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Protective Effect of α-Carbonic Anhydrase CAH3 Against Photoinhibition and Thermal Inactivation of Photosystem II in Membrane Preparations as Compared with α-Carbonic Anhydrase CA4

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Abstract—Photosystem II (PSII) is one of the most vulnerable components of photosynthetic apparatus of the thylakoid membrane to the action of inhibitory factors. The donor side of PSII exhibits high sensitivity to photoinhibition and thermal inactivation, which leads to the loss of O_2-evolving function of the water-oxidizing complex (WOC). The data obtained in this study demonstrated increased stability of WOC activity in the PSII membrane preparations from the wild-type (WT) *Chlamydomonas reinhardtii* compared to the PSII preparations from the *cia3* mutant, which lack α-carbonic anhydrase (CA) CAH3, under conditions of moderate photoinhibition and thermal inactivation. This effect was completely eliminated by adding a CA inhibitor to the PSII preparations from WT. At the same time, addition of active recombinant CAH3 (rCAH3) protein to the preparations from *cia3* restored increased resistance of PSII to these factors. Under the same conditions of photoinhibition and thermal inactivation, the PSII preparations from *Arabidopsis thaliana* demonstrated very low loss of O_2-evolving activity, regardless of the presence or absence of carbonic anhydrase α-CA4, which is similar to CAH3. More pronounced suppression of the O_2-evolving activity in the PSII from *A. thaliana* mutants lacking CA4 was observed only when they were incubated at elevated temperature, indicating the possibility of more significant conformational changes in the WOC proteins of PSII. Despite the clear binding of the rCAH3 to PSII membrane preparations from *A. thaliana*, the enzyme had little effect on the WOC activity in these preparations, suggesting absence of functional interaction between the rCAH3 and PSII from *A. thaliana*. The obtained results indicate different mechanisms of involvement of CAH3 and CA4, both of which are assumed to exist in close association with PSII in live systems, in the PSII functioning.

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Keywords: *Chlamydomonas reinhardtii*, *Arabidopsis thaliana*, carbonic anhydrase CAH3, carbonic anhydrase α-CA4, photosystem II, water-oxidizing complex, O_2-evolving activity, photoinhibition, thermal inactivation

INTRODUCTION

Photosystem II (PSII) is a large multiprotein pigment-containing complex of a thylakoid membrane [1-3], which mediates primary charge separation at the expense of energy of the absorbed quanta of light. At the donor (lumenal) side of PSII this is accompanied by oxidation of water molecules to O_2 and protons (H^+), and at the acceptor (stromal) side – by reduction of quinone electron acceptors Q_A and Q_B [4-6].

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Based on the degree of resistance to the action of the functional inhibiting factors, such as, in particular, excessive illumination and elevated temperature, PSII is the most sensitive element of the photosynthetic apparatus of thylakoid membrane [3, 7-9], hence, search for approaches to maintain high photosynthetic activity of PSII is an important and relevant task.

The mechanism of protecting PSII against excessive illumination involves partial dissipation of the absorbed light energy as heat through the energy-dependent component of non-photochemical chlorophyll quenching, qE, which is mediated by the proteins PsbS and Lhcsr3 (in green algae and mosses) and activity of the xanthophyll cycle [3, 10-13]. They also could re-target part of the absorbed light energy to the more resistant photosystem I through re-distribution of antenna complexes between two photosystems (component of non-photochemical chlorophyll quenching termed state transitions (ST)) [3, 11, 14, 15]. However, in the case of light intensity exceeding total capabilities of protective mechanisms in PSII against excessive illumination, suppression of its photosynthetic activity is observed [9]. This effect is called 'photoinhibition of PSII', and it could be both reversible (under weak or short-term impact), and irreversible process accompanied by the damage of proteins in PSII, such as, in particular, D1 (PsbA), and degradation of reaction centers [3, 7, 9, 16-18]. Mechanisms of rapid repair of damaged PSII exist in live systems, effects of which could be eliminated by using antibiotics, such as chloramphenicol or lincomycin, or by investigating photoinhibition of PSII using isolated membrane preparations [16, 17, 19, 20]. In the process, the rate of PSII photoinhibition depends significantly on the intensity of the acting light [16].

