The photosystem II-associated Cah3 in *Chlamydomonas* enhances the O$_2$ evolution rate by proton removal

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Water oxidation in photosystem II (PSII) is still insufficiently understood and is assumed to involve HCO$_3^-$. A *Chlamydomonas* mutant lacking a carbonic anhydrase associated with the PSII donor side shows impaired O$_2$ evolution in the absence of HCO$_3^-$. The O$_2$ evolution for saturating, continuous illumination (R_{O2}) was slower than in the wild type, but was elevated by HCO$_3^-$ and increased further by Cah3. The R_{O2} limitation in the absence of Cah3/HCO$_3^-$ was amplified by H$_2$O/D$_2$O exchange, but relieved by an amphiphilic proton carrier, suggesting a role of Cah3/HCO$_3^-$ in proton translocation. Chlorophyll fluorescence indicates a Cah3/HCO$_3^-$ effect at the donor side of PSII. Time-resolved delayed fluorescence and O$_2$-release measurements suggest specific effects on proton-release steps but not on electron transfer. We propose that Cah3 promotes proton removal from the Mn complex by locally providing HCO$_3^-$, which may function as proton carrier. Without Cah3, proton removal could become rate limiting during O$_2$ formation and thus, limit water oxidation under high light. Our results underlie the general importance of proton release at the donor side of PSII during water oxidation.

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Introduction

Carbonic anhydrases (CAs) serve different functions in the metabolism of algae and plants. Their role is in carboxylation/decarboxylation reactions, and therefore CA functions in algal carbon-concentrating mechanisms, ion transport, pH homeostasis and in the production of carbon skeletons by mitochondria (for reviews, see Raven, 1995; Moroney *et al*, 2001; Giordano *et al*, 2005). The first intracellular algal α-CA (Cah3) was identified in the green alga *Chlamydomonas reinhardtii* (Karlsson *et al*, 1998). Immunolocalization showed that Cah3 is located on the lumenal side of thylakoid membranes, including those that penetrate the pyrenoid (Mitra *et al*, 2005). Moreover, the Cah3 protein was enriched in a core complex fraction compared with photosystem II (PSII) membrane fragments (Villarejo *et al*, 2002). Cah3 was further found to be functionally associated with the electron donor side of photosystem II (PSII), which is the site of proton release and O$_2$ production. Intact cells, thylakoids and PSII-enriched membranes fragments of a mutant lacking the Cah3 protein (*cia3*) showed impaired water-splitting ability (Villarejo *et al*, 2002).

Although O$_2$ and protons are both produced at the electron donor side of PSII, their release patterns differ greatly. O$_2$ is released only during the terminal transition of a four-electron cycle completed by a metal centre, when this centre returns from its highest (S$_4$) to its lowest (S$_0$) oxidation state. Proton production, on the other hand, is more evenly distributed over several of these state transitions. It is a further complication that release of protons at the catalytic centre is superimposed on transient proton release and uptake caused by electrostatic interactions of peripheral acidic and basic groups with this centre. It is now accepted that proton release at the catalytic centre proper follows a 1:0:1:2 pattern during the transitions sequence S$_0 \to$ S$_1 \to$ S$_2 \to$ S$_3 \to$ S$_4$- - -S$_0$; (Fowler, 1977; Schlodder and Witt, 1999; Junge *et al*, 2002; Dau and Haumann, 2007). The sequence and relation of the transfer of electrons (to Y$_Z$, a redox-active tyrosine residue) and protons (to the aqueous bulk) have recently been described by a nine-step reaction cycle (*I*-cycle; Dau and Haumann, 2006). Evidence for a crucial role of proton release in the S$_3 \to$ S$_4$- - -S$_0$ transition has been presented (Haumann *et al*, 2005; Dau and Haumann, 2008). At low pH, this proton-release step likely limits the yield of dioxygen (Bernat *et al*, 2002). The release of protons during the terminal transition is the slowest step, with the reduction of the metal centre and the release of O$_2$ occurring simultaneously with a typical half-rise time of about 1.3 ms (Joliot, 1968; Kok *et al*, 1970; Haumann and Junge, 1994; Junge *et al*, 2002; Clausen *et al*, 2004). The kinetic H/D-isotope effect of proton transfer (2.4) is larger than that of electron transfer (1.4) (Haumann *et al*, 1997). Thus, if O$_2$ production was rate-limited by proton release, then the limitation would be expected to occur during this terminal step (the $I_0 \to I_1$ transition; Dau and Haumann, 2006).

Evidence that HCO$_3^-$ might be a structural component of the PSII acceptor side between Q$_A$ and Q$_B$, and that it binds to a non-heme iron, were published (Blubaugh and Govindjee, 1988; Diner and Petrouleas, 1990). Possible roles for HCO$_3^-$ on the donor side of PSII (Stemler *et al*, 1974; Villarejo *et al*, 2002; van Rensen and Klimov, 2005) remain controversial, although the proposed functions of HCO$_3^-$ as an electron donor (Warburg and Krippahl, 1958) and a catalyst (Metzner *et al*, 1979) have been ruled out by recent experiment (Clausen *et al*, 2005a; Hillier *et al*, 2006). Furthermore, evidence that HCO$_3^-$ has a structural role (Dasgupta *et al*, 2004; Ferreira *et al*, 2004) remains contradictory (Loll *et al*, 2005; Siegbahn and Lundberg, 2006) and it was this possibility that prompted us to test the effects of HCO$_3^-$ and Cah3 supplementation on the rate of O$_2$ evolution in a Cah3-less mutant. We report here that isolated PSII-enriched membrane fragments derived from the Cah3-less mutant showed a lower rate of O$_2$ evolution than membrane fragments from wild-type (wt) *Chlamydomonas* under exposure to both continuous light and flash light. Our experimental findings support a proposition that the rate limitation of O$_2$ evolution results from a kinetic role of HCO$_3^-$ in the removal of protons produced by the oxidation of water.

Results

The experimental system consisted of isolated PSII membrane fragments from either wt *C. reinhardtii* cells containing the Cah3 protein, wt PSII, or from the Cah3-less mutant (*cia3*), PSII(Cah3−). Both wt and PSII(Cah3−) PSII membrane fragments were suspended in an MES–KOH buffer initially free of inorganic carbon (C$_i$) at pH 5.5. This value is close to (Hager and Holocher, 1994) or slightly below

(Kramer *et al*, 1999) the pH of the thylakoid lumen in the light and lower than the pK_2 of carbonic acid, favouring conversion of HCO$_3^-$ to CO$_2$. PSII activity was measured before and after addition of HCO$_3^-$ and/or overexpressed Cah3. The rate of O$_2$ evolution was first determined under continuous illumination.

The HCO$_3^-$-induced increase in O$_2$ evolution in cia3-mutant PSII membrane fragments is twofold greater in the presence of the Cah3 protein

Expressed per reaction centre (RC), the O$_2$ evolution rate of PSII(Cah3−) fragments in C$_i$-free medium was lower than that of the wt PSII fragments (Figure 1A). This particular study was a logical continuation of our earlier observation (Villarejo *et al*, 2002) that addition of HCO$_3^-$ to PSII(Cah3−) fragments in the O$_2$ electrode chamber led to enhanced O$_2$ release. Two millimolar HCO$_3^-$ stimulated the rate of O$_2$ evolution, on average, by 40%. When overexpressed and purified CA, Cah3, enzyme was added simultaneously with HCO$_3^-$, the stimulation increased twofold and reached the wt PSII rate if calculated per RC (Figure 1A). HCO$_3^-$ alone, at all concentrations and experimental conditions tested (pH, temperature, buffer system etc.), was unable to restore O$_2$ evolution in PSII(Cah3−) to the full wt PSII rate. From Figure 1B and thereafter, 100% corresponds to the O$_2$ evolution rate of PSII(Cah3−) in C$_i$-depleted media (the typical rate was 180–200 µmol O$_2$ (mg Chl)$^{-1}$ h^{-1}).

