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Role of a novel photosystem II-associated carbonic anhydrase in photosynthetic carbon assimilation in *Chlamydomonas reinhardtii*

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Abstract Intracellular carbonic anhydrases (CA) in aquatic photosynthetic organisms are involved in the CO_2-concentrating mechanism (CCM), which helps to overcome CO_2 limitation in the environment. In the green alga *Chlamydomonas reinhardtii*, this CCM is initiated and maintained by the pH gradient created across the chloroplast thylakoid membranes by photosystem (PS) II-mediated electron transport. We show here that photosynthesis is stimulated by a novel, intracellular α-CA bound to the chloroplast thylakoids. It is associated with PSII on the lumenal side of the thylakoid membranes. We demonstrate that PSII in association with this lumenal CA operates to provide an ample flux of CO_2 for carboxylation.

Key words: Carbonic anhydrase; Photosystem II; *Chlamydomonas reinhardtii*; Carbon concentration mechanism

1. Introduction

Photosynthesis in aquatic environments may be limited due to the low solubility and slow diffusion rate of CO_2 in water. However, most unicellular algae have evolved a CO_2-concentrating mechanism (CCM) to compensate for this CO_2 limitation [1]. Carbonic anhydrase (CA), catalyzing the interconversion of CO_2+H_2O and $HCO_3^-+H^+$, has been suggested to play a crucial role in the CCM [1]. In the green alga *Chlamydomonas reinhardtii*, two different intracellular CAs have been identified, a low CO_2-induced mitochondrial CA [2] and a constitutively expressed CA located in the chloroplast thylakoid membranes [3]. The finding of a thylakoid-associated CA is consistent with the hypothesis that HCO_3^- is pumped into the thylakoid lumen, being converted to CO_2 in the acid environment and then diffuses out to the stroma where Rubisco is located [4,5]. It also supports the finding of a PSII-associated CA activity (see [6]).

C. reinhardtii adapts to low Ci conditions by inducing various forms of CAs, which help concentrate Ci internally, resulting in much higher levels than can be obtained by diffusion alone [1–3]. However, the *Cia3* mutant of *C. reinhardtii* cannot grow in atmospheric CO_2. It requires elevated CO_2 for growth [7], which has been ascribed to the lack of an active intracellular α-CA associated with the chloroplast thylakoid membranes [3]. A resumed growth of *Cia3* cells after transformation with the *cah3* gene, a full-length cDNA of α-CA, under low CO_2 conditions [3,7] implies that this thylakoid membrane-bound CA plays a pivotal role in determining the viability of these cells in atmospheric CO_2 through involvement in CCM [4,5].

The aim of this paper was to better understand not only the exact localization in the thylakoid membrane but also the physiological role of the thylakoid lumen CA. Comparing *C. reinhardtii* with and without active α-CA in the thylakoid membranes, we show here for the first time that CA is localized in photosystem (PS) II membrane fractions and that it plays a pivotal role for the proposed thylakoid membrane-mediated CCM.

2. Materials and methods

2.1. Cell growth

Cell wall-less mutants of *C. reinhardtii* with active thylakoid-bound α-CA (CW92) and with inactive CA (*Cia3*/CW15) were grown in batch cultures at 25°C under a continuous irradiance of 150 μmol/m^2/s with 5% CO_2 in air [3].

2.2. Isolation of PSII and PSI fragments

Cells were harvested and washed once in a buffer containing 25 mM HEPES-KOH (pH 7.4). PSII and PSI fragments were isolated from thylakoid membranes using digitonin/Triton X-100 detergents [8].

2.3. 77 K chlorophyll (Chl) fluorescence emission spectra

To obtain 77 K Chl fluorescence emission spectra, thylakoid membranes, PSII or PSI fragments were dark-adapted for 5 min prior to freezing in a clear, acrylic rod sample holder. The sample holder was attached to a fiberoptic-based fluorometer [9], and the sample was immersed in liquid nitrogen. The samples were excited at 433 nm (half band with 2.5 nm) to preferentially excite chlorophyll. Emission spectra were normalized to maximal peak levels.

2.4. Western blotting

Proteins of membrane fragments were solubilized and separated on 8–15% gradient polyacrylamide gels (0.8% bisacrylamide) [3]. Immunoblotting was performed as described in the protocol from Bio-Rad Laboratories. The *Cah3* antibody used was raised in rabbits against an *Escherichia coli* overexpressed *Cah3* polypeptide (Agrisera, Vännäs, Sweden).

