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Carbonic anhydrase CAH3 supports the activity of photosystem II under increased pH

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ABSTRACT

The lumenal carbonic anhydrase (CA) CAH3 from green alga *Chlamydomonas reinhardtii* is the only one CA identified so far in close association with the photosystem II (PSII) multi-subunit protein complex. It was proposed earlier, that CAH3 could facilitate the H^+ removal from the active center of the PSII water-oxidizing complex (WOC) under the light, thereby increasing its activity. In the present work, using PSII enriched membranes from the wild type of *C. reinhardtii* and from the CAH3-deficient mutant cia3, we demonstrate, that the suppression of the photosynthetic activity of PSII by increased pH is more pronounced in preparations from cia3 as compared to the wild type. Experiments with CA inhibitors show that the activity of CAH3 supports the function of PSII and prevents its irreversible inactivation under light upon increased pH. The photosynthetic activity of PSII from cia3 can be restored to the wild type level upon increased pH if an excess of HCO_3^- is added. These findings testify that the main role of CAH3 in the vicinity of PSII is the acceleration of the HCO_3^- dehydration reaction. Measurements of the photoinduced electron transfer rate in PSII from water or from an artificial electron donor indicate, that CAH3 has a direct influence on the WOC function. Based on the data obtained in this work we conclude, that *in vivo* CA-activity of CAH3 may support the photosynthetic activity of PSII at increased pH in the thylakoid lumen and can be observed under the dark to light transition.

1. Introduction

Carbonic anhydrases (EC 4.2.1.1) (CA) are widely spread in nature metalloenzymes which catalyze a slow spontaneous reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$ (see reviews [1–3]). A very high concentration of CO_2 in the ancient atmosphere of Earth [4], probably, contributed to both the involvement of different forms of inorganic carbon (C_i) in physiological processes of organisms as well as to the origin of enzymes that catalyze the interconversion of C_i forms. High CO_2 and HCO_3^- content in the biosphere of the Archean period could have facilitated the evolution of oxygenic photosynthesis from anoxygenic one [4–7], which resulted in the accumulation of O_2 and decrease of CO_2 in the atmosphere. Since CO_2 and O_2 have turned out to be competitors for the active center of Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) (RuBisCO), which is a key enzyme responsible for CO_2 assimilation in the Calvin-Benson cycle, organisms had to create the carbon-concentrating mechanisms (CCM) [8,9]. These mechanisms allowed them to increase the concentration of C_i inside cells by 40–100 times as compared to the environment concentration [10–13]. CAs have become one of the main participants of the CCMs [14–16].

According to recent reports, green alga *Chlamydomonas reinhardtii* has twelve CAs related to evolutionary independent α, β, and γ – families [1,3,17]. Localization and function of most of the CAs are well established and many of them are indeed a part of CCM [3,13,17,18]. However roles of some CAs are under debate up to date. For example, it was considered that the place of localization of β-CA CAH6 is stroma of chloroplasts, where this protein is a part of CCM and where it captures CO_2 leaking from the pyrenoid matrix [16,19]. But recent investigations have shown that the possible location of CAH6 is flagella where this CA may participate in the positive tropism of *C. reinhardtii* to HCO_3^- [20].

The question about the exact role of lumenal CAH3 - the third α-CA found in *C. reinhardtii* [21,22] also remains unanswered. Two different functions have been proposed for CAH3 in the intrathylakoid space. According to the first hypothesis [23,24], CAH3 is associated with the donor side of photosystem II (PSII), where it facilitates the interaction between HCO_3^- and protons transferred from the active site of the PSII

Abbreviations: PSII, photosystem II; WOC, water-oxidizing complex; CA, carbonic anhydrase; C_i, inorganic carbon; CCM, carbon-concentrating mechanism; wt, wild type; DPC, 1,5-diphenylcarbazide; DCPIP, 2,6-dichlorophenolindophenol; EZ, ethoxyzolamide; AZ, acetazolamide; TFMSA, trifluoromethanesulfonamide

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water-oxidizing complex (WOC) to the lumen. Under light conditions, this prevents local acidification near the active site of the WOC and thus increases its functioning. As proposed earlier, this mechanism can explain the decrease of the inhibitory effect of pH 5.5 on the PSII photosynthetic activity in the presence of active recombinant CAH3 [23,24]. The second suggestion is that CAH3 is a participant of CCM, where it facilitates the same reaction but in the area of a pyrenoid. Since CO_2 may easily pass through the thylakoid membrane, it can be delivered directly to RuBisCO [25,26].

The expression levels of many CA genes in *C. reinhardtii* increase significantly under low CO_2 conditions [27–30], indicating that these CAs are part of CCM. In contrast, the adaptation of *C. reinhardtii* to low CO_2 conditions has a negligible influence on both the gene expression level of CAH3 and CAH6 [27–29,31] as well as on the accumulation of these proteins [22,32]. Moreover, in a study of the pyrenoid-negative line of *C. reinhardtii* with a blocked function of CCM it was shown that the levels of gene expression and content of CAH3 and CAH6 proteins in cells are the same as in a wild type (wt) upon adaptation to low CO_2 [33].

Several studies have demonstrated that the amount of CAH3 protein is increased in the area of a pyrenoid when CCM is activated [25,26,31]. It was proposed, that for a transition of CAH3 to the area of a pyrenoid the protein has to be phosphorylated [31]. However, even in this case > 60% (and > 80% when CCM is nonfunctioning) of CAH3 remains in the stromal thylakoids [31] where, as it was clearly shown previously, the protein is associated with a PSII core-complex [23,31,34,35]. Recent works have studied the localization of some of the proposed participants of CCM in *C. reinhardtii* cells using fluorescence microscopy. This was achieved by the transformation of alga cells with the constructions encoding the proteins with a fluorescence tag. It was shown that under low CO_2 conditions (*i.e.* under active CCM) CAH3 has a uniform distribution in the area of a chloroplast without the visible concentration of the protein in the area of a pyrenoid [18,20]. Thus, the facts mentioned above clearly point out that the activity of CAH3 should significantly affect the PSII function.