Addition of Cah3 to PSII(Cah3−) membrane fragments in the presence of HCO$_3^-$ reactivated O$_2$ evolution but this reactivation could be entirely prevented by the addition of either 0.2 µM of the CA-specific inhibitor ethoxyzolamide (EZ) or by removing the substrate HCO$_3^-$ (Figure 1A). In contrast to the addition of Cah3, addition of bovine CA, CAII
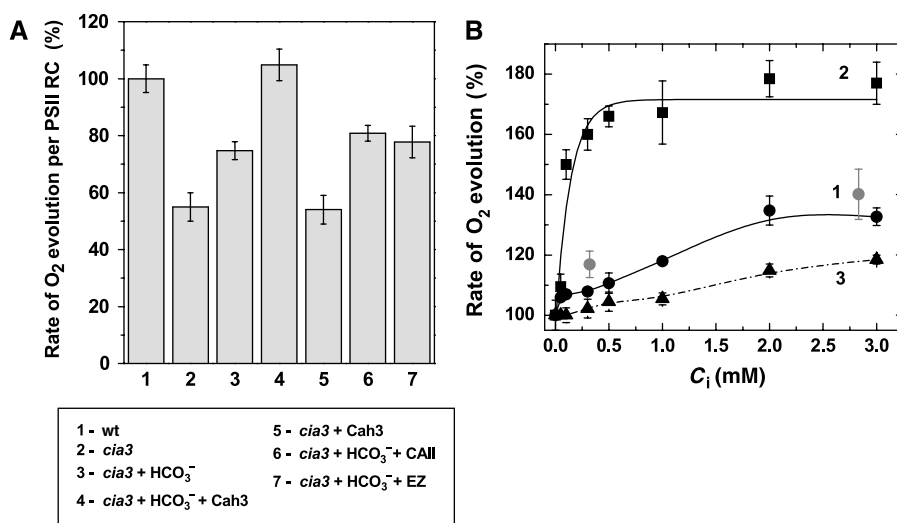

Figure 1 Overexpressed and purified Cah3 protein fully restores O$_2$ evolution in PSII membrane fragments derived from the Cah3-less *cia3* mutant. (**A**) Means ± s.e. of O$_2$ evolution rates expressed as a percentage of the wt rate and per PSII RC in C$_i$-free buffer: (1) PSII from wt cells in C$_i$-free SMS buffer without additions, (2) PSII(Cah3−) without additions, (3) in the presence of 2 mM KHCO$_3$, (4) in the presence of 2 mM KHCO$_3$ and 0.1 µM recombinant Cah3 protein, (5) in the presence of 0.1 µM recombinant Cah3 protein, (6) in the presence of 2 mM KHCO$_3$ and 0.1 µM bovine CAII protein (EC 4.2.1.1) and (7) in the presence of 2 mM KHCO$_3$ and both 0.1 µM recombinant Cah3 protein and 0.2 µM EZ. (**B**) HCO$_3^-$ stimulation of O$_2$ evolution rates in (1) PSII(Cah3−), (2) PSII(Cah3−) reconstituted with 0.1 µM recombinant Cah3 protein and (3) wt PSII. Red circles show O$_2$ evolution rates in the presence of the indicated KHCO$_3$ concentration and 0.1 µM bovine CAII (EC 4.2.1.1). The control (100%) rate of O$_2$ evolution corresponds to the PSII(Cah3−) O$_2$ evolution rate in C$_i$-depleted media and was 180 µmol O$_2$ (mg Chl)$^{-1}$ h^{-1}. The light intensity was 1200 µmol m^{-2} s^{-1} PAR. Chl concentration was 15 µg ml^{-1}. All measurements were conducted at 25°C in the presence of 1 mM DCBQ and 0.5 mM FeCN. Values are means ± s.e. ($n = 3$). A full-colour version of this figure is available at *The EMBO Journal Online*.

(EC 4.2.1.1), under similar conditions did not stimulate O$_2$ evolution above that obtained with HCO$_3^-$ alone (Figure 1A and B), despite its high enzymatic activity in solution.

The O$_2$ evolution rate of PSII(Cah3−) saturated at 0.25 mM HCO$_3^-$ in the presence of added Cah3 enzyme, but in the absence of Cah3 supplementation, saturation was reached first at 2 mM HCO$_3^-$ (Figure 1B). Thus, Cah3 had a twofold greater stimulatory effect at an almost 10-fold lower HCO$_3^-$ concentration compared with HCO$_3^-$ alone. A stimulatory effect on the O$_2$ evolution rate was observed also in wt PSII upon addition of HCO$_3^-$ alone or together with Cah3, but in this case the stimulation never exceeded 15% (Figure 1B). Thus, under continuous illumination, when neither the light intensity nor the electron acceptor side of PSII(Cah3−) were rate limiting, Cah3 in the presence of its substrate, HCO$_3^-$, stimulated the O$_2$ evolution rate twofold (Figure 1A and B; Supplementary Figure S1A and B).

Cah3/HCO$_3^-$ accelerates O$_2$-release kinetics

After dark adaptation of PSII, all Mn$_4$CaTyr clusters of the water oxidizing complex (WOC) are synchronized mainly in the first oxidized state, which is designated S$_1$. Excitation with a train of short light flashes stepwise oxidizes the Mn$_4$CaTyr centres until after the third flash O$_2$ is released. O$_2$ is produced and released only after the centre has reached its fourth oxidation state, S$_4$. Figure 2 shows polarographic
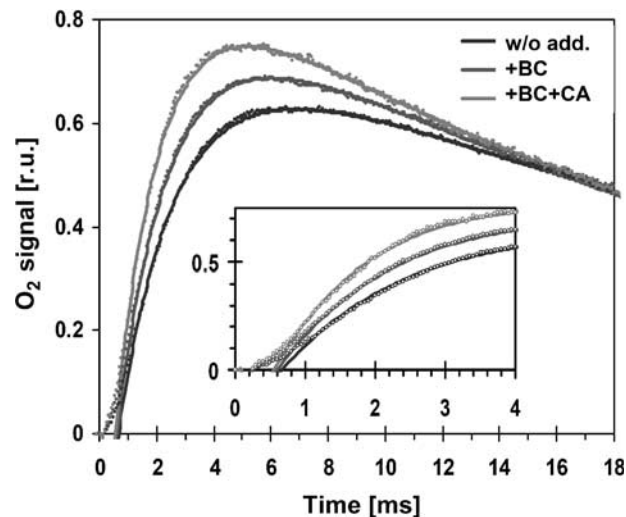