Molecular processes occurring during PSII photoinhibition are associated either with the donor or acceptor sides of PSII [7, 21, 22] and are mediated mainly by formation and accumulation of reactive oxygen species (ROS) [8, 22-25]. The donor side of PSII is more sensitive to photoinhibition, and associated processes occur even under exposure to light of moderate intensity [9, 18, 19, 26, 27].

Significant amounts of oxidized amino acids in the preparations of PSII exposed to light has been found close to the Mn_4CaO_5-cluster of the water-oxidizing complex (WOC) and outlet channels for O_2/ROS [26, 28]. It has been suggested that the ability of Mn ions to photoreception results in disruption of the Mn_4CaO_5-cluster function under illumination and even in removal of Mn ions from the active center of WOC [18]. Incomplete oxidation of H_2O molecules in the damaged Mn_4CaO_5-cluster could facilitate formation of hydrogen peroxide (H_2O_2), which is reduced by Mn^{2+} (Fenton-like reaction) to hydroxyl radical ($OH^{\bullet}$) [22, 29, 30], which, in turn, oxidizes amino acids in its vicinity [26, 28]. Insufficiently effective donation of electrons from WOC to P_{680}^{+} through Tyr_Z under illumination support their cationic forms, which oxidize easily amino acids and pigments in their vicinity [9, 18] causing functional destabilization of PSII.

The degree of PSII photoinhibition increases significantly with increase of temperature [31], however, decrease of photosynthetic activity of PSII at elevated temperatures is observed also during dark incubation of the preparations (thermal inactivation) [32-37]. In the case of thylakoids, decrease of WOC activity has been observed already at 30-40°C [35, 36, 38, 39], which is accompanied by partial removal of the WOC proteins (PsbO, PsbP, PsbQ) from PSII, and degree of this removal increases several folds with temperature increase to 50°C [34, 36, 40, 41]. Simultaneously Mn and Ca ions are removed from the Mn_4CaO_5-clusters [34, 36, 41]. Illumination of the preparations after thermal inactivation is accompanied by formation of ROS both at the acceptor and donor sides of PSII according the mechanisms similar to photoinhibition mechanisms [22]. Similar degradation of the D1 and D2 proteins is also observed [31, 35, 36].

One of the additional factors suppressing activity of Mn_4CaO_5-cluster is local acidification in its close vicinity emerging as a result of obstructed removal of H^+ formed during photoinduced water oxidation into a lumen [4, 42, 43]. This could be facilitated by conformational re-arrangements of the proteins in PSII caused by both increased temperature and oxidative modification of amino acids resulting in destabilization of proton channels [4, 13, 43, 44]. Furthermore, low pH increases significantly the probability of hydrogen peroxide reduction by Mn ions to $OH^{\bullet}$ [22, 30], formation of which increases amount of oxidized amino acids in close vicinity of the Mn_4CaO_5-cluster, thus increasing the degree of conformational rearrangements in the proteins.

It has been suggested previously, that the PSII-associated lumenal α-carbonic anhydrase (CA) CAH3 from green algae *Chlamydomonas reinhardtii* plays a role in stimulation of removal of H^+ from the WOC active center in the case of destabilized proton channels through its dehydratase activity ($H^+ + HCO_3^- \rightarrow H_2O + CO_2$) thus 'neutralizing' protons at the channel exit to lumen [4, 42, 43, 45]. This supported photosynthetic activity of PSII of *C. reinhardtii* [4, 13, 42, 46, 47]. In the *Arabidopsis thaliana* plants, the role of PSII-associated α-CA has been suggested for the CA4 protein, however, according to the authors, contrary to the case of CAH3, this CA catalyzes hydratase direction of the reaction ($H_2O + CO_2 \rightarrow H^+ + HCO_3^-$), which results in additional protonation of lumenal amino acids in the PsbS protein thus initiating qE [48, 49].