The pH value of the intrathylakoid lumen changes during the activity of the photosynthetic apparatus. It decreases under the light to 6.0–5.8 and increases in the darkness or in shadows up to 7.0 [36–39]. A shift from the optimal pH for the WOC function, which is near 6.2–6.5, results in inhibition of its photosynthetic activity [40–43]. Recent investigations of the dependence of CA-activity of recombinant CAH3 on pH have shown that the enzyme has an unusual for α-CA optimal pH level at pH 6.5 [44]. Interestingly, this value closely matches the pH-optimum of WOC [40–43]. Moreover, the pH range where CAH3 demonstrates high activity [44] is matches well with the possible pH values of the intrathylakoid lumen [36–39]. Under increased pH up to 7.0, CAH3 retains > 60% of its activity [44]. This fact allows us to suggest that CAH3 can have an influence on the photosynthetic activity of PSII under the increased pH of the lumen. The data obtained in the present work clearly demonstrate that PSII enriched membrane preparations from the wt of *C. reinhardtii* have the less inhibition of their photosynthetic activity under increased pH as compared to preparations from the CAH3-deficient mutant cia3. It was found, that these differences are due to CA-activity of CAH3, which influences the function of WOC.

2. Materials and methods

2.1. Strains and growth conditions

The cell wall-less mutant CC-503 cw92 mt+ of *C. reinhardtii*, which is usually used as the standard wt in photosynthesis studies, was purchased from the Chlamydomonas Resource Center, University of Minnesota, USA. The cell wall-less and CAH3-deficient double mutant cia3 [23,24,31] was kindly provided by G. Samuelsson (University of Umeå, Sweden).

Both strains were grown photoautotrophycally in minimal medium at 25 °C under aeration with air enriched with 5% CO_2 [23]. Continuous illumination was provided from cool white fluorescent lamps at 100 μmol photons $m^{-2} s^{-1}$.

2.2. Isolation of PSII enriched membrane fragments

Cells of *C. reinhardtii* were collected by centrifugation at 3000*g*, 10 min and washed with chilled buffer A (50 mM Hepes-NaOH (pH 7.8), 350 mM NaCl, 2 mM EDTA). Before disruption 1 mM sodium ascorbate was added to the buffer. Cells were disrupted by passing through a precooled French pressure cell (Thermo scientific) at 28 MPa (4000 Psi). Whole cells and large debris were harvested with a low-speed spin (500 g, 5 min), and then membranes were pelleted at 7000 g, 30 min. The pellet was resuspended in chilled buffer B (50 mM Hepes-NaOH (pH 7.8), 3 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 200 mM sucrose, 1 mM sodium ascorbate) and precipitated at 7000 g, 30 min. The upper green layer of the pellet, which contained primarily thylakoid membranes, was carefully resuspended by paint brush in chilled buffer C (20 mM MES-NaOH (pH 6.5), 15 mM NaCl, 5 mM $MgCl_2$, 300 mM sucrose) to a chlorophyll concentration of 3–4 mg ml^{-1}. For the PSII enriched membrane fragments (BBY-particles [45]) isolation, 20% (w/w) Triton X-100 solution was added to the thylakoid membranes (Triton X-100 to chlorophyll ratio was as 20:1 (w/w) and the final concentration of chlorophyll was 2 mg ml^{-1}), and the suspension was incubated for 20 min on ice under gentle stirring in the dark. BBY-particles were harvested by centrifugation at 40000*g*, 35 min and washed thrice in the buffer C. Finally, the pellet was resuspended in the buffer C containing 20% glycerol to a chlorophyll concentration of 2–3 mg ml^{-1}, homogenized and frozen at −80 °C. All steps were carried out at 4 °C and green dim light.

2.3. Chlorophyll concentration

The concentration of total chlorophyll was determined spectroscopically after extraction in 80% acetone [46].

2.4. Carbonic anhydrase activity measurements

Carbonic anhydrase activity was determined upon addition of ice-cold CO_2-saturated water to the reaction mixture, containing 25 mM veronal buffer (pH 8.6), 50 mM KCl and 15 mM $MgCl_2$ as it was described previously [47]. The rate of pH change at 2 °C due to the CO_2 hydration reaction was measured by Mettler Toledo InLab 413 pH electrode and cpX-2 pH/ion meter (Institute of Biological Instruments RAS, Pushchino). The carbonic anhydrase activity was expressed in Wilbur-Anderson Units (WAU) [48] and calculated as WAU = $(t_0\text{-}t)/t$, where t_0 and t are the time for the pH change from 8.3 to 7.8 in a buffer without additions (control) and in a buffer containing BBY-particles, respectively. The value of WAU activity was calculated per mg of chlorophyll.

2.5. O_2-evolving activity measurements

The rate of photosynthetic oxygen evolution was measured at 25 °C with a Clarke-type electrode, in a 1-ml cell (Hansatech Instruments Ltd., Norfolk, United Kingdom). Measurements were carried out in medium, containing either 20 mM MES-NaOH (pH 5.0–7.0) or 20 mM MOPS-NaOH (pH 6.5–7.9) and 35 mM NaCl, 400 mM sucrose. 1 mM potassium ferricyanide and 0.2 mM 2,6-dichloro-*p*-benzoquinone (Fig. S1) were used as electron acceptors. The chlorophyll concentration during measurements was 10 μg ml^{-1}.

2.6. Electron transfer rate measurements

Photoinduced electron transfer from water or from the electron

donor 1,5-diphenylcarbazide (DCP) to the electron acceptor 2,6-dichlorophenolindophenol (DCPIP) was measured spectroscopically by the decrease in photoinduced absorption at 600 nm as a result of DCPIP reduction. The assay was performed in the same medium as it was done in the measurements of oxygen evolution at chlorophyll concentration of 10 μg ml^{-1}. A freshly prepared DPC, dissolved in dimethyl sulfoxide solution, was used at concentration of 1 mM. Different extinction coefficients of 50 μmol DCPIP were used for pH 6.5 and 7.0 due to the dependence of DCPIP properties on pH [49] (Fig. S2).