Figure 2 Flash-induced raw polarographic transients recorded with a Clark-type O$_2$ electrode in C$_i$-free SMS buffer with PSII(Cah3−) membranes, blue symbols; PSII(Cah3−) membranes in the presence of 1 mM KHCO$_3$, green symbols and PSII(Cah3−) membranes reconstituted with 0.7 μM recombinant Cah3 protein and 1 mM KHCO$_3$, red symbols. The lines result from simulations involving a delayed onset of the exponential rise, an either mono- or bi-exponential rise and an exponential decay. The following parameters were used in the simulations shown (delay//first rise time and amplitude//second rise time and amplitude//decay time): without additions (blue)—670 μs//1.8 ms, 65%//6 ms, 35%//24 ms; with HCO$_3^-$ (green)—600 μs//1.8 ms, 84%//4 ms, 16%//24 ms; with HCO$_3^-$ and Cah3 (red)—550 μs//1.6 ms, 100%//not required//24 ms. Unless HCO$_3^-$ and Cah3 are simultaneously supplemented, a bi-exponential simulation of the O$_2$ rise is required (see Supplementary data). However, due to extensive parameter correlations, various combinations of amplitudes and time constants can result in simulations of similar quality. A full-colour version of this figure is available at *The EMBO Journal Online*.

recordings of O$_2$ release of dark-adapted PSII(Cah3−) upon release of the third flash. A typical half-rise time of O$_2$ release is 1.3 ms (Clausen *et al*, 2004). In agreement with steady-state O$_2$ evolution, O$_2$ rise in PSII(Cah3) membranes suspended in C$_i$-free buffer and exposed to flash light was accelerated by the addition of Cah3 or just HCO$_3^-$ (Figure 2). In the wt PSII, on the other hand, there was no noticeable effect of these additions on the rise of O$_2$ (data not shown). The half-rise time of wt PSII was 1.3 ms, whereas in PSII(Cah3−) it was 1.9 ms. In the presence of HCO$_3^-$, the apparent half-rise time decreased by about 20% and even further when HCO$_3^-$ and Cah3 were added simultaneously. The amplitude of flash-induced polarographic recordings of O$_2$ release was not influenced significantly by Cah3/HCO$_3^-$, implying that the number of active centres remained unaffected.

The above description of the O$_2$-release kinetics by a single half-rise time is oversimplified. The O$_2$ electrode signal indeed comprises (i) an initial delay (lag phase, $t < 600$ μs) reflecting mostly a delayed O$_2$ release by PSII (Clausen *et al*, 2004), (ii) a rise in O$_2$ concentration reflecting O$_2$ formation by PSII and (iii) a decay due to O$_2$ diffusion within the electrode cell. For PSII(Cah3−) supplemented with HCO$_3^-$ and Cah3, the rise phase is well described by a single exponential. However, without HCO$_3^-$/Cah3, a bi-exponential simulation of the rise phase of O$_2$ release is required for good agreement, where the slower phase contributes by 10–30% to the total amplitude of the rise (Supplementary Figure S2, data simulation). The simulations *inter alia* show that the time constants of the major rise phase are unaffected by HCO$_3^-$ addition and are essentially identical to the values determined by the delayed fluorescence measurements described below. Therefore, the acceleration of O$_2$ release by HCO$_3^-$ and Cah3 supplementation is mostly explainable by a decrease in the contribution of a minor slower component to the exponential rise of O$_2$ release; a minor contribution presumably stems from a reduction in the duration of the initial lag phase.

Cah3/HCO$_3^-$ affects the PSII donor side

HCO$_3^-$ binds to the non-heme iron at the acceptor side of PSII (Ferreira *et al*, 2004; Loll *et al*, 2005). After formate treatment, HCO$_3^-$ addition is required to restore Q$_A$→Q$_B$ electron transfer (Blubaugh and Govindjee, 1988; Diner and Petrouleas, 1990). It was therefore important to clarify whether or not the stimulatory effect of HCO$_3^-$ in PSII(Cah3−) was caused by an acceptor-side effect. To address this question, we measured flash-induced changes in the yield of prompt chlorophyll (Chl) fluorescence, which reflect the re-oxidation of reduced Q$_A$ by electron transfer to Q$_B$ (Dau, 1994; Steffen *et al*, 2001). As is evident, there was no difference in the decay kinetics between various samples (Figure 3). This observation strongly suggests that the Cah3/HCO$_3^-$ effect is not associated with changes at the acceptor side of PSII(Cah3−) (no formate treatment). The increase in Chl fluorescence yield (ΔF) under continuous light also supports a limitation at the donor side (Supplementary Figure S2).

Cah3 stimulates O$_2$ evolution through its substrate/product bicarbonate as a carrier facilitating proton transfer

Cah3, an α-type CA with a rate of about one million turnovers per second (Lindskog, 1997), speeds up the reaction

$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. It is conceivable that Cah3 facilitates the removal of H^+ at the donor side of PSII and, by doing so, facilitates H_2O oxidation. This would be consistent with the data in Figures 1 and 2; Supplementary Figure S2 showing that Cah3 enzymatic activity was required
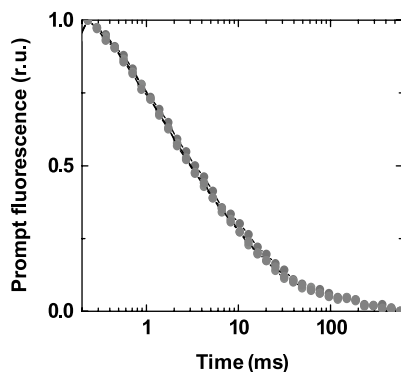

Figure 3 Flash-induced changes in the yield of prompt Chl fluorescence measured in a pump-probe experiment as described under Materials and methods. The time course was detected after the third flash was applied to dark-adapted PSII membrane fragments isolated from the *cia3* mutant in C_i-free SMS buffer. PSII(Cah3−) membranes, blue; PSII(Cah3−) membranes in the presence of 1 mM KHCO$_3$, green and PSII(Cah3−) membranes reconstituted with 0.07 μM recombinant Cah3 protein in the presence of 1 mM KHCO$_3$, red. Chl concentration was 10 μg ml^{-1}. See Buchta *et al* (2007) for more details on the prompt fluorescence measurements. A full-colour version of this figure is available at *The EMBO Journal Online*.

to obtain full reactivation of water oxidation. In agreement with earlier reports (Clausen *et al*, 2005a; Hillier *et al*, 2006), we disregard the possibility that HCO$_3^-$ is directly involved in the electron donation in PSII. Instead, our hypothesis is that HCO$_3^-$ accelerates proton removal from the vicinity of the WOC, which is essential for the energetics of water oxidation (Krishtalik, 1986; Haumann and Junge, 1994; Dau and Haumann, 2007; Renger, 2007).

This hypothesis was tested using the amphiphilic pH indicator/buffer neutral red (NR), which has been shown to be an efficient H^+ donor/acceptor through bimolecular interactions at the thylakoid membrane surface (Auslander and Junge, 1975; Junge *et al*, 2002). A clear stimulation of O$_2$ evolution by this dye was obtained. In the presence of 20 μM NR, the rate of O$_2$ evolution of PSII(Cah3−) increased to the same extent as with 2 mM HCO$_3^-$ (Figure 4A). At higher concentrations of NR (40 μM), the stimulation was comparable to that of HCO$_3^-$ and Cah3 added simultaneously. At optimal NR concentrations, there was no further stimulation by 2 mM HCO$_3^-$. A decrease of the O$_2$ evolution rate was observed at NR concentrations exceeding 50 μM (data not shown). In wt PSII, no significant increase in the O$_2$ evolution rate was found by the addition of NR. The fact that NR mimics the effect of HCO$_3^-$ and Cah3 in the water oxidation reaction supports the conclusion that Cah3 accelerates O$_2$ evolution by facilitating the removal of protons from the WOC.

