah3 has been isolated and its gene sequenced [16]. The gene sequence exhibited homology to the  $\alpha$  class of CAs. The presence of a thylakoid leader sequence and results from immunoblotting showed that this protein is localised to the lumen of the thylakoids in this green alga. Its calculated size from sequence data is 29.5 kDa. The Cah3 enzyme has been shown to be a requirement for growth at ambient  $\text{CO}_2$  being isolated from a mutant screen for this phenotype [17,18]. CA activity has also been identified in the PSII fractions of other green algae (*Tetradon minimum* and *Chlamydomonas noctigama*) [19]. These preparations contain a 30 kDa protein that cross reacts with the Cah3 antibody. These data support the idea of a separate CA protein associated with PSII.

A curious junction between the two hypotheses occurred when it was shown that a 33 kDa protein from C4 plants cross reacted with the CA antibody raised against the Cah3 protein from *C. reinhardtii* [20]. Those authors found the most CA activity and antibody binding to a 30 kDa protein in the 1 M  $\text{CaCl}_2$  wash fraction, i.e. the soluble fraction of PSII that contains the extrinsic PSII components. It was implied that the MSP (PsbO protein) has a CA activity. However, the significance of the cross reaction is unclear; the MSP sequence [21] shows that it is not an  $\alpha$  CA like Cah3 [16].

The idea that CA activity is associated with a PSII component has been examined by attempting to localise the source of the activity to either the soluble or membrane bound components of PSII. Typically a salt-washing procedure is used such as 1–2 M NaCl to remove PsbU and PsbV or PsbP and PsbQ. A 1–2 M  $\text{CaCl}_2$  wash is used to remove the MSP. The membrane and soluble fractions resulting from this treatment are assayed to identify the distribution of the activity. The isolated 33 kDa MSP has been argued to possess CA activity [22]. However, the same work also concluded that there was an additional membrane bound CA activity. This work was carried out on C3 plant preparations with care taken to minimise contamination from stromal  $\beta$  CAs, echoing similar work in C4 plants [20].

In another set of experiments data indicated that the soluble extrinsic fraction of PSII was devoid of CA activity, albeit without the desalting to remove the 1 M  $\text{CaCl}_2$  [23]. Instead the CA activity was localised within the membrane fraction. The CA activity of this fraction was also suggested to be increased by the addition of detergent, which was interpreted to alleviate a hydrophobic barrier impeding access to the CA site. This result was somewhat loosely claimed to explain a faster hydration rate than dehydration rate; as  $\text{CO}_2$  is more permeable through hydrophobic barriers than charged bicarbonate. The same experiments [23] have also shown that the solubilised membrane fraction binds an immobilised sulphonamide (a CA inhibitor) column.

A similar investigation claimed that the membrane associated CA activity was not from a contaminating stromal CA based on immunoblotting against  $\beta$  CA [24]. Treatments with salt washing, membrane disruption, lipophilic and hydrophilic sulphonamides supported the idea that the CA activity was in the membrane. It was also noted that activity purified with PSII.

While a specific physiological role for CA and PSII is still lacking, the concept that the CA activity might be coupled to photochemistry and via inference electron transport of PSII has received some attention in the literature. This notion is supported by the studies mentioned above and where CA inhibitors have been reported to reduce the oxygen evolving activity of PSII preparations [25,26] and in cases where the CA activity has been examined as a function of light exposure [27].

In the current work we sought to isolate the PSII associated CA activity using a highly specific and sensitive MIMS (membrane inlet mass spectrometry) based assay [28–30]. This assay has a number of key advantages that include direct observation of the reaction at chemical equilibrium via the isotopic reaction instead of merely the chemical reaction, and the ability to calculate rate constants for the (de)hydration reactions not just a net velocity. With this technique we have examined what role the CA activity has in PSII function.

## 2. Materials and methods

### 2.1. PSII-enriched membrane fragment preparation

Spinach membranes enriched in PSII (BBYs) were isolated from thylakoids by a 5% Triton X-100 treatment [31]. The detergent treated material was washed with buffer and the protein stored as beads at  $-80^\circ\text{C}$  until use.

### 2.2. PSII core preparation

The PSII core preparation was isolated from BBY PSII-enriched membrane fragments using 1.5% *n*-dodecyl- $\beta$ -D-maltoside and purified on a POROS HQ column (PerSeptive Biosystems) under a linear flow of  $1800\text{ cm h}^{-1}$  by elution with a linear gradient of 0–50 mM  $\text{MgSO}_4$ . The PSII core complexes were then dialyzed against 20 mM BIS-TRIS (pH 6.5), 400 mM sucrose, 20 mM  $\text{MgCl}_2$ , 5 mM  $\text{CaCl}_2$ , 0.03% (w/v) *n*-dodecyl- $\beta$ -D-maltoside and concentrated with an Amicon concentration cell. The core samples were stored as small beads at  $-80^\circ\text{C}$ . Rates of  $\text{O}_2$  evolution were  $\sim 1500\text{ }\mu\text{mol O}_2\text{ (mg of Chl)}^{-1}\text{ h}^{-1}$  in the presence of 500  $\mu\text{M}$  2,6 dichloro-benzoquinone and 1 mM ferricyanide [25,32].