2.7. Western blot analysis

Proteins separation of BBY-particles from wt and mutant cia3 was carried out by electrophoresis under denaturing conditions in a 16% polyacrylamide gel [50] in Mini-PROTEAN 3 Cell (BioRad). The samples were loaded on a gel at an equal amount of chlorophyll content, as shown in Fig. 2. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Amersham, Protran, 0.45 μm NC) using the Mini Trans-Blot Cell wet blotting system (BioRad). The membrane was incubated overnight at 4 °C with anti-rabbit primary antibodies against D1 and CAH3 proteins produced by Agrisera (Sweden) (AS05 084 and AS05 073, respectively). Donkey anti-rabbit antibodies labeled with horseradish peroxidase (GE Healthcare) were used as secondary antibodies in a dilution of 1:5000. The antibody-antigen conjugates were detected by a Pierce ECL Plus Western Blotting kit (Thermo Scientific) and the gel documentation system ChemiDoc (BioRad). Quantification of bands on the blots was performed by Image J software.

2.8. Determination of C_i in solutions

The total content of C_i (CO_2, HCO_3^-, CO_3^{2-}) in buffer solutions was determined as described previously [51]. The concentration of CO_2 evolving from 3 ml of a buffer after the addition of 1 ml 5 M HCl in a closed vial of known volume was measured by a gas chromatography system KristalLux 4000 (Meta Chrom, Yoshkar-Ola, Russia).

2.9. Determination of cytochrome b559

Determination of the total content of Cyt b599 in BBY-particles was made by measurement of absorption changes at 559 nm (ΔA_{559}) from reduced minus oxidized difference spectra in the region of 540–580 nm [52,53]. Before the measurements the samples were diluted in a buffer, containing 20 mM MES-NaOH (pH 6.5), 35 mM NaCl, 400 mM sucrose, to chlorophyll concentration of 100 μg ml^{-1}. Complete oxidation of sample suspension was achieved by the addition of 50 mkM potassium ferricyanide, and the reduction was performed by addition of a few grains of dithionite.

3. Results

3.1. The pH dependence of O_2-evolving activity of BBY-particles from wt and cia3

As it is shown in Fig. 1, the O_2-evolving activity of BBY-particles isolated from wt and cia3 mutant of *C. reinhardtii* depends on pH. The obtained curves have optimum – plateaus in the interval of pH 6.2–6.5 where preparations from wt and cia3 show the same O_2 evolution rate equal to 280 μmol O_2 (mg Chl h)$^{-1}$. Under shifting pH to acidic or alkaline side inhibition of the O_2-evolving activity was observed. In the acidic area there was no difference between curves related to wt or cia3. In contrast, under increasing pH a significant difference was observed. Already at pH 6.65 the O_2-evolving activity of preparations from cia3 was suppressed more than in wt. The maximum distinction in the inhibition was achieved at pH 7.0 (Fig. 1). In this case the O_2 evolution rate in BBY-particles from wt decreased by ~10%, but for

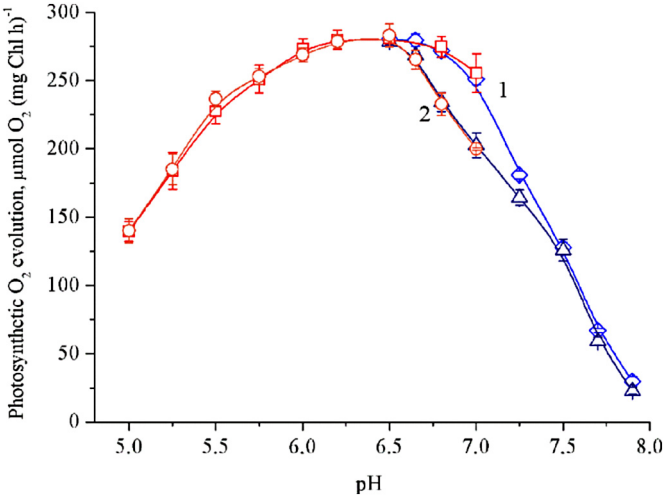

Fig. 1. The pH dependence of the photosynthetic O_2 evolution rate. Curve 1 (marked by squares (MES-NaOH) and rhombs (MOPS)) is BBY-particles from wt, and curve 2 (marked by circles (MES-NaOH) and triangles (MOPS)) is BBY-particles from cia3. The measurements were carried out in a medium containing 20 mM MES-NaOH (pH 5.0–7.0) or MOPS (pH 6.5–7.9), 35 mM NaCl and 400 mM sucrose. Each point on the curves is an average of at least 3 separate experiments (at least 5 separate experiments were done for pH 6.5–7.0) with subsequent calculation of standard deviation.

BBY-particles from cia3 this value was near 30%, *i.e.* the difference between wt and cia3 was about 20%. Interestingly, with a further increase of pH this difference diminished and disappeared completely at pH above 7.5.

According to the results of western-blot analysis, BBY-particles from wt and cia3 contained an equal amount of D1 protein at the same chlorophyll content (Fig. 2, A and B). As it is known, D1 is a subunit of the PSII core-complex [54]. Thus, BBY-particles from wt and cia3 had the same chlorophyll/PSII ratio. This was further supported by the observation of equal content of Cyt b559 in the preparations (Fig. 2, C), if the same concentrations of chlorophyll were used. Cyt 559 is another subunit of the PSII core-complex [54]. At the same time, only preparations from wt showed a clear signal of CAH3 (Fig. 2, A), and the observed CA-activity was near 31 WAU (mg Chl)$^{-1}$ (Fig. 2, D). This allows us to speculate that the obtained difference in inhibition of the O_2-evolving activity between BBY-particles from wt and cia3 under increased pH was due to the CA-activity of CAH3.