Additional independent support for a proton limitation in PSII(Cah3−) comes from isotope-exchange experiments
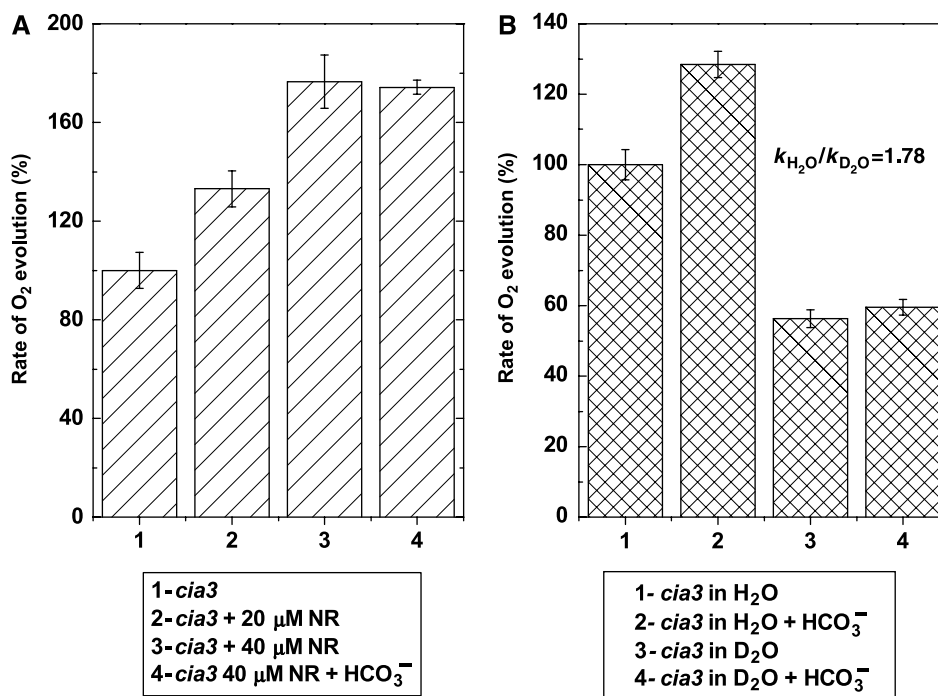

Figure 4 Bicarbonate stimulates O$_2$ evolution in PSII(Cah3−) by facilitating proton transfer away from WOC. (**A**) Stimulatory effect of the amphiphilic pH indicator NR on the O$_2$ evolution rate of membrane fragments from *cia3*. PSII(Cah3−), 1; PSII(Cah3−) in the presence of 20 μM NR, 2; in the presence of 40 μM NR, 3 and in the presence of 40 μM NR and 2 mM KHCO$_3$, 4. The initial rate of O$_2$ evolution (indicated as 100% level in the figure) was 183 μmol O$_2$ (mg Chl)$^{-1}$ h^{-1}. The assay medium was depleted of endogenous C_i and the Chl concentration was 15 μg ml^{-1}. All measurements were conducted at 25°C in the presence of 1 mM DCBQ and 0.5 mM FeCN. Values are means ± s.e. ($n = 4$). (**B**) Rate of O$_2$ evolution in PSII membrane fragments from the *cia3* mutant measured in H$_2$O and D$_2$O media. PSII(Cah3−) in SMS buffer, 1; PSII(Cah3−) in SMS buffer in the presence of 1 mM KHCO$_3$, 2; PSII(Cah3−) in D$_2$O-SMS buffer, 3 and PSII(Cah3−) in D$_2$O–SMS buffer in the presence of 1 mM KHCO$_3$, 4. The k_{H_2O}/k_{D_2O} in the PSII(Cah3−) was 1.78 and 1.15 in wt PSII. Values are means ± s.e. ($n = 6$). The initial rate of O$_2$ evolution (indicated as 100% level) was 190 μmol O$_2$ (mg Chl)$^{-1}$ h^{-1}.

(Figure 4B). The kinetic isotope effect k_{H_2O}/k_{D_2O} in the mutant was 1.78, whereas in it was only 1.15 the wt PSII. Importantly, stimulation of O_2 release by HCO_3^- in PSII(Cah3−) in an H_2O-based buffer was 37%, whereas no statistically significant stimulation could be detected in a D_2O-based buffer.

To investigate more directly the influence of HCO_3^- on the rate of proton transfer from the Mn complex to the lumenal bulk phase, measurements of delayed Chl fluorescence (DF) were conducted. Results obtained previously by time-resolved DF and X-ray measurements (Haumann *et al*, 2005; Buchta *et al*, 2007) and other methods (Rappaport *et al*, 1994) suggest that in the O_2 evolution transition ($S_3 \rightarrow S_4 \rightarrow S_0$), an S_4 intermediate is formed by transfer of a proton from the Mn complex to the lumenal bulk phase (for a detailed discussion, see Dau and Haumann, 2007). This step of removing a proton from the Mn complex precedes electron transfer and dioxygen formation itself. It is likely that this proton-removal step is an essential prerequisite for dioxygen formation (Dau and Haumann, 2005, 2008).

The third nanosecond-laser flash applied to dark-adapted PSII membrane fragments induces $S_3 \rightarrow S_4 \rightarrow S_0$ transition. In the time course of DF excited by the third flash (Figure 5), the $S_3 \rightarrow S_4$ transition ($t < 1$ ms) is reflected in the decay from the initial value at 10 µs to the plateau reached at about 1 ms. The subsequent DF decay in the millisecond time domain reflects the reduction of the $Y_Z^{\bullet +}$ radical and the simultaneous dioxygen formation step itself (Zankel, 1971; Grabolle and Dau, 2005; Clausen *et al*, 2005b; Buchta *et al*, 2007). In PSII(Cah3−) fragments suspended in C_i-depleted media, the rate of proton removal in the S_4 formation step was reproducibly found to be accelerated by about 25% by the addition of HCO_3^- (visual inspection of DF; Figure 6) but not so in the wt PSII (not shown). A tri-exponential simulation of the DF time courses (Buchta *et al*, 2007) revealed that the corresponding time constant changed from 200 to 150 µs upon HCO_3^- addition, whereas in the wt it was close to 150 µs and was unaffected by HCO_3^-. However, the time constant

assignable to $Y_Z^{\bullet +}$ reduction and O_2 formation is apparently unaffected by HCO_3^- addition (time constant of 1.9 ± 0.1 ms, with and without HCO_3^-).

As in the $S_3 \rightarrow S_4 \rightarrow S_0$ transition, in the $S_2 \rightarrow S_3$ transition electron transfer likely is also preceded by an essential step of removing a proton from the Mn complex, as proposed by Dau and Haumann (2006), and reviewed elsewhere (Dau and Haumann, 2007). The $S_1 \rightarrow S_2$ transition does not involve any proton removal from the Mn complex. As shown in Figure 8, in the $S_2 \rightarrow S_3$ transition (but not in $S_1 \rightarrow S_2$ transition), the DF decay was also accelerated by the addition of HCO_3^- (by ∼20%). Thus, the DF analysis supports a role of HCO_3^- in the removal of protons from the Mn complex.