2.5. Room temperature Chl fluorescence and O_2 exchange measurements

Chl *a* fluorescence induction was measured at 37°C using a pulse amplitude-modulated fluorometer (PAM Chlorophyll fluorometer, Walz, Effeltrich, Germany) with the PAM 103 accessory and a Schott KL 1500 lamp (Schott, Mainz, Germany) to provide saturating flashes. A PAM-compatible system of cuvette, magnetic stirrer, oxygen electrode and Björkman type actinic lamp (Hansatech, King's Lynn, UK) were used for the simultaneous measurement of fluorescence and oxygen evolution. Cells washed in CO_2-free MES-KOH (pH 5.5) (3 μg Chl/ml) were dark-adapted for 5 min in the cuvette. Fluorescence yield, F_o, was determined by illuminating the dark-

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adapted cells with a low-intensity light (0.1 μmol/m^2/s) modulated from a light-emitting diode. A 1 s flash of saturating white light (8000 μmol/m^2/s) was given to determine the maximal fluorescence in dark-adapted cells (F_m). When steady-state fluorescence (F) was achieved after the onset of actinic light, a saturating pulse was given to determine the maximal fluorescence in the light adapted cells (F_m'). Photochemical efficiency of PSII (ΦPSII) was estimated as $1-F/F_m'$ [10]. The actinic light intensity was varied from 50 to 1500 μmol/m^2/s. To measure PSII-mediated O_2 evolution in intact cells, 5 mM $K_3Fe(CN)_6$ and 2 mM 2,5-dichloro-1,4-benzoquinone (DCBQ) were sequentially added 2 min before illumination with actinic light.

2.6. Computer simulation

We employed a modification of the Monte Carlo method with the stochastic procedure comprising the processes of PSII-facilitated HCO_3^- diffusion into lumen, CA-driven HCO_3^-/CO_2 conversion in the lumen, non-catalyzed conversion in the stroma, CO_2 assimilation by the Calvin cycle, as well as feedback end-product down-regulation of PSII via the pH gradient across the thylakoid membrane. The transition probabilities, representing the rates of corresponding processes, were dynamically adjusted to accommodate several orders of magnitude difference in the rates.

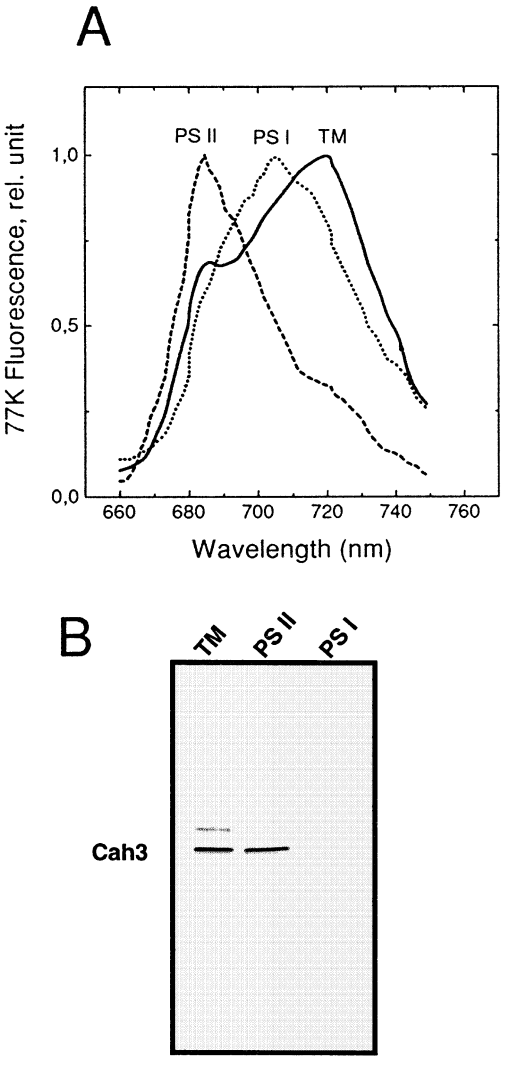

Fig. 1. A: 77 K chlorophyll emission spectra of subcelluar fractions of thylakoid membranes (TM), PSII and PSI fragments from *C. reinhardtii* (CW92). B: Immunoblot analyses of thylakoid membranes (TM), PSII and PSI fragments from *C. reinhardtii* (CW92). 15 μg protein was loaded and a CA-specific antibody (*Cah3*) was used. Molecular mass markers are not shown.