In addition, presence of different sources of CA activity near the WOC of PSII has been suggested in

a number of previous studies [49-53], including effects of CA on WOC functioning [54-56]. However, the data on effects of CA or CA activity on the resistance of PSII to photoinhibition and thermal activation are practically absent at present, which emphasizes importance and novelty of such studies.

Use of membrane preparations of PSII from *C. reinhardtii* and *A. thaliana* in this study including both wild-types (WT) and mutants lacking CAH3 and CA4, respectively, allowed to examine participation of these CAs in preservation of O_2-evolving function of WOC in PSII during photoinhibition and thermal inactivation. Moreover, use of the moderately high light intensities and temperatures during the long-term exposure provided the possibility to avoid rapid and destructive changes in the preparations, which could mask the effects of CAs [31, 35, 36]. Addition of the highly active recombinant CAH3 protein (rCAH3) [57] to PSII of *C. reinhardtii* and *A. thaliana* provided the opportunity to investigate both specificity of the action of this CA on photosynthetic activity of PSII from algae, and evaluate capability of the rCAH3 to bind to PSII preparations from *A. thaliana* both containing (WT) and lacking the CA4 protein (mutants).

MATERIALS AND METHODS

Membrane preparations isolated from a green algae *C. reinhardtii* and *A. thaliana* plants and enriched with PSII served as objects of the study.

A CC-503 strain was used as a standard WT *C. reinhardtii*, and a *cia3* mutant – as a strain without CAH3 in a thylakoid lumen [42, 43]. Algae cultures were cultivated under conditions described in the previous study [4] at 25°C with continuous illumination with cold white light (LED 6500 K) of ~90 μmol photons·m^{-2}·s^{-1} intensity and aeration with air containing 5% CO_2. In the case of *A. thaliana* plant ecotype Columbia-0 (Col) was used as a WT, and plants of homozygous lines 8-8 and 9-12 – as mutants with knockout of the gene encoding α-CA4 (*At4g20990*) [49]. Plants were grown for 5-6 weeks in a growth chamber at 19°C and short photoperiod (8-h day/16-h night) with illumination intensity ~120 μmol photons·m^{-2}·s^{-1} [58]. Isolation of membrane preparations enriched with PSII was carried out according to the protocols described previously for *C. reinhardtii* [4] and *A. thaliana* [49]. Maximum rates of O_2 evolution were ~280 μmol O_2·mg^{-1} chlorophyll·h^{-1} for PSII preparation from *C. reinhardtii* and ~310 μmol O_2·mg^{-1} chlorophyll·h^{-1} – for PSII preparations from *A. thaliana*. These values were accepted as 100% in the measurements. Isolated preparations of PSII containing 10% of glycerol were stored at −70°C.

Total chlorophyll concentration was measured using spectrophotometry in acetone extracts (80%) of PSII preparations [59].

Rate of photoinduced evolution of O_2 was measured in a 1-ml cell with Clark electrode (Hansatech, United Kingdom) at 25°C, illumination with saturating red light (~2300 μmol photons·m^{-2}·s^{-1}; λ > 600 nm) in the presence of electron acceptor potassium ferricyanide (1 mM) and 2,6-dichloro-*p*-benzoquinone (0.2 mM [60]), as has been described previously [44].