Purified Cah3 protein stoichiometrically rebinds to mutant PSII membrane fragments and restores their activity

According to the functional analysis presented above, lumenal Cah3 is a protein with the molecular architecture required to bind to a specific site of PSII, where its function is accomplished. To test its rebinding ability, we conducted western blot analysis using Cah3-specific antibodies. The single 29-kDa band corresponding to Cah3 is clearly visible in the lane containing wt PSII, while no cross-reaction occurred with PSII(Cah3−). The same samples were examined using PsbO-specific antibodies (Figure 7A). Following reactivation of the O_2-evolving activity of PSII(Cah3−) in the presence of overexpressed and purified Cah3, the samples
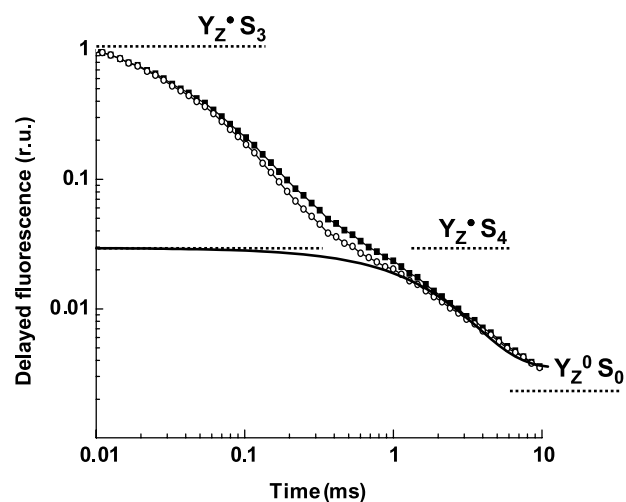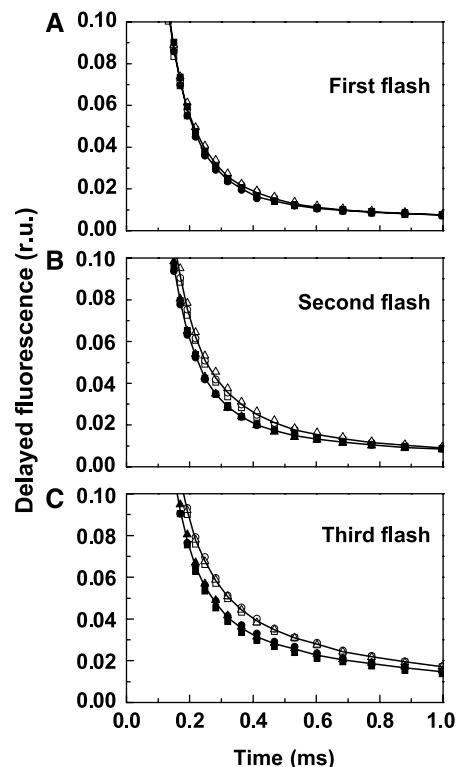

Figure 5 Decay of DF following the third flash, with correction for the influence of Q_A re-oxidation. PSII(Cah3−) in C_i-free SMS buffer without additions, open symbols; PSII(Cah3−) in C_i-free SMS buffer in the presence of 1 mM KHCO₃, filled symbols. Chl concentration was 10 µg ml⁻¹. For details of the DF measurement and its analysis, see Buchta *et al* (2007).

Figure 6 Time course of DF for the first three flashes (**A–C**) applied to dark-adapted PSII(Cah3−) isolated from the *cia3* mutant in C_i-free SMS buffer. Open symbols, without additions; filled symbols, in the presence of 1 mM KHCO₃. The figure shows three independent replicates for each treatment and the lines are the best-fit curves for each group of three replicates within a treatment. All curves are normalized at 10 µs.
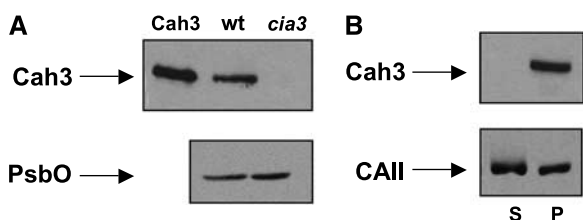
Figure 7 Stimulation of O$_2$ evolution of PSII membrane fragments caused by binding of Cah3. (**A**) Immunoblot analysis of PSII membrane fragments from the wt and the *cia3* mutant (*cia3*). The recombinant Cah3 protein was loaded as a positive control (Cah3). (**B**) Immunoblot analysis of PSII(Cah3−) after reconstitution with recombinant Cah3 protein, followed by centrifugation to obtain pellet (P) and soluble (S) fractions. The antibodies used were against Cah3, PsbO and CAII. Lanes *cia3*, wt and P were loaded with 7 μg of Chl and Cah3 along with 1.2 μg Cah3 protein.

were centrifuged to separate the membrane-bound protein from the soluble fraction (Figure 7B). Both pellet and supernatant were subjected to western blot analysis using Cah3-specific antibodies. All Cah3 protein detected was in the pellet (lane P). It is therefore concluded that the Cah3 protein binds to PSII(Cah3−). The K_D for binding of Cah3 was about 0.1–0.2 μM, indicating specific binding. K_D was calculated at ratios from 0.1 to 5 Cah3 per PSII RC. Increasing the ratio further caused unspecific binding. The reactivation of O$_2$-evolving activity was also tested with bovine CAII (see Figure 1A and B), and the sample was separated into membrane-bound and soluble fractions after the O$_2$ assay. Importantly, CAII also had a different distribution than Cah3 between pellet and supernatant (Figure 7B). Much less CAII was bound to the membrane phase compared with Cah3. It was not possible to determine the K_D for bovine CAII because CAII was evenly distributed between pellet and soluble fraction at all concentrations. In agreement with the rebinding experiments, the greatest stimulation of mutant O$_2$ evolution by Cah3 was observed at a 1:1 protein to PSII RC ratio (Supplementary Figure S3).

Discussion

Our starting point was the striking observation that in the Cah3-deficient mutant assayed in C$_i$-free media, the rate of O$_2$ evolution per PSII under continuous light was approximately half of that of the wt, and that complete restoration to the wt rate in the mutant was only achieved after addition of over-expressed Cah3 together with HCO$_3^-$ (Figure 1A and B). This observation raises the question of what important function could Cah3 have within the PSII complex to so strongly enhance O$_2$ release?

One possibility that we could exclude is that the Cah3 protein itself fulfils a structural function within the PSII multiprotein complex. Such a function would remain after addition of low concentrations of the CA-specific inhibitor EZ, a rather small molecule that binds to the active site of Cah3 and with no influence on the structure of the enzyme (Huang *et al*, 1998). As is demonstrated in Figure 1A, the stimulatory effect obtained by Cah3 disappeared after the addition of this inhibitor. Even in the presence of 1 mM HCO$_3^-$, no stimulatory effect was found for EZ-inhibited Cah3. The lack of stimulation by the Cah3 enzyme in the absence of its substrate, HCO$_3$ (Figure 1A), is further corroborating evidence that there is no

structural effect of this protein on the O$_2$-evolving activity of PSII. The specific association of Cah3 with PSII is proven by the low K_D of about 0.1 μM and a maximal stimulation of O$_2$ evolution at a 1:1 ratio of Cah3 to PSII. In contrast, bovine CAII did not show any specific binding over a broad concentration range and resulted in no stimulation of O$_2$ evolution despite its high activity (Figure 1A and B; Supplementary Figure S3). Thus, on the basis of the results presented here and earlier results (Karlsson *et al*, 1998; Villarejo *et al*, 2002), we feel confident to propose that lumenal Cah3 is a PSII-specific CA and that it restores O$_2$ evolution by exerting a locally directed, rather than a general, CA activity in the lumen bulk at low HCO$_3^-$ concentration.