### 2.3. Membrane inlet mass spectrometry-carbonic anhydrase assay

The isotope preparation and carbonic anhydrase assay was carried out as specified in [28] with an Isoprime (UK) mass spectrometer. For these experiments a sample cuvette of 150  $\mu\text{l}$  was used and 0.5  $\mu\text{l}$  of 400 mM  $\text{NaH}^{13}\text{C}^{18}\text{O}_3$  was added per reaction. The MIMS cuvette was illuminated via a fibre optic connected to a light source (Schott KL-1500, Germany). This cuvette when not being illuminated was completely dark. The electron acceptors used in the CA assay were the same as the oxygen assay buffer. The  $^{18}\text{O}$  enriched bicarbonate was produced by dissolving 96%  $^{13}\text{C}$  enriched  $\text{NaH}^{13}\text{CO}_3$  in 95–98%  $^{18}\text{O}$ -water (Isotec, USA) and allowing the solution to isotopically equilibrate for >24 h.

### 2.4. Data fitting and analysis

The rate constants for  $\text{CO}_2$  hydration ( $k_1$ ), dehydration ( $k_2$ ) and the MIMS membrane consumption ( $k_{\text{leak}}$ ) were solved by minimisation of the residuals from a model outlined below (Eqs. 2–8). Least squares data fitting was performed with Excel (Microsoft) using the inbuilt solver function for optimizing nonlinear problems according to the generalized reduced gradient (GRG2) algorithm. For the experimental conditions performed in this paper the CA rates were explicitly solved by the simultaneously fitting the background and the experimental data. The differential equations contain terms  $B_3$ ,  $B_2$ ,  $B_1$ , and  $B_0$  representing triple, double, single and non- $^{18}\text{O}$  labelled bicarbonate and  $C_2$ ,  $C_1$  and  $C_0$  representing double, single and non-labelled  $\text{CO}_2$  signals (i.e.  $^{13}\text{C}^{18,18}\text{O}_2$ ,  $^{13}\text{C}^{18,16}\text{O}_2$ ,  $^{13}\text{C}^{16,16}\text{O}_2$ ). The model is then fit against  $\text{CO}_2$  signals measured by MIMS. The background hydration/dehydration rates are designated  $^b k_1$  and  $^b k_2$  respectively. The data fitting and analysis for the control conditions were otherwise performed as previously [28]. The solutions for  $k_1$  and  $k_2$  are then valid for the experiment at a given pH and ionic strength, for which we attempted to maintain, yet will be different under different conditions.

$$\frac{d[C_0]}{dt} = \left( k_2 \left( B_0 + \frac{1}{3} B_1 \right) + ^b k_2 \left( B_0 + \frac{1}{3} B_1 \right) \right) - (k_1(C_0) + ^b k_1(C_0)) - k_{\text{leak}}(C_0) \quad (2)$$

$$\frac{d[C_1]}{dt} = \left( k_2 \frac{2}{3} (B_1 + B_2) + ^b k_2 \frac{2}{3} (B_1 + B_2) \right) - (k_1(C_1) + ^b k_1(C_1)) - k_{\text{leak}}(C_1) \quad (3)$$

$$\frac{d[C_2]}{dt} = \left( k_2 \frac{1}{3} (B_2 + B_3) + ^b k_2 \frac{1}{3} (B_2 + B_3) \right) - (k_1(C_2) + ^b k_1(C_2)) - k_{\text{leak}}(C_2) \quad (4)$$

$$\frac{d[B_0]}{dt} = (k_1(C_0) + ^b k_1(C_0)) - (k_2(B_0) + ^b k_2(B_0)) \quad (5)$$

$$\frac{d[B_1]}{dt} = (k_1(C_1) + ^b k_1(C_1)) - (k_2(B_1) + ^b k_2(B_1)) \quad (6)$$

$$\frac{d[B_2]}{dt} = (k_1(C_2) + ^b k_1(C_2)) - (k_2(B_2) + ^b k_2(B_2)) \quad (7)$$

$$\frac{d[B_3]}{dt} = -(k_2(B_3) + ^b k_2(B_3)) \quad (8)$$

The rate constants for  $\text{CO}_2$  hydration and dehydration can also be expressed as a measure of the change in  $^{18}\text{O}$  enrichment of the  $\text{CO}_2$  species (i.e.  $^{18}\text{O}$  mole fraction). This analysis was performed concurrently with the above kinetic evaluation and is expressed as the  $^{13}\alpha$  as follows:

$$^{13}\alpha = \frac{2 \times C_3 + C_2}{2 \times (C_3 + C_2 + C_1)} \quad (9)$$

This parameter is useful for tracking the overall speed of the reaction relative to the background. The rate at which  $^{18}\text{O}$  is removed from the  $^{13}\text{C}$  species or the

slope when plotted versus time can be used to show the relative rate of CA for a series of conditions [29,30].