In the experiments with photoinhibition, PSII membrane preparations diluted to concentration 15 μg of chlorophyll per ml in a buffer containing 20 mM MES (pH 6.5 or 7.0), 35 mM NaCl, and 400 mM sucrose were incubated in a thermostated cell at 20°C under illumination with red light (λ > 600 nm) of intensity 1200 μmol photons·m^{-2}·s^{-1}; 1-ml aliquots of a suspension were sampled for measuring the rate of O_2 evolution after 0-, 15-, 30-, 45-, and 60-min incubation. Simultaneously a control sample was incubated in the dark under the same conditions and rate of O_2 evolution was measured at the same time intervals (time shift in parallel measurements was 5 min). Thermal inactivation of PSII was carried out according to the similar protocol, with samples incubated in the dark at 33°C, while the reference sample was incubated at 20°C.

Recombinant protein rCAH3 with high CA activity (~8300 WAU·mg^{-1}) was prepared and purified as described in the recent publication [57]. Initial used concentration of rCAH3 was 0.71 mg·ml^{-1}. Prior to photoinhibition or thermal inactivation rCAH3 protein [57] was added to dilutions of PSII at the ratio 0.2 μl·ml^{-1} (or 142 ng·ml^{-1}), unless stated otherwise in the legends to figures. To perform Western blotting, membrane preparations of PSII from control and experimental samples were precipitated in microtubes by centrifugation at 12,000*g* for 5 min. Samples diluted in a loading buffer (50 mM Tris-HCl (pH 6.8); 3% SDS; 0.005% bromophenol blue; 10% sucrose; 5% mercaptoethanol) were loaded onto a polyacrylamide gel (PAAG) at the ratio 2 μg of chlorophyll per lane.

Western blotting was carried out using electrophoresis in a denaturing 12.5% SDS-PAAG followed by transfer of proteins onto a PVDF-membrane (Immun-Blot PVDF Membrane, 0.2 μm; Bio-Rad, USA) in a Mini-PROTEAN 3 Cell (Bio-Rad) with a module for wet blotting Mini Trans-Blot (Bio-Rad), as described previously [44]. Membranes blocked with a 5% skim milk powder solution were incubated overnight at 4°C with primary rabbit antibodies against CAH3 protein (Agrisera, Sweden; AS05 073) at 1 : 2000 dilution, and next for 1 h at room temperature with secondary antibodies against rabbit proteins labelled with horseradish peroxidase (GE Healthcare, USA) at dilution 1 : 5000.

Visualization was carried out using a Pierce ECL Plus reagent kit (Thermo Scientific, USA) and a Chemi-Doc XRS+ system (Bio-Rad). Staining of major protein bands on a membrane was carried out by short-term (3-5 s) immersion of a membrane into a 0.2% Ponceau solution in 5% glacial acetic acid and membrane washing with distilled water until clear background was reached [44].

CA-Activity of rCAH3 protein added to PSII preparations from *C. reinhardtii cia3* was measured according to previously described protocol [57] using a 25 mM Tris-buffer (pH 8.5) at 0°C. Activity was presented in Wilbur-Anderson Units (WAU) per 1 mg of rCAH3 protein or total chlorophyll. Membrane fraction of PSII containing rCAH3 was precipitated from 5 ml of suspension by centrifugation at 10,000*g* for 5 min and resuspended in an incubation buffer (pH 6.5) to final volume of 100 µl, which was introduced to a reaction mixture for measuring CA activity. Incubation buffer (100 µl) without PSII was used as a negative control.

RESULTS

To investigate participation of CAH3 and CA4 in maintenance of PSII activity under conditions of moderate photoinhibition, membrane preparations of PSII isolated from the WT *C. reinhardtii* (CC-503) and *A. thaliana* (Col), as well as from the *C. reinhardtii cia3* and *A. thaliana* 8-8 and 9-12 mutants not containing CAH3 and CA4, respectively, were subjected to illumination with light of moderate intensity for one hour. This exposure caused loss of O_2-evolving activity