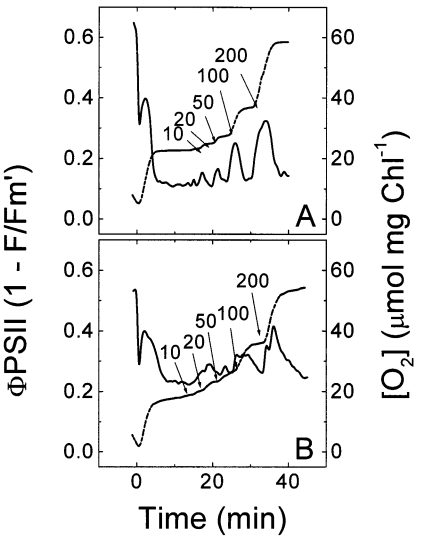

Fig. 2. Simultaneous measurements of PSII efficiency (estimated as $1-F/F_m'$, solid line) and O_2 evolution (dotted line) in dark-adapted cells of *C. reinhardtii* (CW92, A) and the *Cia3*/CW15 mutant (B). Cells resuspended with CO_2-free 20 mM MES-KOH (pH 5.3) were dark-adapted for 5 min and then subjected to continuous illumination by light saturating for photosynthesis (750 μmol photons/m^2/s). When cells showed no net O_2 evolution, various concentration of HCO_3^- were added where indicated by arrows labeled with numbers in μM.

3. Results and discussion

To localize precisely the CA of the chloroplast thylakoid membranes, PSII- and PSI-containing fragments were isolated from *C. reinhardtii* cells by differential centrifugation [8]. Only PSII-containing fractions, as characterized by 77 K Chl fluorescence emission spectra [9], cross-reacted with the CA-specific antibody [3] in wild type cells (Fig. 1). CA associated with PSII (PSII-CA) is likely to reside on the lumenal side of the thylakoid membrane since the amino acid sequence contains thylakoid lumen-targeting domains [3].

The very close association of a CA with PSII in the thylakoid lumen indicates that PSII-CA is closely connected to either the CCM [4,5] or the function of PSII [7]. We do not believe that it is directly linked to the function of PSII. Instead we postulate that the physiological role of this PSII-CA is to deliver a high and steady flux of CO_2 to the carbon assimilation process. To test this hypothesis, properties of both oxygen evolution and Chl *a* fluorescence were studied when varying amounts of HCO_3^- were added to a cell wall-less mutant of *C. reinhardtii* with (CW92) or without (*Cia3*/CW15) an active PSII-CA [3]. A cell wall-less strain of *C. reinhardtii* was used in the present study because it makes it easy to wash away surplus HCO_3^- and chemicals are also readily taken up since there are almost no uptake barriers linked to the cell wall in these mutants. Furthermore, fractionation of cells is simple since there is no cell wall to break. Cells grown in 5% CO_2 in air were used instead of low CO_2-grown cells to exclude possible involvement of other CAs since under such high CO_2 growth conditions PSII-CA is still present [3], while other CAs localized in the mitochondria and periplasm are not induced [2,6].

The HCO_3^- concentration in intact cells was reduced by washing cells three times in CO_2-free buffer (MES-KOH,