### 2.5. Standard carbonic anhydrase assay conditions

The assay buffer consisted of 1.2 M betaine, 100 mM HEPES pH 7.5, 40 mM NaCl. At 20  $^\circ\text{C}$ . BBY samples were added to a final concentration of 0.5 mg/ml chlorophyll. The reaction volume was 150  $\mu\text{l}$ .

### 2.6. Standard oxygen assay conditions

Measurements were made with an oxygen electrode (Hansatech Oxytherm, UK) modified to contain a side illumination port. The buffer used was 100 mM HEPES pH 7.5, 40 mM NaCl with 500  $\mu\text{M}$  phenyl-p-benzoquinone and 1 mM ferricyanide. The chlorophyll concentration was 10  $\mu\text{g/ml}$ . All assays were at 20  $^\circ\text{C}$ .

### 2.7. DCMU and ethoxzolamide titrations

Standard assay conditions were used and activity was titrated with the inhibitors 3-(3,4-dichlorophenyl)-1,1-methylurea (DCMU) dissolved in ethanol, and ethoxzolamide (EZ) in DMSO. To measure the effect on oxygen evolution three separate assays were carried out at each inhibitor concentration. To determine the effect on the CA activity three separate sequential additions covering the examined concentration range were carried out. All inhibitor assays used material from the same BBY prep.

## 3. Results

The CA equilibrates bicarbonate with carbon dioxide in an extremely rapid reversible reaction. The rate can be directly monitored via a process of isotopic equilibration beginning with introduction of  $^{18}\text{O}$  labelled bicarbonate as substrate. The isotopic exchange assay produces data as shown in Fig. 1 and reports an initial phase of *chemical equilibrium* with the peak of  $m/e=49$  ( $\text{C}^{18,18}\text{O}_2$ ) at approximately 50 s and a subsequent phase of *isotopic equilibration* where the other  $\text{CO}_2$  species at  $m/e=47$  ( $\text{C}^{18,16}\text{O}_2$ ) and 45 ( $\text{C}^{16,16}\text{O}_2$ ) are created from isotopic exchange. The fit to these data in Fig. 1A is black while the data itself are in red dashes. The data in Fig. 1 are fit to a time range over three orders of magnitude and is indicative of a highly accurate description of the equilibrium system. In Fig. 1B the same data are used to plot against time the  $^{18}\text{O}$  enrichment of  $^{13}\text{CO}_2$  species which is given as  $^{13}\alpha$ . The natural log of  $^{13}\alpha$  when plotted against time is linear, and is a direct measure of the rate of change of  $^{18}\text{O}$  enrichment [29]. Using this method of analysis the slope of the line in this figure expresses the overall rate of the reaction: the steeper the slope, the faster the CA reaction. This  $^{13}\alpha$  analysis is highly suited for relative comparisons between different samples. In summary, both approaches in Figs. 1A and B report directly the rates of CA activity and show the effect of the  $^{18}\text{O}$  being reversibly shuttled from  $\text{H}^{13}\text{CO}_3^-$  to  $^{13}\text{CO}_2$ , and with a proportion of the label moving to  $\text{H}_2\text{O}$  where it is assumed to be infinitely dilute.

A range of experimental conditions were tested with the MIMS assay to determine the localisation of CA within PSII (see Table 1). As the bicarbonate- $\text{CO}_2$  exchange reaction has an appreciable background contribution manifest in all measurements derived from non-enzymatic origin, it was necessary to explicitly subtract the background rates of hydration and