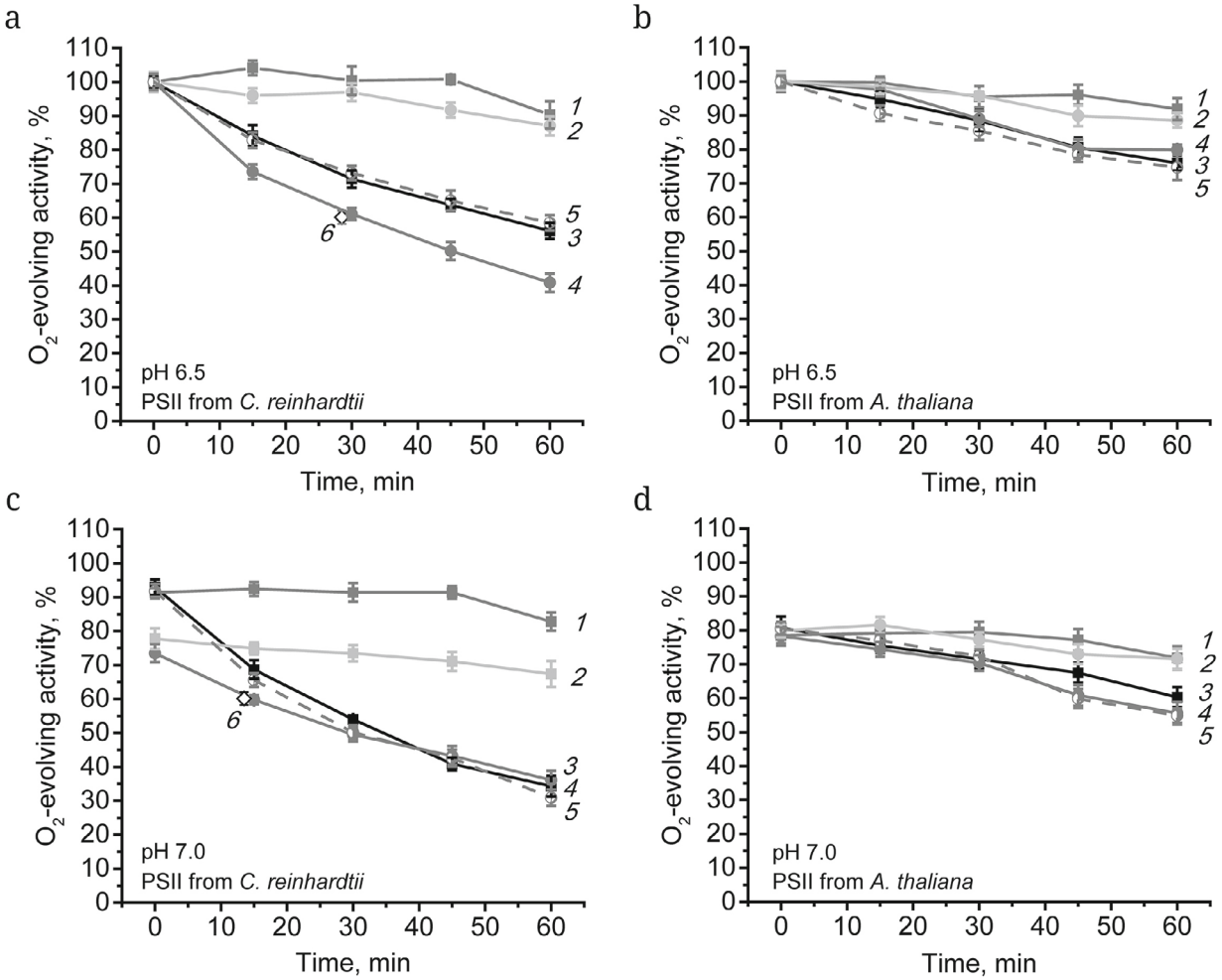

Fig. 1. Change in the rate of O_2 evolution by the membrane PSII preparations isolated from *C. reinhardtii* (a, c) and *A. thaliana* (b, d) during 1-h incubation at 20°C in the dark (*1*, *2*) and under continuous illumination with red light (*3*-*6*). Measurements were carried out at pH 6.5 (a, b) and 7.0 (c, d). *1*, *3* – PSII preparations from WT (*C. reinhardtii* CC-503 and *A. thaliana* Col, respectively); *2*, *4*, *5* – PSII preparations from mutants *C. reinhardtii cia3* and *A. thaliana* 8-8, respectively; *5* – rate of O_2 evolution in the presence of 0.2 µl·ml^{-1} (142 ng·ml^{-1}) of rCAH3; *6* – rate of O_2 evolution by the PSII preparations from *C. reinhardtii* CC-503 in the presence of 1 µM ethoxyzolamide. Curves obtained from the PSII preparations from *A. thaliana* 9-12 identical to the ones from 8-8 are omitted for clarity of presentation. Results are presented as a mean ± standard deviation of the mean (n = 3-5).

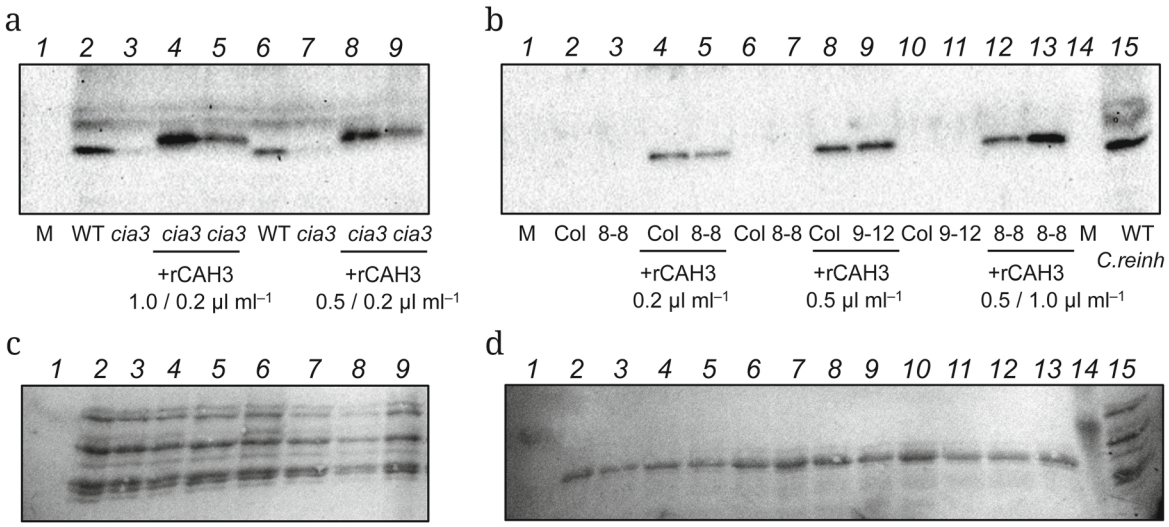

Fig. 2. Results of Western blot analysis of the PSII preparations from *C. reinhardtii* (a) and *A. thaliana* (b) using primary antibodies against CAH3. Recombinant protein rCAH3 was added to the PSII preparations in different concentrations shown under the underlined lanes (*4* and *5*, *8* and *9*, *12* and *13*). c and d) Membranes (a) and (b), respectively, with major proteins stained using Ponceau solution.

by the PSII preparations from *C. reinhardtii* even at pH 6.5 optimal for WOC (Fig. 1). In the case of WT the rate of O_2 evolution decreased by ~16% after 15-min illumination, by ~29% – after 30 min, and after one hour – by ~44%. The decrease of activity was more pronounced in the PSII preparations from *cia3* – ~27% after 15-min illumination, ~39% – after 30 min, and ~59% – after one hour of photoinhibition (Fig. 1a). The control samples containing PSII preparation from the WT and from the *cia3* mutant and incubated at close temperature (20°C) in the dark did not exhibit suppression of the O_2-evolving activity. Only 10% decrease of the rate of O_2 evolution in these preparations was observed after 1 h of incubation.