3.2. The influence of CA inhibitors on O_2-evolving activity at increased pH

To confirm that the results described above were due to the CA-activity of CAH3, the influence of three CA inhibitors: trifluoromethanesulfonamide (TFMSA), ethoxyzolamide (EZ) and acetazolamide (AZ) on the O_2-evolution activity of BBY-particles at increased pH was investigated. These inhibitors differ in their physical and chemical properties and have been previously used in studies with PSII enriched preparations [47,51].

As it can be seen in Fig. 3, in the presence of 0.5 μmol TFMSA, EZ or AZ the O_2-evolving activity of preparations from wt at increased pH was inhibited stronger than in the absence of these CA inhibitors. At the same time, the obtained curves almost completely matched the curve related to cia3. In contrast, the addition of 0.5 μmol EZ to the BBY-particles from cia3 had no influence on the O_2 evolution rate at pH 7.0. These observations are in agreement with our hypothesis that it is the CA-activity of CAH3 that supports the function of WOC under increased pH.

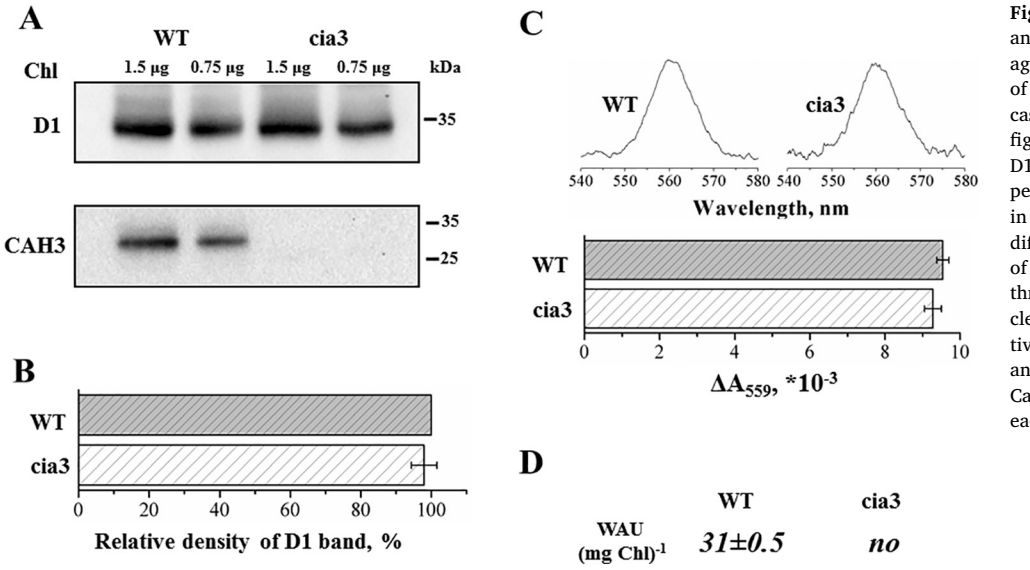

Fig. 2. Results obtained from immunoblot-analysis (A) by using primary antibody against D1 and CAH3 proteins. The amount of Chl, loaded on the gel, was the same in the case of D1 and CAH3 and is indicated in the figure. (B) – quantification of density of the D1 band on the blots from five separate experiments. The results were normalized to wt in each blot. (C) – reduced minus oxidized difference spectra of Cyt b559 and the value of ΔA at 559 nm on the spectra from at least three separate measurements in BBY-particles from wt and cia3. The value of CA-activity in BBY-particles from wt and cia3 (D) is an average of nine separate measurements. Calculation of standard deviation is shown in each case.

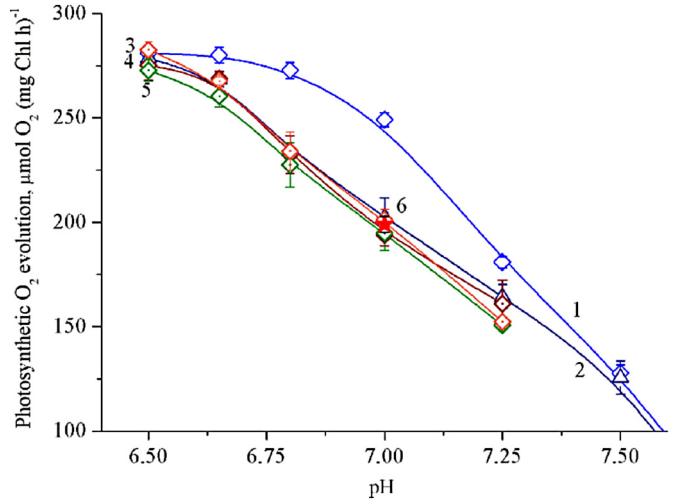

Fig. 3. Inhibition of the O_2 evolution rate with increasing of pH in a medium in the absence (curves 1 and 2) or in the presence of CA inhibitors. Curve 1 (marked by rhombs) and curve 2 (marked by triangles) are BBY-particles from wt and mutant cia3, respectively. Curves 3 (marked by orange rhombs), 4 (marked by brown rhombs) and 5 (marked by green rhombs) are BBY-particles from wt in the presence of 0.5 μM TFMSA, EZ and AZ, respectively. Point 6 (marked by a red star) is BBY-particles from cia3 in the presence of 0.5 μM EZ. The measurements were carried out in the medium containing 20 mM MOPS, 35 mM NaCl and 400 mM sucrose. Each point on the curves is an average of at least 3 separate experiments (at least 5 separate experiments for pH 6.5–7.0) with subsequent calculation of standard deviation.