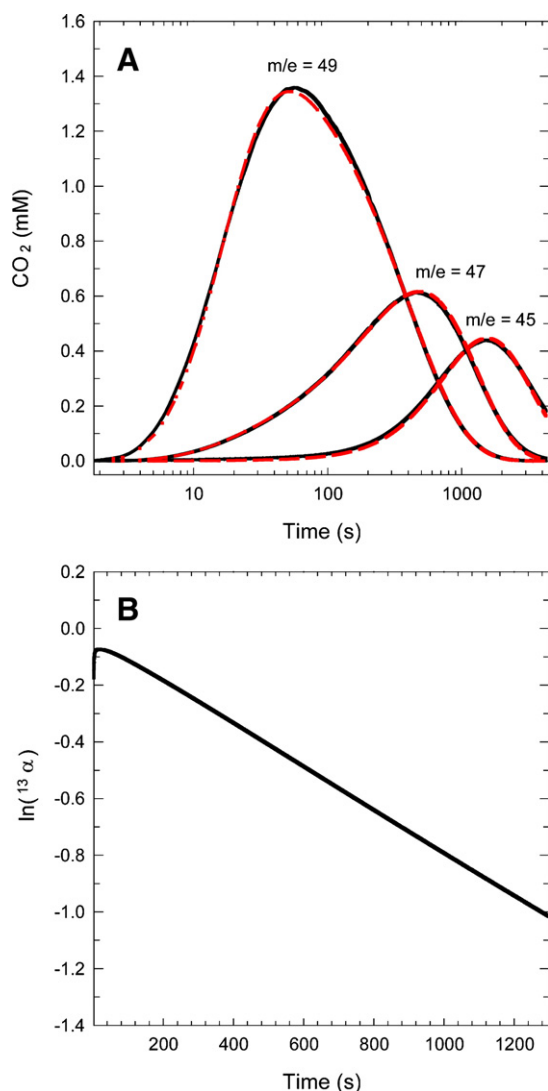


Fig. 1. The membrane inlet mass spectroscopy (MIMS) based carbonic anhydrase assay presenting (A) raw traces of changes in the concentration of <sup>13</sup>CO<sub>2</sub> species changes with time at  $m/e=49$  ( $C^{18,18}O_2$ ), 47 ( $C^{18,16}O_2$ ) and 45 ( $C^{16,16}O_2$ ) (dashed red lines) and the a fit of the data (black lines), and (B) the natural log of the <sup>13</sup>α as a function of time. The measurements were performed on BBYs at 20 °C.

dehydration via a global fitting approach. Representative background rates  $k_1$  and  $k_2$  with values of  $9.1 \text{ s}^{-1}$  and  $0.4 \text{ s}^{-1}$  are reported in Table 1 for the assay buffer. The background uncatalysed rate is both temperature and pH dependent and all efforts were made to control this variable. The <sup>18</sup>O enrichment rate derived from the <sup>13</sup>α line is also included for all measurements. These are expressed relative to the buffer only condition where the <sup>13</sup>α is equal to 1.0. Intact spinach BBY samples were assayed to represent the initial state of the system before modification. Unadjusted for reaction centre concentration the relative <sup>13</sup>α slope shows that this reaction was 92 times faster than buffer alone. This corresponds to specific (de) hydration rates adjusted for reaction centre concentration,  $k_1$  and  $k_2$ , of  $2300 \text{ s}^{-1}$  and  $91 \text{ s}^{-1} \mu\text{mol}^{-1}$  respectively. These values are clearly indicative of some form of CA activity associated with PSII. To examine the contribution of the soluble

fraction the extrinsic proteins were removed from the BBYs by a 1 M CaCl<sub>2</sub> wash. This reduced the associated CA activity. The relative <sup>13</sup>α slope was 2.9 that of background rates, representing a thirty fold decrease, while the specific rates  $k_1$  decreased to  $190 \text{ s}^{-1} \mu\text{mol}^{-1}$  and the  $k_2$  to  $5.6 \text{ s}^{-1} \mu\text{mol}^{-1}$ , representing a 12–16 fold decrease. The extrinsic proteins released in the 1 M CaCl<sub>2</sub> wash were measured but after dialysis showed no activity (data not shown). Finally, purified PSII core particles that contain reduced chlorophyll antenna proteins yet bind the MSP protein were compared. The PSII core preparations were highly active for oxygen evolution (data not shown) yet showed minimal CA activity with the specific hydration term  $k_1 < 0.1 \text{ s}^{-1} \mu\text{mol}^{-1}$  and dehydration term  $k_2 < 0.01 \text{ s}^{-1} \mu\text{mol}^{-1}$  plus no net increase in the <sup>13</sup>α rate over the background. This result indicates that for this type of PSII core preparation all CA activity was removed from the oxygen evolving component, so the activity was either localised to a removed protein or was deactivated during the purification (Fig. 2).

The extrinsic MSP protein of PSII was specifically examined as a purified recombinant *Thermosynechococcus elongatus* MSP. The assay of this protein found that there was no CA activity with  $k_1$  and  $k_2$  rates equivalent to background rates i.e.  $k_1 < 0.1$  and  $k_2 < 0.01 \text{ s}^{-1} \mu\text{mol}^{-1}$ . The <sup>13</sup>α slope for the isolated PSII particles and the recombinant *T. elongatus* MSP came out to be measurably slower than the background. The reason for this is unclear, but may be due to a mild pH shift via the protein's buffering capacity or change in ionic strength in the buffer despite the high buffering capacity present. A final comparison was made with purified α CA from bovine erythrocytes. The assay revealed rates  $k_1$  at 190,000 and  $k_2$  at  $2,300 \text{ s}^{-1} \mu\text{mol}^{-1}$ , which was two orders of magnitude greater than the intact BBYs protein. Our findings then were unable to show an association of CA activity with highly purified PSII protein or with recombinant MSP under our conditions.