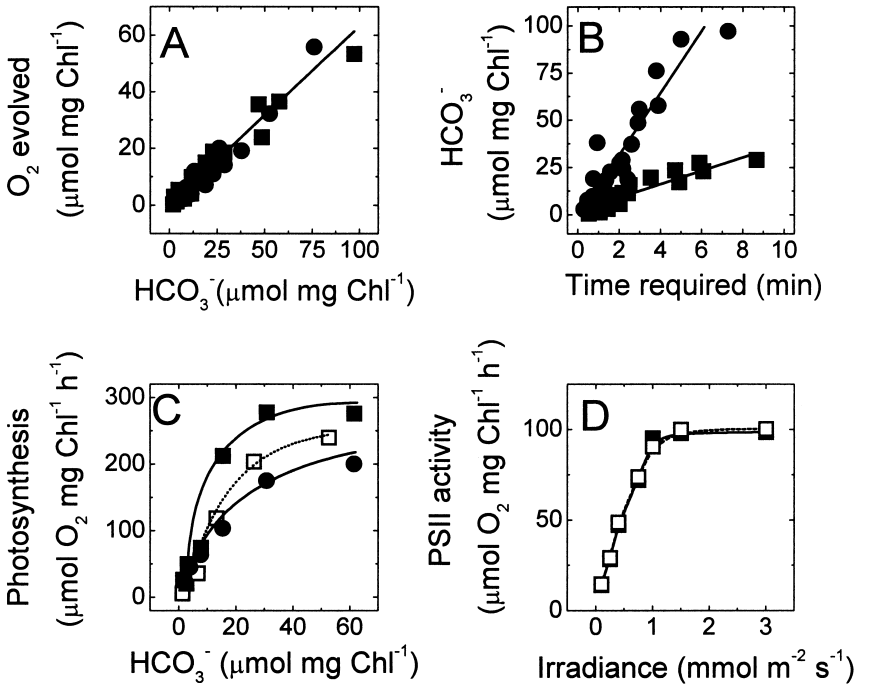

Fig. 3. Linear correlation between the amount of evolved O_2 and added HCO_3^- concentration (A) and time required to deplete added HCO_3^- (B) in CW92 cells at limiting (■, 100 μmol photons/m^2/s) and saturating (●, 750 μmol photons/m^2/s) light irradiances. Data for *Cia3* cells are not presented because of difficulties in determining the time for CO_2 depletion. C: Rate of photosynthetic O_2 evolution versus HCO_3^- concentration in CW92 cells with (□) or without (■) an internal CA inhibitor (10 μM ethoxyzolaminde; EZ) and in *Cia3* cells (●). Measuring irradiance was 750 μmol photons/m^2/s. D: PSII activity versus irradiance in CW92 cells treated with (□) or without (■) 10 μM EZ in the presence of 1 mM HCO_3^-, 5 mM $K_3Fe(CN_6)$ and 2 mM DCBQ.

pH 5.3). Upon illumination of the rinsed cells after dark acclimation with light saturating for photosynthesis, CW92 cells steadily evolved O_2 after an initial lag period. This phase was followed by a total inhibition of net O_2 evolution (Fig. 2A), when CO_2 released in respiration equalled CO_2 fixed in photosynthesis. The cessation of net O_2 evolution in this case is due to the depletion of HCO_3^- since O_2 evolution was resumed immediately after HCO_3^- addition (Fig. 2A). Furthermore, the higher the concentration of HCO_3^- added, the longer the time period needed for depletion and, hence, the higher the amount of O_2 evolved (Fig. 3A). The time for depletion and the HCO_3^- concentration(s) added were directly proportional to each other (Fig. 3B), and the linear correlation is indicative of a mechanism enabling CW92 cells to maintain constant rates of CO_2 assimilation down to very low concentrations of CO_2. The photochemical efficiency of PSII [10] changed in parallel with O_2 evolution, which emphasizes the close link between PSII photochemistry and CO_2 dependent O_2 evolution (Fig. 2A).

In the case of the *Cia3* CA mutant, the depletion of HCO_3^- occurred much more gradually due to the reduced rate of O_2 evolution at steady-state photosynthesis and no pronounced 'cut-off' effects of O_2 evolution was observed, as seen in the CW92 cells (Fig. 2B). However, at light limiting photosynthesis (100 μmol/m^2/s) where PSII activity is limiting the photosynthetic O_2 evolution, no considerable differences were found between the two cell strains (data not shown). CW92 cells treated with a CA inhibitor, ethoxyzolamide (EZ) [6], inhibiting both internal and external CAs, showed comparable rates of photosynthetic O_2 evolution to *Cia3* (Fig. 3C). This reduced rates of utilization of HCO_3^- in EZ-treated CW92 cells at saturating light irradiance or *Cia3* cells with inactive PSII-CA (Fig. 3C) would result in a several-fold increase in internal Ci concentration [7]. This suggests the PSII-CA is directly involved in controlling the Calvin cycle activity by supplying CO_2 to Rubisco. Given that the only difference between CW92 and *Cia3* is the presence of an active PSII-CA in the former, PSII-CA apparently helps to provide a steady supply of CO_2 for the assimilation process. Indeed, it is strongly supported by a finding that the EZ treatment in CW92 cells had no effect on the PSII-mediated O_2 evolution in the presence of an artificial electron acceptor of PSII, DCBQ (Fig. 3D).

We therefore propose that the thylakoid membrane-mediated CCM [4,5] is driven by PSII and catalyzed by PSII-CA, thus providing ample supply of CO_2 for the carboxylation process. The proposed HCO_3^- pump [4,5] would be fuelled by the pH gradient created by the photosynthetic electron transport (Fig. 4A). In the dark, we assume that HCO_3^- in the lumen is in equilibrium with the stromal HCO_3^-, which, in turn, is in equilibrium with external Ci. Upon illumination, the photosynthetic electron transport generates translocation of protons from the stroma into the lumen, causing an increase in the stromal pH and a decrease in the lumenal pH to values below that of the HCO_3^--CO_2 pK_a of 6.3. In the acidic lumen, dehydration of HCO_3^- to CO_2 and H_2O will be favored, thus increasing the CO_2/HCO_3^- ratio at equilibrium. The lumenal CO_2 will diffuse spontaneously into the stroma along its concentration gradient where it will enter the carbon reduction cycle. The lowered HCO_3^- concentration in the lumen will stimulate the influx of HCO_3^- from the stroma. Therefore, we postulate that the pH gradient formed by the