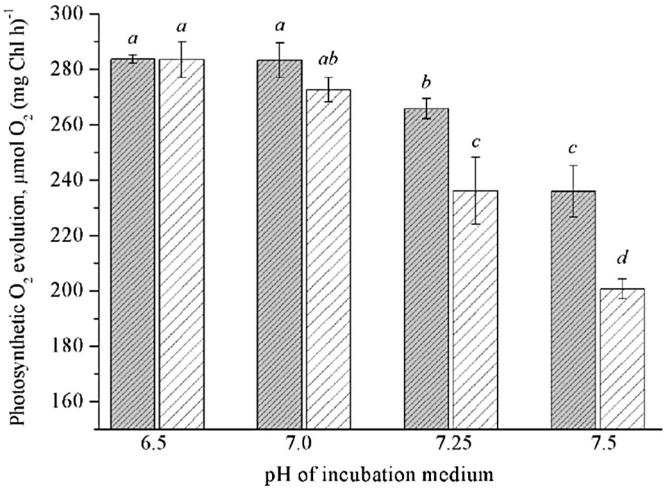

Fig. 4. Reversibility of O_2-evolving activity inactivation upon incubation of preparations in medium at pH 6.5, 7.0, 7.25 and 7.5 in BBY-particles from wt (grey columns) and cia3 (white columns). Before measuring the O_2 evolution rate, the samples were pelleted and resuspended in a buffer containing 20 mM MOPS (pH 6.5), 35 mM NaCl and 400 mM sucrose. Each value is an average of 3–4 separate experiments with subsequent calculation of standard deviation. The letters above bars indicate a statistically significant difference between the values of each experiment, $p < 0.05$.

3.3. Reversibility of the O_2-evolving activity inhibition at increased pH

Fig. 4 shows the O_2 evolution rate of BBY-particles from wt or cia3 after incubation in buffers with increased pH and subsequent re-suspension in a buffer with pH 6.5. It is obvious that the irreversible inhibition of the WOC function is almost completely absence at pH levels up to 7.0 in both preparations. Although, a negligible loss of O_2-evolving activity was observed in some PSII from cia3, but it was statistically insignificant.

With a greater shift of pH the appearance of PSII with irreversible inhibited WOC was observed in preparations from both wt and cia3. The amount of these PSII increased with pH. Moreover, the difference between preparations from wt and cia3 was proportional to the pH increase (Fig. 4).

Thus, the presented results clearly show that the observed inhibition of the O_2-evolution function of WOC at pH up to 7.0 (Fig. 1) is fully reversible. Furthermore, under further increase of pH, CAH3 reduces the negative influence of pH on the WOC function.

3.4. Stimulation of the O_2-evolving activity in BBY-particles from cia3 at pH 7.0 by bicarbonate

As it was proposed previously, the possible function of CAH3 on the donor side of PSII is an acceleration of the reaction of HCO_3^- contained in the lumen with H^+ produced in the active center of the WOC as a result of photosynthetic water oxidation [23,24].

Since HCO_3^- is a substrate for the reaction, in the absence of CAH3 (in case of cia3) the spontaneous interaction of HCO_3^- with H^+ could be accelerated by adding of an excess of bicarbonate. That in turn would stimulate the activity of WOC in preparations from cia3. At pH 7.0, when the difference in inhibition of the O_2-evolving activity between

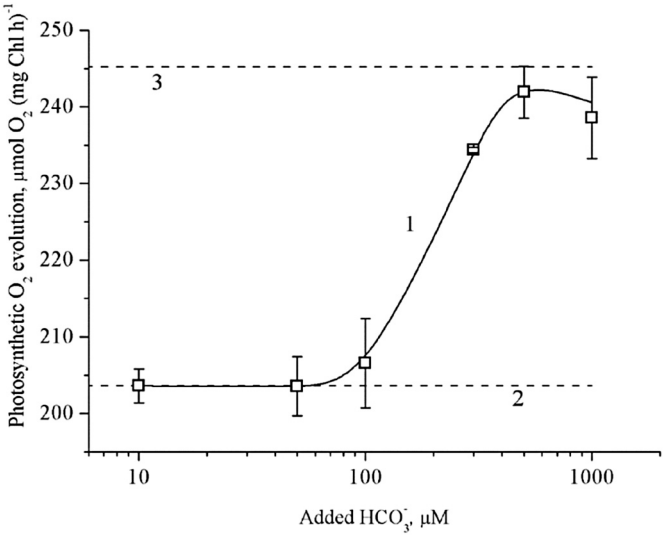

Fig. 5. Dependence of the O_2 evolving activity of BBY-particles from cia3 at pH 7.0 on concentration of added $NaHCO_3$ (curve 1). Lines 2 and 3 are the O_2 evolution rate of BBY-particles from cia3 and wt, respectively in the absence of additions. The measurements were carried out in the medium containing 20 mM MOPS (pH 7.0), 35 mM NaCl and 400 mM sucrose. Each point on the curve is an average of at least 3 separate experiments with subsequent calculation of standard deviation.

BBY-particles from wt and cia3 was maximal (Fig. 1), the addition of HCO_3^- indeed increased the O_2 evolution rate in preparations from cia3. The increase started at concentrations of bicarbonate > 100 μM (Fig. 5), and reached a maximum at 500 μM HCO_3^-, where the O_2 evolution rate almost achieved the level of wt. These findings support our hypothesis that the activity of CAH3 in BBY-particles is necessary for the acceleration of the reaction of HCO_3^- with H^+ in the vicinity of PSII.

3.5. Comparison of the influence of HCO_3^- and EZ on the O_2-evolving activity in BBY-particles at pH 6.5 and 7.0

A series of comparative experiments were carried out in order to study the influence of HCO_3^- and EZ on the O_2 evolution rate in BBY-particles from wt and cia3 at pH 6.5 and 7.0. The results are summarized in Table 1.