Table 1

Carbonic anhydrase activity assayed by isotopic exchange using a membrane inlet mass spectrometer using standard assay conditions at 20 °C

Sample	Protein added		<sup>13</sup> α	Carbonic anhydrase rate constants	
	mg ml <sup>-1</sup> <sup>a</sup>	nmol <sup>b</sup>		$k_1 \text{ s}^{-1} \mu\text{mol}^{-1}$	$k_2 \text{ s}^{-1} \mu\text{mol}^{-1}$
Spinach BBYs	1.2	0.7	92	2300	91
Spinach BBYs (- MSP) <sup>c</sup>	1.2	0.7	2.8	190	5.6
Spinach PSII particles	0.05	0.1	0.9	<0.1	<0.01
<i>T. elongatus</i> MSP	0.7	1.4	0.8	<0.1	<0.01
Bovine CA	$0.36 \times 10^{-2}$	$1.3 \times 10^{-2}$	36	190,000	7500
Buffer	0	0	1.0	9.1	0.4

The derived hydration and dehydration rate constants are  $k_1$  and  $k_2$ , and <sup>13</sup>α is a measure of the decrease in <sup>18</sup>O mole fraction in <sup>13</sup>CO<sub>2</sub> with time.

<sup>a</sup> mg/ml quantities for either chlorophyll in the case of PSII samples, or protein for MSP and CA samples.

<sup>b</sup> Moles of reaction centre based on 220 chlorophyll per BBY sample, 35 chlorophyll per PSII core sample and 1 MSP per PSII.

<sup>c</sup> The MSP and other extrinsic proteins were removed by a 1 M CaCl<sub>2</sub> wash.



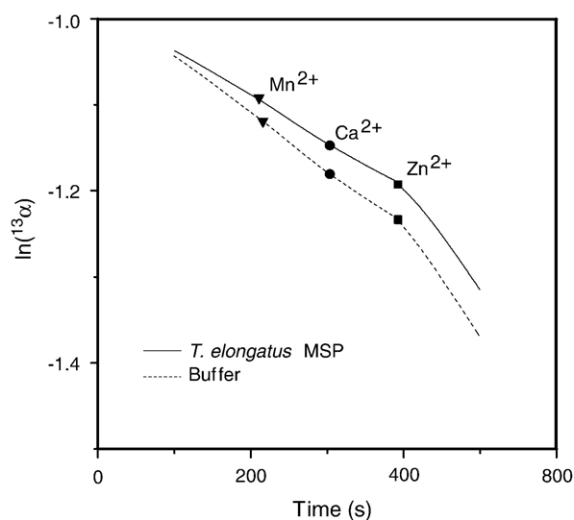


Fig. 2. Effect of Mn, Ca, and Zn addition on carbonic anhydrase activity of recombinant manganese stabilising protein. The  $^{18}\text{O}$  labelled bicarbonate is injected at time zero and following a short phase of chemical equilibrium a constant negative slope is achieved. Sequential addition of metal ions was performed every 90 s to a final concentrations of 50 mM. The solid line represents the recombinant *T. elongatus* manganese stabilising protein, the dashed line is buffer only background. All measurements were performed at 20 °C.

The possible involvement of divalent cations was then examined with the recombinant *T. elongatus* MSP in case the CA activity was impaired by loss of such a cofactor. The results shown in Fig. 2 show effects of cations on the  $^{13}\alpha$  rate with addition of 50 mM  $\text{MnCl}_2$  slightly increased the slope, indicative of a slight acceleration of the reaction. A subsequent addition of 50 mM  $\text{CaCl}_2$  fractionally reduced the slope and the final addition of 50 mM  $\text{ZnCl}_2$  significantly increased the reaction. However, these changes in rate were quite independent of the presence of the recombinant *T. elongatus* MSP being entirely mirrored in the background rates. The results like those in Table 1 do not support any CA activity associated with the

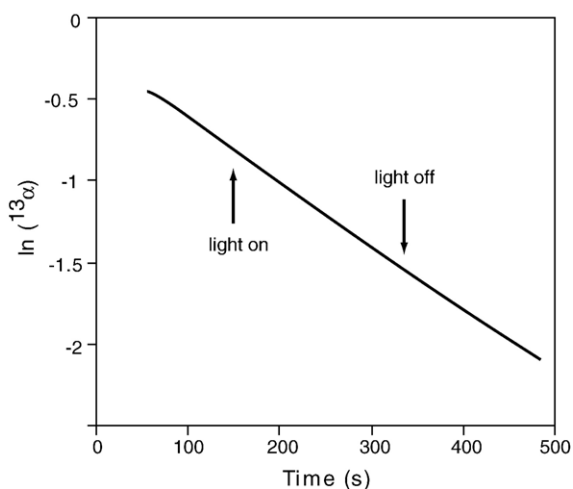


Fig. 3. Effect of light on carbonic anhydrase activity. Under the standard assay conditions at 20 °C spinach BBYs were exposed to light for 3 min. The upward arrow indicates when the illumination began and the downward arrow indicates when it stopped.

MSP of PSII. However, CA activity found as an intrinsic CA might still be important for PSII function.

The relationship between the PSII associated CA activity and that of PSII function was examined by performing the CA assay under illumination conditions in Fig. 3. For this experiment a spinach BBY preparation was assayed in the dark for CA activity and then exposed to actinic light for three min before being returned to the dark. The slope of the  $^{13}\alpha$  line remained unaffected by the light indicating that the rate of CA activity was independent of the lighting conditions of the reaction, whereas  $\text{O}_2$  production increased in the light (data not shown). While this result indicates the CA activity *per se* does not correlate with any electron transport activity of PSII, it is possible that a constant residual CA activity may be required for PSII function.