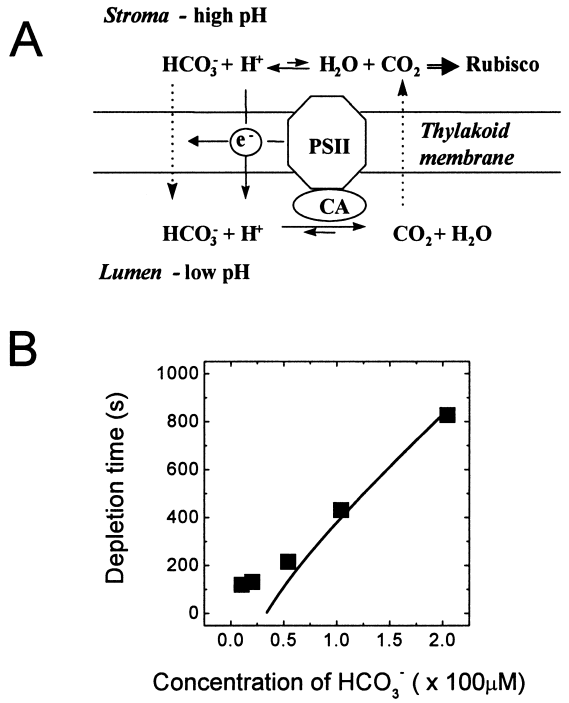

Fig. 4. A: A diagram showing the involvement of PSII carbonic anhydrase in a CO_2-concentrating process, which is generated and maintained by PSII activity. See text for description. B: Depletion times vs. added bicarbonate concentration: theory (solid line) compared to experimental data (squares).

photosynthetic electron transport generates a flux of Ci in which (i) stromal HCO_3^- enters the acidic lumen, (ii) HCO_3^- is dehydrated to CO_2 and H_2O, and (iii) CO_2 diffuses out from the lumen to be fixed by the carboxylating enzyme, Rubisco, in the photosynthetic carbon reduction process.

However, an uncatalyzed dehydration of HCO_3^- to CO_2 and water in the lumen would be rate-limiting for CO_2 fixation by Rubisco [5]. Furthermore, some of the CO_2 diffusing out of the lumen would be spontaneously converted to HCO_3^- in the alkaline stroma, thus reducing the amount of CO_2 available for carboxylation. These two limitations would be eliminated by a lumenal CA and the lack of stromal CA. A lumenal CA would increase the rate of dehydration of HCO_3^- to CO_2 with several orders of magnitude, making the rate of formation of CO_2 much faster than the rates of CO_2 diffusion and carboxylation [5]. The higher rates of O_2 evolution (and consequence rapid depletion of HCO_3^-) observed in cells with an active PSII-CA than in cells with an inactive PSII-CA by mutagenesis or chemical inhibition (Fig. 3C) strongly argue for the involvement of a PSII-CA in enhancing the CO_2 diffusion process.

For this inorganic carbon pump hypothesis to be plausible, the movement of HCO_3^- and CO_2 through the thylakoid membranes must not be limiting for CO_2 fixation. However, this is not likely to be the case since biological membranes are known to be relatively permeable to CO_2 and the rate of CO_2 diffusion is determined by the CO_2 gradient [5]. Furthermore, the rate of HCO_3^- flux into the lumen, as driven by its concentration gradient at the expense of ATP synthesis, would be sufficient to avoid a rate-limiting supply of CO_2 for carboxylation [4,5].

A simple model was suggested to depict a thylakoid CO_2 pump which includes five processes: (1) HCO_3^- diffusion from stroma to lumen and back, (2) CO_2 diffusion from stroma to lumen and back, (3) HCO_3^-/CO_2 interconversion in the stroma, (4) HCO_3^-/CO_2 interconversion in the lumen, facilitated by CA, and (5) CO_2 consumption by the Calvin cycle and four pools of HCO_3^- and CO_2, i.e. stromal and lumenal HCO_3^- and CO_2 (Rojdestvenski et al., in preparation). Due to the order of magnitude differences in the rates of the above five processes [5,11], the solution depends on only three parameters: V_{max} and K_m of the Calvin cycle, and pH value in the lumen. Our results (Fig. 4B) show a good correspondence between the theory and the experiment for the inorganic carbon depletion times.

Therefore, we suggest that a PSII-driven electron transport in *C. reinhardtii* is a feedfoward regulator of CO_2 assimilation and a PSII-CA greatly amplifies the flux of CO_2 available for carboxylation.

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