At the optimal pH for the WOC function, equal to 6.5 (Fig. 1), the O_2 evolution rate in BBY-particles from both wt and cia3 showed the same level in the absence of additions as well as in the presence of 500 μM HCO_3^-, 0.5 μM EZ or 500 μM HCO_3^- plus 0.5 μM EZ (Table 1). A completely different situation was observed at pH 7.0. In this case, as also shown above, the inhibition of the O_2 evolution rate was 20% higher in preparations from cia3 as compared to wt (Fig. 1, Table 1). The addition of 500 μM HCO_3^- had no influence on the O_2 evolution rate in BBY-particles from wt, but it stimulated the O_2-evolving activity in BBY-particles from cia3 up to wt level (Table 1). In contrast, the addition of 0.5 μM EZ to BBY-particles from wt inhibited their O_2-evolving activity to the cia3 level and had no effect on the O_2-evolving activity of preparations from cia3 (Table 1).

An interesting effect of EZ on the O_2 evolution rate was noted. In the presence of EZ, the addition of 500 μM HCO_3^- stimulated the O_2 evolution rate in preparations from cia3 to the wt level. However, upon the addition of 500 μM HCO_3^- to the preparations from wt, which were inhibited beforehand by EZ, a minor part of the WOC remained non-functional. If one assumes the O_2 evolution rate in the absence of additions to be 100% (Table 1), EZ inhibited it to ~77%, and the subsequent addition of HCO_3^- recovered the O_2-evolving activity only up to ~92%. The inhibition of CAH3 by EZ somehow prevented the interaction of HCO_3^-, contained in a solution, with H^+ transferred from the active site of WOC, and that resulted in the decrease of the maximum level of the O_2-evolving activity which PSII could reach.

Table 1
The influence of HCO_3^- and EZ on the O_2 evolution rate in BBY-particles from wt and cia3 at pH 6.5 and 7.0. Each value is an average of at least 3 separate experiments with calculation of standard deviation.

Additions	Photosynthetic O_2 evolution, μmol O_2 (mg Chl h)$^{-1}$			
	pH 6.5		pH 7.0	
	WT	cia3	WT	cia3
Without additions (control)	280 ± 4	279 ± 3	249 ± 5	203 ± 9
+500 μM HCO_3^-	277 ± 4	276 ± 3	247 ± 4	245 ± 3
+0.5 μM EZ	275 ± 7	275 ± 3	194 ± 5	199 ± 4
+0.5 μM EZ, 500 μM HCO_3^-	280 ± 5	276 ± 2	231 ± 5	248 ± 9

3.6. Photoinduced reduction of DCPIP from water and DPC by PSII at pH 6.5 and 7.0

To demonstrate that the effects observed above were due to the function of CAH3 on the donor side of PSII, experiments on photoinduced reduction (photoreduction) of a well-known electron accepter DCPIP by PSII from water or from an electron donor DCP were carried out. The results are presented in Table 2.

At pH 6.5, preparations from wt and cia3 in the absence of additions showed the same values of DCPIP photoreduction by PSII from water. That result agreed with the data obtained earlier during O_2-evolving measurements (Fig. 1, Table 1). Additionally, no differences were observed upon addition of 0.5 μM EZ, 500 μM HCO_3^-, or 500 μM HCO_3^- plus 0.5 μM EZ. In the presence of 500 μM DPC, the DCPIP photoreduction by PSII increased by about 20%, both in BBY-particles from wt and cia3 (Table 2). This indicated that the preparations both from wt and cia3 contained the same proportion of PSII with irreversibly inactivated WOC (~20%).

At pH 7.0 the inhibition of the DCPIP photoreduction by PSII from water was observed. This effect was stronger in BBY-particles from cia3 (Table 2). At the same time, in the presence of 500 μM DPC the rate of the DCPIP photoreduction by PSII increased in preparations both from wt and cia3 up to an equal and, probably, maximum level. In this case,

Table 2
The rate of DCPIP photoreduction from water and DPC by BBY-particles from wt and cia3 at pH 6.5 and 7.0. Each value is an average of at least 3 separate experiments with calculation of standard deviation.

Additions	DCPIP photoreduction, μmol DCPIP (mg Chl h)$^{-1}$			
	pH 6.5		pH 7.0	
	WT	cia3	WT	cia3
Without additions (control)	37.3 ± 1	37.0 ± 2	23.2 ± 1	16.3 ± 1
+500 μM DPC	45.0 ± 2	46.1 ± 2	32.5 ± 1[a]	31.9 ± 1
+0.5 μM EZ	36.7 ± 1	37.4 ± 2	16.4 ± 2	16.9 ± 1
+0.5 μM EZ, 500 μM DPC	45.3 ± 3	45.8 ± 3	31.9 ± 2	32.3 ± 1
+500 μM HCO_3^-	37.6 ± 1	36.6 ± 1	23.1 ± 1	22.4 ± 1
+0.5 μM EZ, 500 μM HCO_3^-	37.0 ± 1	36.2 ± 1	20.4 ± 1	22.8 ± 1
+0.5 μM EZ, 500 μM HCO_3^-, 500 μM DPC	45.3 ± 2	45.5 ± 1	32.3 ± 1	31.6 ± 1

[a] This value was taken as 100% in Table 3.

Table 3
Calculation of the DCPIP photoreduction rate from water and DPC at pH 7.0. Here the value in the presence of DPC is considered to be 100% (marked by asterisk in Table 2). Each value is an average of at least 3 separate experiments with calculation of standard deviation.

Additions	DCPIP photoreduction, %	
	pH 7.0	
	WT	cia3
Without additions (control)	71.3 ± 3	50.2 ± 4
+500 μM DPC	100 ± 4[a]	98.2 ± 3
+0.5 μM EZ	50.4 ± 5	52.1 ± 4
+500 μM HCO_3^-	71.1 ± 3	68.9 ± 3
+0.5 μM EZ, 500 μM HCO_3^-	62.8 ± 4	70.1 ± 3

[a] Includes ~20% of PSII with inactivated WOC (see text).

the addition of EZ or HCO_3^- had no effect (Tables 2). If one assumes this value to be 100% for pH 7.0 and subtracts 20% related to PSII with inactivated WOC from it (as is described above), the DCPIP photoreduction by PSII from wt and cia3 reveals inhibition level of ~10% and ~30%, respectively (Table 3). These values were the same as the one observed in the O_2-evolving activity measurements (Table 1).