If the activity was independent of light then perhaps it was related to supporting the oxygen evolving activity of PSII in some other, indirect fashion. We sought to test the correlation in Fig. 4 by examining the rate of CA activity and the rate of oxygen evolution. For this comparison five independent preparations of spinach BBYs were tested under the standard oxygen assay and CA assay conditions. For each sample we found that the BBY preparations had similar rates of oxygen evolving activity, around  $300 \mu\text{mol O}_2 (\text{mg Chl})^{-1}$  at 20 °C, but their associated CA activity varied by one order of magnitude: between 2.5 and 25 times the background rate. The inference from this result was the oxygen evolving activity appeared to be independent of the magnitude of CA activity. However, this experiment does not exclude the possibility that the residual  $2.5\times$  background rate of CA activity might be the level of activity required for PSII function. To test this it would be necessary to demonstrate independent titrations of PSII activity and CA activity.

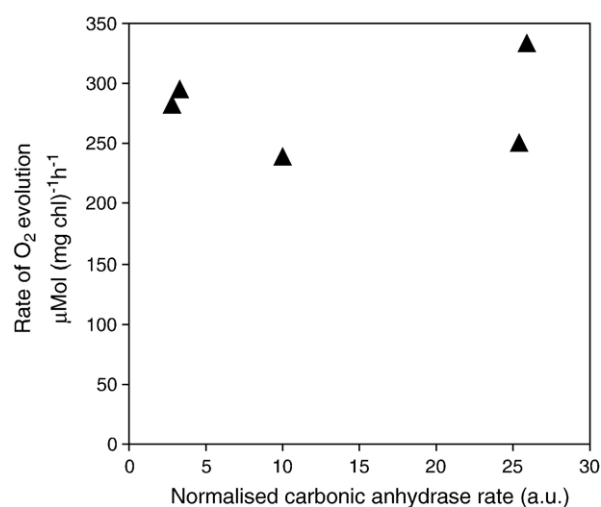


Fig. 4. Correlation between oxygen evolving activity and carbonic anhydrase activity. Under the standard assay conditions at 20 °C spinach BBYs were assayed for carbonic anhydrase activity and for oxygen evolution activity. The rates of carbonic anhydrase reactions were expressed as a function of the background uncatalysed reaction rate (see Table 1) and sampling point represents a unique BBY preparation.

Titration of oxygen evolving activity in PSII samples is shown in Fig. 5 and is progressively inhibited to zero by the addition of 0–10 mM DCMU. Over the same range the CA activity was unaffected. This finding, therefore, shows no apparent association between the chemistry of water oxidation and the ability for the associated CA activity with PSII samples. The result is in general agreement with the illumination experiment in Fig. 4. It remained then to test if inhibited CA activity would affect PSII function. To test any dependence of the PSII activity on CA activity the associated CA activity was gradually inhibited by the addition of 0–2 mM EZ in Fig. 6. The results showed that CA activity was inhibited by as much as 95%, a level equivalent to  $1.4\times$  the background uncatalysed rate, yet the oxygen evolving activity was almost entirely unaffected by this level of inhibition. There was at most a 5–10% reduction in oxygen evolving activity at the higher concentrations, perhaps due to non-specific interactions of EZ