The PSII preparations from *A. thaliana* demonstrated under the same conditions higher resistance (Fig. 1b). Rate of O_2 evolution by the PSII preparations from Col decreased by ~5% after 15 min of incubation, by 11% – after 30 min, and by ~24% – after 1 h. This was almost 2-fold lower in comparison with the values obtained for the PSII from WT *C. reinhardtii*. The curves of suppression of the O_2-evolving activity by the PSII in the membrane preparations isolated from the *A. thaliana* mutants 8-8 and 9-12 were identical and showed decrease by ~3% after 15 min of incubation, by ~11% – after 30 min, and by ~21% – after 1 h, which was practically the same as for the curve obtained for the PSII from Col (Fig. 1b). In the process, decrease of WOC activity by 10% was observed for the control samples incubated for 1 h in the dark.

In order to confirm role of CAH3 in maintenance of activity of PSII of *C. reinhardtii* under conditions of moderate photoinhibition, active recombinant protein rCAH3 was added to the PSII from *cia3* prior to the start of illumination (at the ratio 0.2 µl·ml^{-1} (142 ng·ml^{-1})), and a known inhibitor of CA, ethoxyzolamide (EA) was added to the PSII preparations from WT (at concentration 1 µM). Long-term illumination of such PSII preparations resulted in elimination of the inhibition of O_2-evolving activity of WOC in the PSII from *cia3* in comparison with the PSII from WT, while the PSII preparations from WT, on the contrary, demonstrated more significant suppression of the rate of O_2 evolution, similar to one observed for the PSII from *cia3* (Fig. 1a). Addition of rCAH3 to the PSII preparations from the *A. thaliana* 8-8 and 9-12 mutants did not affect significantly the degree of reduction of O_2-evolving activity by the preparations (Fig. 1b).

Western blot analysis using primary antibodies against CAH3, as expected, demonstrated binding of the rCAH3 protein with the PSII preparations from the *cia3 C. reinhardtii* (Fig. 2a) with the band intensity increasing with the increase of the amount of recombinant protein added to the PSII preparations. This indicated the possibility of non-specific binding of a fraction of rCAH3 with the membrane fraction, however, the effect of maintenance of the PSII activity during photoinhibition implied functional interaction between the WOC in the PSII preparations and rCAH3 molecules. At the same time, intensive binding of the rCAH3 with the PSII preparations from *A. thaliana* was also observed, moreover, binding was observed for both the preparations from Col, and from the mutant lines 8-8 and 9-12 (Fig. 2b). Intensity of the band was also higher in the cases, when larger amounts of recombinant proteins were added to the samples.

This indicated strong non-specific binding of the rCAH3 protein with the membrane fraction of PSII independent on the presence of the native CA4 protein. However, absence of the effect of the added rCAH3 on the activity of PSII from the 8-8 and 9-12 mutants indicated either impossibility of functional interaction of the WOC in the PSII from *A. thaliana* with CAH3, or insufficiently strong stress exposure in the case of PSII from *A. thaliana* under used illumination conditions to reveal the role of CA.