The oxidation of Y$_Z$ is very fast (rise time <1 μs) and the detected rise time of O$_2$ evolution is independent of the rate constants at the acceptor side of PSII. That we observed an influence of Cah3 and HCO$_3^-$ on this rise (Figure 2) but not on the prompt fluorescence decay kinetics (Figure 3) are strong arguments for the localization of the HCO$_3^-$ effect at the electron donor side. Whereas rise was accelerated by HCO$_3^-$, the magnitude of the laser flash-induced O$_2$ pulse was largely unaffected, implying a constant number of active PSIIs. In conclusion, five different experimental approaches: (i) O$_2$ evolution and (ii) variable fluorescence (ΔF) for continuous illumination (Figure 1; Supplementary Figure S2), (iii) O$_2$ liberation, (iv) prompt and (v) delayed fluorescence kinetics detected after flash excitation (Figures 2, 3, 5 and 6) provide consistent coherent results and support a direct function of HCO$_3^-$ and Cah3 at the PSII donor side.

Warburg and Krippahl (1958) proposed that CO$_2$ is the source of O$_2$ in photosynthesis. Later, this hypothesis was modified by Metzner *et al* (1979), who suggested that HCO$_3^-$ acted as the H$_2$O supply to PSII. In two recent papers on O$_2$ evolution in cyanobacteria and green plants (Clausen *et al*, 2005a; Hillier *et al*, 2006), it was corroborated that H$_2$O, and not HCO$_3^-$, is the donor of electrons and the sole source of O$_2$. However, a structural role of HCO$_3^-$ at the donor side of PSII, for example, in reconstitution and stabilization of the Mn$_4$CaTyr cluster, has remained a possibility (Dasgupta *et al*, 2004; van Rensen and Klimov, 2005). In the results wt PSII exhibited no measurable stimulation when HCO$_3^-$ was added to the C$_i$-depleted sample buffer, while PSII(Cah3−) was stimulated. This suggests that the wt binds HCO$_3^-$ with a much higher affinity than PSII(Cah3−). Under our experimental conditions, HCO$_3^-$ could not be removed from wt PSII and therefore we cannot exclude that there is a tight HCO$_3^-$-binding site at the PSII donor side (Dasgupta *et al*, 2004; van Rensen and Klimov, 2005). This site could be arginine 357 of the CP43 protein, which was suggested to be an HCO$_3^-$ ligand (Ananyev *et al*, 2005; Hwang *et al*, 2007). However, it appears likely that HCO$_3^-$ either leaves the Mn cluster after photo-assembly or is tightly bound and cannot be removed by bubbling with CO$_2$-free gas in spinach thylakoids (Shevela *et al*, 2007). Because it is now excluded that HCO$_3^-$ is the primary electron donor to PSII, and because Cah3 or HCO$_3^-$ does not likely exerts its effects only through a structural function, the possibility of HCO$_3^-$ acting as a local proton acceptor/carrier came into focus.

To test this hypothesis, we used NR, an amphiphilic buffer known to bind protons on the surface of thylakoid membranes (Auslander and Junge, 1975; Junge *et al*, 2002). The stimulatory effect of NR on the rate of O$_2$ evolution was as

great that as observed for the joint addition of HCO$_3^-$ and Cah3 to PSII(Cah3−), whereas there was no NR effect observed in wt PSII (Figure 4A). We note that NR effectively restores H$_2$O oxidation in PSII(Cah3−), whereas other buffers with suitable pK_a do not (data not shown). As opposed to these buffers, NR is amphiphilic and aligns along the membrane surface, where it accepts protons directly from the donor(s) instead of accepting protons from the bulk phase of the lumen.

The rate of O$_2$ evolution in PSII(Cah3−) was also modified when exchangeable protons were replaced by deuterons (Figure 4B). The significant kinetic isotope effect (K_{H_2O}/K_{D_2O} of 1.78) suggests that in C$_i$-depleted media and in the absence of Cah3 activity, the water oxidation is rate limited by a process involving proton translocation. In contrast, in wt PSII the replacement of H$_2$O by D$_2$O causes only minor changes ($\leqslant 15\%$) in the O$_2$ evolution rate, similar to the report by Sinclair and Arnason (1974) for *Chlorella* cells (K_{H_2O}/K_{D_2O} of 1.29). In the D$_2$O-based buffer solution, only an insignificant HCO$_3^-$-induced increase of the O$_2$ evolution rate was found in PSII(Cah3−). There are several conceivable explanations for the latter finding, for example, slow or inefficient deuteronation of HCO$_3^-$ or changed pK-values in D$_2$O (upshift by 0.2–0.7 units; Glasoe and Long, 1960) could impair the role of HCO$_3^-$ as a proton carrier in the Cah3-less PSII. The kinetics of electron transfer is virtually the same in H$_2$O and D$_2$O (Haumann *et al*, 1997; Christen and Renger, 1999). In marked contrast, proton release from the WOC, especially in the terminal step of the water oxidation reaction, exhibits greater isotope effects (Haumann *et al*, 1997; Karge *et al*, 1997). The result obtained for PSII(Cah3−) in a D$_2$O-based solution at pH 5.5 revealed a K_{H_2O}/K_{D_2O} value consistent with proton transfer being the rate-limiting step of the water oxidation reaction in the absence of Cah3 activity and HCO$_3^-$.

Further support for a role of the Cah3/HCO$_3^-$ system in proton removal from the Mn complex comes from our time-resolved measurements of DF after laser-flash excitation (Figures 5 and 6). In the S$_2 \rightarrow$ S$_3$ and S$_3 \rightarrow$ S$_4 \rightarrow$ S$_0$ transitions of the WOC, an essential proton-release step has been suggested to precede electron transfer (Haumann *et al*, 2005; Dau and Haumann, 2007). The corresponding proton movement towards the lumenal bulk is directly reflected in the DF time courses (Buchta *et al*, 2007). The DF decays indeed suggest that in PSII(Cah3−), HCO$_3^-$ addition is required for restoration of the proton-removal rate to the wt level. These time-domain experiments demonstrate acceleration of proton removal at the PSII donor side, and thus support the suggested role of HCO$_3^-$ as a proton acceptor and/or carrier.

A stimulating effect on O$_2$ evolution was obtained by adding HCO$_3^-$, Cah3 or both of them together under continuous light and excitation with short light flashes (Figures 1, 2, 5 and 6; Supplementary Figure S2). The lower stimulation detected for flash illumination, in comparison with continuous illumination, can be explained in several ways. We favour the explanation that under continuous light the protons produced in PSII(Cah3−) will saturate the proton acceptors. When subjected to a small number of short flashes, saturation of acceptors will not take place and therefore the stimulatory effect by HCO$_3^-$ and Cah3 is less pronounced.