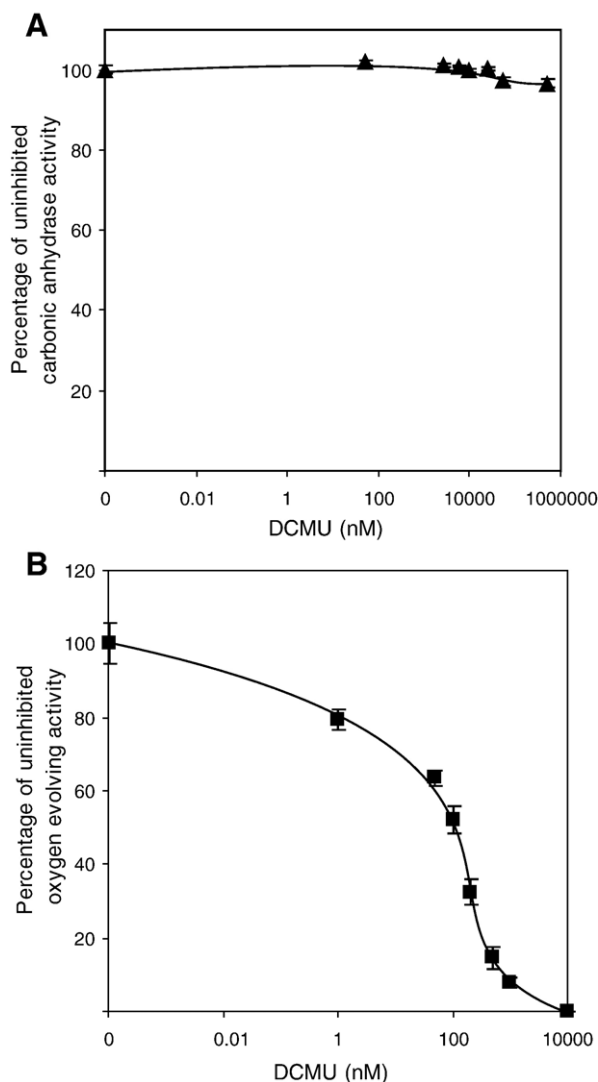


Fig. 5. The DCMU titration of oxygen evolving activity in spinach BBY material was used to measure (A) the percentage carbonic anhydrase activity and (B) the percentage of oxygen evolving activity. The error bars indicate  $\pm 1$  S.E. For the carbonic anhydrase assay the error bars are smaller than the symbols. All measurements were performed at 20 °C.

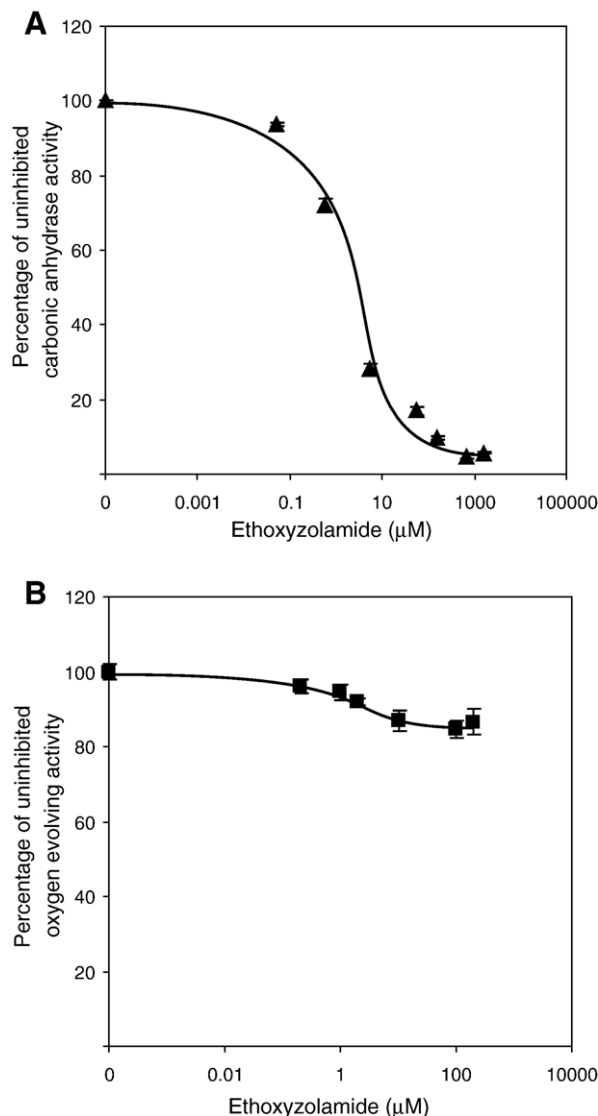


Fig. 6. Ethoxzylamide titration of carbonic anhydrase activity in spinach BBY material was used to measure (A) the percentage carbonic anhydrase activity and (B) the percentage of oxygen evolving activity. The error bars indicate  $\pm 1$  S.E. For the carbonic anhydrase assay the error bars are smaller than the symbols. All measurements were performed at 20 °C.

with PSII. Collectively, the data do not support any requirement for CA activity for PSII function.

#### 4. Discussion

The aim of this work was to specifically examine CA activity associated with PSII and to determine if PSII had any requirement for that activity. Our approach was to first attempt to locate the CA activity in the membrane or soluble fractions of PSII and then determine if activity in those fractions was associated with or required for PSII function. An important aspect of our investigation was the requirement for a CA activity assay that would be both quantitative and would also operate under chemical equilibrium conditions. The MIMS based method developed more than 60 years ago is the method of choice for studying CA and has been widely implemented

[28–30,33–36]. Other techniques that are based on radiocarbon counting of  $^{14}\text{CO}_2$  [37] and pH transient measurements [23,26] have also been used to study CA activity with PSII, but the techniques are less sensitive, require extensive signal averaging and do not operate under chemical equilibrium conditions. The MIMS approach as we will discuss further below measures a signal that is sustained over long time scales and was recently extended to numerically solve the overall rate constants for the hydration/dehydration reactions [28]. This approach simplifies to some extent the proposed intermediate steps of carbonic anhydrase [38] to a single hydration and dehydration reaction. However, the MIMS approach is a quantitative assessment of the overall kinetic steps of hydration and dehydrations used to describe the bicarbonate– $\text{CO}_2$  exchange reactions.