The addition of 0.5 μM EZ to BBY-particles from wt decreased the rate of the DCPIP photoreduction by PSII from water to the cia3 level. At the same time there was no effect of EZ on the rate of the DCPIP photoreduction by BBY-particles from cia3. Addition of 500 μM HCO_3^- has had no influence on the rate of the DCPIP photoreduction by BBY-particles from wt, while in the case of preparations from cia3 the increase of the rate was observed (Tables 2 and 3). Upon addition of 0.5 μM EZ to preparations from cia3, subsequent addition of 500 μM HCO_3^- led to the increase of the DCPIP photoreduction rate by PSII from water up to the wt level. Different results were obtained for preparations from wt. The addition of 0.5 μM EZ decreased the DCPIP photoreduction rate from water by PSII from ~70 to ~50% (level of cia3), and the subsequent addition of 500 μM HCO_3^- restored the DCPIP photoreduction only up to ~63% (Table 3).

Thus, these results are fully consistent with the data observed during the measurements of O_2-evolving activity. This allows us to conclude that the differences detected in the photosynthetic activity of BBY-particles from wt and cia3 upon increased pH were due to the influence of CA-activity of CAH3 on the PSII activity, particularly due to a direct influence on the WOC function.

4. Discussion

In the present work we have demonstrated that BBY-particles isolated from wt *C. reinhardtii* and from the mutant cia3 grown under identical conditions have the same photosynthetic activity at optimal pH 6.5 per equal amount of chlorophyll (Figs. 1, 3, 4 and Tables 1 and 2), as well as the same chlorophyll/PSII ratio (Fig. 2). Also, measurements of the DCPIP photoreduction by PSII from DPC have shown that preparations from wt and cia3 had the same amount of PSII (~20%) with inactivated WOC (Table 2). We propose, that this is the fraction of assembling or/and degrading PSII in which WOC is non-active. In previously published data [23] however, it was shown that the chlorophyll/PSII ratio was 1.6 times higher in wt as compared to cia3, and that PSII from cia3 had twice higher stimulation effect on DCPIP photoreduction by DPC as compared to PSII from wt. Our data do not confirm these differences between BBY-particles from wt and cia3. Also we showed that the addition of CA-inhibitors or HCO_3^- at pH 6.5 had absolutely no effect on photosynthetic activity in BBY-particles both from wt and cia3 (Figs. 1, 3 and Tables 1 and 2). These results prove, that the influence of the CA-activity of CAH3 on PSII function in BBY-particles in such conditions was absent or negligible.

It is known, that the increase of pH from the optimal range for the WOC function (6.2–6.5) leads to an inhibition of the photosynthetic activity of PSII [40–43]. The same effect was also observed in our work using PSII from *C. reinhardtii* (Fig. 1). However, the neutral pH could be reached in intrathylakoid lumen in the darkness or in the shadow [36–39]. Upon an increase of pH we observed a difference in the values of the photosynthetic activity inhibition of PSII in BBY-particles from wt and cia3. The difference was maximal at pH 7.0 and was near 20% (Fig. 1 and Tables 1, 2, 3). As it was shown in experiments with three CA-inhibitors (Fig. 3), this difference was due to CA-activity of CAH3, which was only present in the preparations from wt (Fig. 2), and somehow supported PSII function at increased pH. Moreover, the results of the DCPIP photoreduction by PSII from water or DPC (Tables 2, 3) clearly showed that CAH3 directly influenced the WOC function.

As considered, the main reason for inhibition of the PSII photosynthetic activity upon increasing pH is the release of Cl^- ions (or its substitution by OH^-) from their sites of localization near the WOC active center caused by deprotonation of Cl^--binding groups [40,42]. It is also proposed that Cl^- ions together with negatively charged carboxyl groups of amino acid residues and molecules of water form proton channels which provide the effective removal of H^+ from the active center of the WOC to the lumen space during photosynthetic water oxidation process [54–58]. The extraction of Cl^- may destruct these channels and decrease their efficiency, as it was shown, for example, by computer simulation [58], that in turn results in an inhibition of the WOC function. Changes in the relative position of amino acid residues participating in the formation of these proton channels also may lead to the decrease of H^+ transferring efficiency, even if Cl^- ions remain in the sites of their localization. This effect can result from conformational rearrangements of protein globules in the case of pH shifting, as it was shown for one of the major protein of the WOC – PsbO [59–61], including the PsbO protein from *C. reinhardtii* [62].

We believe that in our experiments the main contribution to the observed suppression of the PSII photosynthetic activity upon increase of pH up to 7.0 was due to changes in relative position of amino acid residues, rather than from the Cl^- extraction from the WOC. This suggestion is supported by the reversibility of inhibition of the PSII O_2-evolving activity after preincubation of BBY-particles at increased pH. As it is seen in Fig. 4, the preparations from both wt and cia3 saved full activity after their preincubation at pH 7.0. However, the extraction of Cl^- from the WOC could cause irreversible inactivation the O_2-evolving function of PSII, since the reverse binding of Cl^- to their site of location in PSII takes > 30 min [56]. Moreover, high Cl^- content in a medium can prevent its extraction by increased pH. As it was shown previously, the presence of > 25 mM Cl^- may extend the optimum – plateau of the PSII O_2-evolving function up to pH 7.0 [43] where its maximal activity is promoted [43,63]. In the present work, all buffers contained 35 mM NaCl, which should keep Cl^- in their binding sites. The irreversible inactivation of the WOC in BBY-particles (Fig. 4), as well as an absence of the differences in an inhibition level of the O_2-evolution activity between preparations from wt and cia3 (Fig. 1) were observed in our experiments only at pH higher than 7.25, as we believe due to Cl^- extraction from the WOC. Even in this case, irreversible inactivation of PSII was stronger in preparations from cia3 (Fig. 4).