In each water oxidation cycle, four protons are transferred from the Mn complex to the lumenal bulk phase; the sequence and distinct properties of the four steps of removal

of 'intrinsic' protons from the Mn complex have been discussed elsewhere (Junge *et al*, 2002; Dau and Haumann, 2006, 2008). The results reported here consistently suggest that HCO$_3^-$ may promote more efficient proton removal from the Mn complex (but not the proton production at the catalytic site itself), and we propose that it does so in each of the four proton-removal steps. However, the various experiments reported here relate differently to each of the four distinct proton-removal steps. In the S$_2 \rightarrow$ S$_3$ and S$_3 \rightarrow$ S$_4 \rightarrow$ S$_0$ transitions, proton removal from the Mn complex has been proposed to precede electron transfer to the Y$_Z$ radical, and a specific Cah3/HCO$_3^-$ influence on these proton-removal steps is suggested by the DF transients (Figure 6). In the S$_3 \rightarrow$ S$_4$ transition, the preceding proton transfer delays Mn reduction and O$_2$ formation (Haumann *et al*, 2005). Thus, the Cah3/HCO$_3^-$ influence on the time course of O$_2$ release (Figure 2) could result from an effect on this specific proton movement step. However, the deceleration of this proton removal step in the absence of Cah3 and HCO$_3^-$ is clearly too small to explain the severe limitation in the O$_2$ evolution rate detected for continuous illumination. Earlier time-resolved proton-release studies (Haumann *et al*, 1997) suggest that in the S$_4 \rightarrow$ S$_0$ transition a second proton is removed from the Mn complex concomitantly with (in H$_2$O at pH 7.4) or after (in D$_2$O or at pH $\leqslant 6.3$) the kinetically indistinguishable events of electron transfer to Y$_Z$, Mn reduction and O$_2$ release. Consequently, at lowered pH and in D$_2$O, this delayed proton removal may be the slowest step in the water oxidation cycle and this could limit the O$_2$ evolution rate under continuous, saturating illumination. Evidently, in the absence of Cah3 and HCO$_3^-$, this proton release is delayed with respect to dioxygen formation and thus affects the rate of O$_2$ evolution under continuous light. However, this delay remains undetectable in flash-induced DF and in the major rise phase detected in the O$_2$-release experiments.

Figure 8 illustrates the possible sequence of events in the S$_3 \rightarrow$ S$_0$ transition initiated by the third flash of saturating light (Dau and Haumann, 2007, 2008), which in the following sections is discussed with regard to the HCO$_3^-$ influence on O$_2$ formation in the Cah3-less mutant. The formation of Y$_Z^{\bullet +}$ is followed by a proton-removal step ((1) in Figure 8), which is accelerated by HCO$_3^-$ addition by about 50 µs, as suggested by the time-resolved DF and O$_2$-release measurements (contribution to the lag preceding the O$_2$ rise, T_{lag1}). However, the electron transfer to Y$_Z^{\bullet +}$ (2) remains unaffected by HCO$_3^-$ ($T_{\text{ox}}^{\text{main}} \approx 1.9 \pm 0.1$ ms in the DF and O$_2$ signal). The subsequent O$_2$ formation step (3) is faster than the preceding ET step ($k_{\text{ET}} \ll k_{\text{O=O}}$) and thus is not directly detectable in any kinetic study. O$_2$ formation is followed by an O$_2$-release step (4); here this step is assumed to be reversible (Clausen and Junge, 2004) such that a sizable fraction of O$_2$ remains bound. O$_2$ release could also contribute to the lag in the O$_2$ signal (T_{lag2}) (Clausen *et al*, 2004). The final step in Figure 8 involves the release of a proton (5), which, for PSII(Cah3−) in the absence of HCO$_3^-$, is slower than the Y$_Z^{\bullet +}$ reduction and O$_2$ formation ($k_{\text{DP2}} < k_{\text{ET}}$). This explains the biphasic O$_2$ release suggested by the time-resolved polarographic measurements, because the slow proton release in conjunction with the reversible O$_2$-release step implies a contribution of the slow phase to the O$_2$ signal ($T_{\text{ox}}^{\text{slow}}$). Upon addition of HCO$_3^-$ and Cah3 to the mutant PSII, the final proton-release step accelerates ($k_{\text{DP2}} < k_{\text{ET}}$) and becomes faster than Y$_Z^{\bullet +}$ reduction/O$_2$
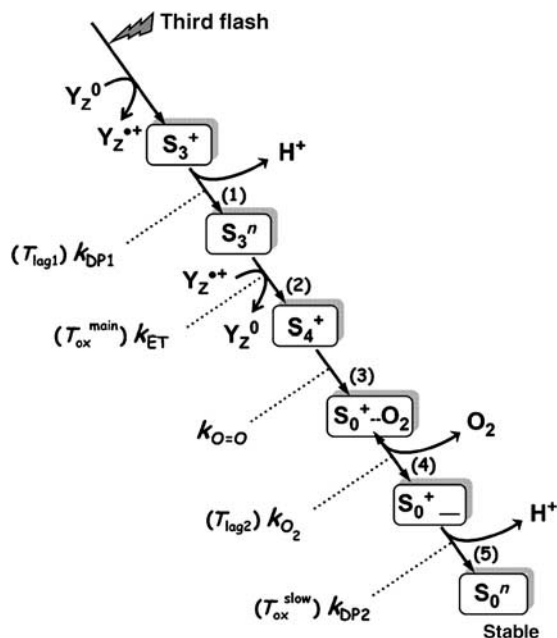
Figure 8 Proposed sequence of events induced by the third flash of saturating light applied to dark-adapted PSII. The initial oxidation of Y_Z, a specific redox-active tyrosine residue, is followed by the removal of a proton from the Mn complex (step 1) and only subsequently by electron transfer from the Mn complex to $Y_Z^{\bullet+}$ (step 2). The latter ET step is kinetically indistinguishable from O_2 formation and the associated reduction of the Mn complex (step 3). The subsequent dioxygen release (step 4) is assumed to be reversible. Finally, a second proton is removed from the Mn complex (step 5), a process that may involve a significant rearrangement of the Mn ligand environment. Steps (1) and (5) are assumed to be accelerated by the Cah3/HCO$_3^-$ system. The given time constants refer to possible contributions to the O_2-release signal, which accordingly may reflect not only T_{ox}^{main} but also T_{lag1} and T_{lag2} as well as T_{ox}^{slow} (the latter only in PSII(Cah3−) in the absence of HCO$_3^-$ or Cah3). In the nomenclature used here, the S-states refer to states of the Mn complex. The states S_3, S_4 and S_4' of Haumann *et al* (2005) correspond to states S_3^+, S_3^n and S_4^+, respectively. The subscripts indicate the number of oxidizing equivalents accumulated by the Mn complex, and the superscripts refer to the relative charge of Mn complex, which is assumed to be either neutral (n) or single positive charge (+).

formation, and thus invisible in the O_2 signal. We conclude that, assuming a specific HCO$_3^-$ influence on the two proton-release steps, the reaction sequence of Figure 8 provides a consistent and plausible explanation of the time-resolved DF and O_2 data on $S_3 \rightarrow S_0$ transition.