In the first experiments we assayed two PSII preparations that represented two levels of purity. Spinach BBYs are thylakoid membrane fragments that are enriched in PSII to levels of ~95%, contain relatively intact PSII complexes, and were shown in our measurements to contain CA activity at levels significantly greater i.e. 92 times that of background (Table 1). This finding is in agreement with our earlier work [28] and work using other techniques [23,27,36]. However, when we examined the highly purified PSII core preparation produced after partial detergent solubilisation of PSII from the membrane we were unable to find any specific CA activity in PSII core preparations (Table 1). This finding was contrary to other reports [22–24]. Also, other evidence has been presented that the CA activity is associated with the extrinsic MSP [22]. We performed analysis of the initial PSII BBY membrane fragments that were treated to dissociate the extrinsic proteins by 1 M  $\text{CaCl}_2$  washing. We found there to be no activity detected in the wash fraction itself (data not shown) and no activity in recombinant MSP forms of this protein (Table 1). We did however, find a reduction in the intrinsic CA activity in the BBY sample (Table 1). These data therefore suggest the reduction in CA activity was either associated with the removal of a co-purified CA protein, or was destroyed during the treatment arising from the dissociation of the extrinsic proteins. It is unlikely that the 1 M  $\text{CaCl}_2$  destroyed the activity as it has been previously shown not to affect activity in other CAs [39]. This finding does not support any direct association of CA and PSII implying the CA activity could be attached to PSII, the membrane itself or even be physically localised between the membranes. We finally then examined recombinant *T. elongatus* MSP and attempted chemical rescue experiments by adding  $\text{Mn}^{2+}$ ,  $\text{Ca}^{2+}$ , or  $\text{Zn}^{2+}$  (Fig. 2) and again found no stimulation of CA activity.

It appears from our findings that CA activity does not co-purify with PSII and there appears to be no association of CA activity with the MSP from PSII. This behaviour is thus suggestive that any CA activity is present as a contaminant rather than an intrinsic capacity of PSII. However, this interpretation was dismissed by Lu et al. [22] and Moskvina et al. [24] on the basis of there being multiple activities present and the failure to detect  $\beta$ -CA protein following immunoblotting. These authors indicated a form of CA activity did indeed purify with PSII. Results from Lu et al. [22] were also indicative that

recombinant MSP was able to exhibit a CA activity especially in the presence of Mn. However, there appears no indication that the MSP contains a zinc binding site from the crystal structures [8,9] and the use of Mn as the catalytic metal would at this point be entirely without precedent. However, it is possible that the diversity of CAs is greater than that currently realised and the MSP may be a novel class.

Why there is such discrepancy between our MIMS analysis and some other approaches is unclear. A variation between sample preparation techniques may be one interpretation for the difference. Another more likely prospect is the difference may lie in the methods used for CA assay. The authors above have based their analysis on measurements of a relatively small pH transient that requires extensive signal averaging and is resolved for a few seconds. Similarly the radiocarbon experiments are based on a short burst of enzymatic activity that is also averaged ( $n=40$  [22]). The short times that these techniques are implemented over are in marked contrast to the MIMS approach that is measured over longer times, i.e. 1–5000 s. We feel that based on these findings the functional significance of a CA enzymatic process that is only manifested over times shorter than ~10 s could be questioned. Another finding in contrary to the short <10 s measurements mentioned above is the actual period of chemical equilibrium. The MIMS experiments indicate chemical equilibrium is only reached after ~50 s at 20 °C (Fig. 1) and a constant rate of CA exchange follows for many tens of minutes. This result markedly contrasts the short transient phenomena recorded by the other groups and suggests more work is needed to examine the possible differences of the approaches.

If we then consider that the CA activity is not an integral component of PSII, the activity may therefore be derived from a separate CA protein that is associated with PSII function. This then continues to pose the question as to what function there would be for the CA activity and PSII. To examine this we correlated CA activity vs. oxygen evolving activity. We found oxygen evolution is inhibited without impairing CA function (Fig. 5). Furthermore, the reverse shows that CA activity can be essentially totally (95%) inhibited without inhibiting the oxygen evolving activity (Fig. 6).

Our results therefore are indicative that any CA activity associated with PSII is found in non-stoichiometric quantities. This suggests for the spinach C3 photosynthesis system we studied that the CA is not required for optimal PSII function. While spinach may not need a CA activity, this potentially could be a different situation in other species such as *C. reinhardtii* where the PSII has CA physically attached [40]. However, given the overall structural and mechanistic similarity between different PSII in different photoautotrophs the water splitting chemistry appears to be entirely conserved [41]. Therefore this association of CA may have other functions outside from the specifics of water splitting [42].

The role of these CAs is more likely to be associated primarily with a carbon concentration mechanism supplying sufficient  $\text{CO}_2$  to rubisco, possibly in accordance with the Raven hypothesis which proposes that bicarbonate is transported to the thylakoid lumen to be converted into  $\text{CO}_2$  by CA,

assisted by the acid pH. The CO<sub>2</sub> is then available to rubisco on diffusion through the thylakoid membrane [43]. The possibility that bicarbonate may play a structural role either as a cofactor in the water oxidising complex or in a less specific role on the donor side remains an equally valid proposal [44].

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