As it was mentioned above, the increase of pH up to 7.0 can change the relative position of amino acid residues that form proton channels, making the H^+ transfer between them more difficult. It is known, that as the result of the photosynthetic water oxidation, $4H^+$ are released per one molecule of O_2. Therefore, at the rate of O_2-evolution equal to 280 μmol O_2 (mg Chl h)$^{-1}$ (Fig. 1, Table 1) the rate of proton release is about 75H^+/s per PSII. Thus, even a slight decrease in efficiency of proton removal from the WOC active center to the lumen space can negatively impact the water oxidation reaction and the O_2-evolution. The increased concentration of H^+ in the outlet of the channels to the lumen space due to low diffusion of protons along a thylakoid membrane [39,64] could reduce the gradient of H^+ in the channel and, respectively, decrease the efficiency of H^+ transfer. At pH 7.0,

recombinant CAH3 has a CA-activity close to its maximal [44] and HCO_3^- is the dominant form of C_i (> 80% (Fig. S3)). We propose that in such conditions CAH3 can accelerate the bicarbonate dehydration reaction ($H^+ + HCO_3^- \rightarrow H_2O + CO_2$) leading to the fast neutralization of H^+ at the proton channels outlet, and assisting the removal of protons from the active center of WOC. As a consequence, the inhibition or the absence of CAH3 (*i.e.* a decrease in the rate of the bicarbonate dehydration reaction) should lead to suppression of the PSII photosynthetic activity under increased pH (Fig. 4, Tables 1, 2, 3), and at the same time the addition of excess of HCO_3^- (*i.e.* an acceleration of the spontaneous dehydration of bicarbonate) should stimulate the PSII photosynthetic activity (Fig. 5, Tables 1, 2, 3), which is in perfect agreement with the results of our work.

In experiments performed at pH 7.0 with preparations from wt we observed that inhibition of CAH3 by EZ prevented full restoration of the suppressed photosynthetic activity of WOC by HCO_3^-. Base on this unexpected effect we propose, that the molecule of CAH3, localized and oriented specifically near the donor side of PSII, can act as a barrier between the outlet of proton channel(s) and a medium. In this case, even under an excess of bicarbonate the inhibited globule of the CAH3 protein would decrease the efficiency of interaction between HCO_3^- and H^+, transferred by channel(s). This would lead to an inability of full restoration of the PSII photosynthetic activity. The protein of CAH3 is absent in BBY-particles from cia3, therefore HCO_3^- can easily interact with H^+ resulting in 100% recovery of the PSII photosynthetic activity (Tables 1, 2, 3). This effect is of interest and requires further study.

At pH 6.5 the photosynthetic activity of BBY-particles from wt and cia3 was the same and did not depend on the presence of CA-inhibitors (Figs. 1, 3 and Tables 1, 2) or added bicarbonate (Tables 1, 2). That means that the activity of CAH3 does not affect the WOC function and the amount of HCO_3^- in the medium is enough for optimal PSII activity. It was detected, that the content of C_i in the buffer at pH 6.5 can reach 380 μM with the relevant part of HCO_3^- about 200 μM (Fig. S4). At the same time, at pH 7.0 the content of C_i in the buffer increases up to 460 μM with the relevant part of HCO_3^- about 380 μM (Fig. S4). In this case, in contrast to preparations from wt, BBY-particles from cia3 required for the maximal photosynthetic activity additional about 500 μM C_i (Fig. 5) or ~400 μM HCO_3^- (Fig. S4), *i.e.* a three to four fold increase of HCO_3^- concentration as compared to pH 6.5. Moreover, at pH 6.65, already 250 μM HCO_3^- in the medium (Fig. S4) was not enough to keep the high level of the photosynthetic activity of PSII in the absence of CAH3 (cia3) or upon inhibition of CA-activity of the protein (Figs. 1, 3). Thus, it is clear that CA-activity of CAH3 decreases the necessary concentration of HCO_3^- for supporting the maximal photosynthetic activity of PSII in BBY-particles upon increased pH.

According to previously published data, the concentration of C_i inside cells of *C. reinhardtii* grown in laboratory conditions can reach 400–600 μM [11,13], that is 220–300 μM HCO_3^- at pH 6.5 or 320–480 μM HCO_3^- at pH 7.0 (Fig. S3). Based on this fact, on the estimated content of C_i in our buffers, and on the results of the experiments described above, it follows that *in vivo* CAH3 almost always has enough amount of HCO_3^- for supporting the maximal photosynthetic activity of PSII. Moreover, this role of CAH3 becomes more significant for the WOC function if the pH of the medium is increased from the optimal value, equal to 6.2–6.5 up to 7.0.

The dependence of the WOC function on pH in preparations from wt *C. reinhardtii*, which was obtained in this study (Fig. 1), is fully matched with the dependencies, which were previously reported for green algae as well as for higher plants [40,42,43]. Nevertheless, the effect of the CA-activity on the pH dependence of the WOC function was shown here for the first time. The association of the CAH3 protein with PSII also has been found in other green algae [35] indicating that the described role of CAH3 may be common for all of them. The algae are very mobile organisms and can quickly move from deep waters to the illuminated surface or from the shaded to bright light areas. Taking it into account, the proposed function of CAH3 can be seen as an evolutionary adaptation, which supports the photosynthetic activity of PSII at increased pH of the thylakoid lumen, *i.e.* after adaptation to the dark or shade. In spite of the fact, that carrier of CA-activity in PSII from higher plants has not been identified so far, the influence of that activity on the PSII function has been shown [1,47,51]. This inspires an investigation of the effect of the CA-activity on the pH dependence of the WOC function in the PSII-complexes isolated from higher plants, and compare the results with the data of the present work.

Transparency Document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

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