The negative consequences, if protons were not removed fast enough, should be more pronounced for saturating continuous illumination than for excitation by a small number of light flashes. In the absence of Cah3 and added HCO$_3^-$, photodamage occurred fast with a half time of 17 s even though the light intensity was as low as $200 \, \mu mol \, m^{-2} \, s^1$, photosynthetic active radiation (PAR) (Supplementary Figure S4). Photo-inhibition becomes more pronounced at acidic pH, as shown elsewhere (Spetea *et al*, 1997). A lack of HCO$_3^-$ would, according to our hypothesis, lead to an increased concentration of protons in PSII that could increase the risk of photodamage at the donor side. To compensate for the slowed donor-side reactions, which result from less efficient proton removal at the donor side, and the impaired stability of PSII, the *cia3*-mutant cells accumulate more PSII at standard growth conditions than does the *wt* (Villarejo

et al, 2002). As a consequence, electron supply from PSII is not rate limiting when whole-cell photosynthesis is measured (Hanson *et al*, 2003). The difference between wt and *cia3*-mutant cells *in vivo* was revealed under high-light conditions, where an increase in the accompanied protons release is predicted (Villarejo *et al*, 2002; Hanson *et al*, 2003). On the other hand, the *cia3* thylakoids were impaired in ATP synthesis due to an altered proton gradient (van Hunnik *et al*, 2002), probably reflecting that Cah3 also have an important function in maintaining an optimal H$^+$/HCO$_3^-$/CO$_2$ balance. In comparison with higher plants (Kanervo *et al*, 2005), the light reactions of the photosynthetic apparatus in green algae are less well studied (Wilson *et al*, 2006). Several photosynthetic mutants of *C. reinhardtii* have been valuable tools in gaining an understanding of light adaptation in this model organism (Rochaix, 2002). Non-photochemical quenching is composed of two components, state transition and ΔpH-driven quenching. In *C. reinhardtii*, the state transition component is smaller than in higher plants (Finazzi *et al*, 2006). Thus, a reduced ΔpH feedback on the PSII activity in algae may require an additional fine-tuning of the lumenal pH through regulation of CA activity. For *C. reinhardtii* we propose that Cah3 is one component out of several, involved in regulating activity at the donor side of PSII.

So far *C. reinhardtii* is, to our knowledge, the only organism with a characterized CA associated with PSII. CA activity has also been found in PSII preparations from higher plants (Moubarak-Milad and Stemler, 1994; Clausen *et al*, 2005a; Hillier *et al*, 2006; McConnell *et al*, 2007), although no PSII-associated enzyme has yet been characterized. An in-depth analysis of the thylakoid membrane proteome of *Arabidopsis thaliana* chloroplasts (Friso *et al*, 2004) revealed an α-CA homologue At4g20990. However, a database search of higher plant genomes did not identify any close homologue to Cah3. Nevertheless, 35 candidates putatively located in the chloroplast can be proposed from the data (Supplementary Table T1; Supplementary data). The reactions at the PSII donor side have been inferred to be almost invariant to the evolution from cyanobacteria to higher plants (Karge *et al*, 1997). However, although all photosynthetic organisms share the primary mechanism for water oxidation, differences may occur between how this is facilitated. In the cyanobacteria studied, no CA activity associated to PSII has been detected (Hillier *et al*, 2006). While HCO$_3^-$ is also clearly required at the donor side of cyanobacterial PSII (Carrieri *et al*, 2007), the HCO$_3^-$ concentration around PSII is kept high by active HCO$_3^-$ transport in these cells. Different strategies to keep the HCO$_3^-$ concentration high around PSII therefore seem to have evolved. We suggest based on our and others results (Hillier *et al*, 2006; Carrieri *et al*, 2007), that PSII-associated CA first appeared in eukaryotic unicellular algae. However, the data currently available do not facilitate a clear-cut conclusion of the role of CA in water oxidation in higher plants.

Irrespective of the still open question whether in higher plants a Cah3 homologue is associated with PSII, the results reported here relate to questions that are of general importance for photosynthetic water oxidation. Dioxygen and protons are reaction products of water oxidation at the donor side of PSII. Evidence has been reported that the dioxygen-release step may be critical for the energetics, and thus the efficiency, of photosynthetic water oxidation (Clausen and Junge, 2004). Our findings suggest that also proton removal

from the catalytic Mn complex of PSII is a process that can potentially limit photosynthetic dioxygen formation seriously, at least for continuous, saturating illumination at the lumenal 'working pH' of about 5.5. The PSII-associated Cah3/HCO$_3^-$ system likely does not eliminate completely, but presumably reduces this limitation in some or even all organisms of oxygenic photosynthesis. We also note that the findings reported here provide circumstantial, but relevant, support to recent considerations (Dau and Haumann, 2007, 2008) on the crucial role of distinct proton-removal steps for energetics and mechanism of photosynthetic water oxidation (Krishtalik, 1986), thereby supporting a shift of focus in PSII research from the electron transfer reactions towards the role of the associated proton movements.

Materials and methods

Isolation of PSII membrane fragments

PSII membrane fragments were usually prepared simultaneously from 20 l algal cultures of strains *cw92* and *cia3* of *C. reinhardtii* using a 1:20 Chl-to-Triton ratio (w/v) (Berthold *et al*, 1981). The typical O$_2$ evolution rate of PSII membrane fragments was 170–190 μmol (mg Chl)$^{-1}$ h^{-1}. For more experimental details, see Supplementary data.

Expression and purification of recombinant Cah3

The plasmid pET32-Xa-cah3 encodes a 394-amino-acid long fusion protein in which the mature Cah3 is linked to a 156-amino-acid N-terminal extension containing His$_6$-tag thioredoxin and a Factor Xa cleavage site. The construct was verified by DNA restriction digestion analysis and DNA sequencing. The recombinant fusion protein was purified using a fast-performance liquid chromatography system (Pharmacia Biotech, Uppsala, Sweden) by affinity chromatography on a HisTrap Chelating HP column with 5 ml bed volume (Amersham Biosciences AB, Uppsala, Sweden). For more experimental details, see Supplementary data.

PSII activity measurements

Steady-state O$_2$ evolution was measured with a Hansatech Clark-type O$_2$ electrode (CB1D; Hansatech, Norfolk, UK) in media containing 50 mM MES–KOH (pH 5.5), 400 mM sucrose and 35 mM NaCl (SMS buffer). The media was depleted of endogenous CO$_2$/HCO$_3^-$ by bubbling for 1 h with CO$_2$-free air. CO$_2$-free air was generated by passing air through a solution of 50% KOH and a 20-cm layer of ascarite (5–20 mesh particle size; Sigma-Aldrich, Seelze, Germany). Chl concentration during the measurements was 10 μg ml^{-1}. All measurements were conducted at 25°C in the presence of 1 mM 2.b-dichloro-p-benzoquinone (DCBQ) and 0.5 mM FeCN as electron acceptors.

Flash-induced release of O$_2$ was measured polarographically with a bare Pt electrode as described earlier (Clausen and Junge, 2004; Clausen *et al*, 2004). Both prompt and delayed time-resolved measurements of Chl fluorescence were conducted according to the procedure by Buchta *et al* (2007). For the DF measurements, dark-adapted samples were exited by a train of saturating nanosecond-laser flashes (FWHM ∼5 ns, λ = 532 mn) with a 0.7-s interval between each flash.

Prompt Chl fluorescence was measured as follows. The sample was excited with nanosecond-laser pulses as described above. The time course of the prompt fluorescence was measured with a commercially available instrument (FL 300; Photon Systems Instruments, Brno, Czech Republic) using a pump-probe technique with logarithmically spaced probe pulses (six pulses per decade, 100 μs to 690 ms, λ$_{max}$ = 615 nm). The temperature of the sample was 24.5°C.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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