Non-optimal pH is another stress factor affecting O_2-evolving activity of PSII [4, 61-63]. As has been shown previously, shift of the medium pH from 6.5 to 7.0 caused reversible suppression of WOC activity in the PSII of *C. reinhardtii*, furthermore, reduction of the O_2-evolving activity was more pronounced (by ~20%) in the PSII from *cia3*, due to the absence of CA activity of CAH3 in close vicinity of WOC [4]. Suspension of the membrane preparations of PSII form *C. reinhardtii* in the medium with pH 7.0 reproduced the same results. The rate of O_2 evolution before illumination for the PSII from WT and *cia3* were ~93% and ~74% of the respective values observed at pH 6.5 (Fig. 1c). The control samples incubated in the dark demonstrated maintenance of this difference during time, although a minor gradual decrease of the O_2-evolving activity of the PSII from *cia3* was observed, while a sharp drop of activity to ~83% was observed in the case of WT PSII after 45 min of incubation. Moderate photoinhibition of the preparations of PSII under these conditions caused more pronounced loss of O_2-evolving activity in comparison with pH 6.5. In particular, the rate of O_2 evolution in the PSII from WT was lower by ~7% before illumination and decreased further by ~31% after 15 min, by ~46% – after 30 min, and by ~65% (versus ~44% at pH 6.5) – after 1 h of illumination. The O_2-evolving activity of the PSII from *cia3* was decreased by ~26% before illumination and next decreased by ~40% after 15 min, by ~50% – after 30 min, and by ~64% (versus ~59% at pH 6.5) – after 1 h of illumination.

Comparison of the decrease of O_2-evolving activity of WOC in the PSII from WT and *cia3* at pH 7.0 demonstrated larger declining slope in the case of PSII from WT during first 30 min of illumination, which next reached the values observed for the PSII from *cia3*. After 30 min of photoinhibition both curves were very similar (Fig. 1c). Higher activity of WOC in the PSII from WT for the first 30 min could be explained by the CA activity of CAH3, which was confirmed by the decrease of the rate of O_2 evolution in the PSII preparations from WT to the level of PSII from *cia3* on addition of EA. Moreover, addition of rCAH3 to the PSII preparations from *cia3* resulted in the curve of decrease of the O_2-evolving activity similar to the one obtained for the PSII from WT (Fig. 1c).

Unlike in the case of PSII from *C. reinhardtii*, the PSII preparations from *A. thaliana* at pH 7.0 demonstrated reduced by ~20% rate of O_2-evolution, in comparison with pH 6.5, for all variants (Fig. 1d). At the same time, differences with the control samples were minimal during first 30 min of photoinhibition. After 30 min the O_2-evolving activity decreased by ~10% for the PSII from Col and by ~15% for the PSII from 8-8 and 9-12 mutants. Addition of rCAH3 to the PSII preparation from 8-8 and 9-12 mutants did not affect the shape of the curve of suppression of the rate of O_2 evolution during photoinhibition indicating lack of functional interaction between the enzyme and the WOC of *A. thaliana*.

To investigate participation of CAH3 and CA4 in protection of PSII against moderate thermal inactivation, PSII preparations from *C. reinhardtii* and *A. thaliana* were incubated at 33°C in the dark at pH 6.5 or 7.0.

At pH 6.5 activity of WOC in the PSII from WT *C. reinhardtii* did not decrease significantly during the first 15 min of incubation (by ~10%), however, after 30 min this decrease reached ~30%, and after 1 h – ~60% (Fig. 3a). The curve of the loss of O_2-evolving activity by the PSII from *cia3* was smoother and the values of decrease were ~22% after 15 min and ~38% after 30 min of incubation. After 45 min of incubations the curves for the PSII from WT and PSII from *cia3* practically coincided indicating loss of the protective role of CAH3. It should be mentioned that the addition of rCAH3 to the preparation of PSII from *cia3* stimulated the rate of O_2 evolution, however, during the first 15 min of incubation, when the difference between the curves obtained for the PSII from WT and PSII from *cia3* was the largest, this stimulation was only ~50% (Fig. 3a).

In the case of PSII preparations from *A. thaliana* the curve of decrease of O_2-evolving activity by the PSII from Col differed only slightly from the control curves up to 45 min of incubation, but displayed sharp drop by ~22% after 1 h (Fig. 3b). The rates of O_2 evolution in the PSII preparations from 8-8 and 9-12 mutants started to decrease after 30 min of incubation by ~18% and ~27%, respectively, observed after 45 min and 1 h. Addition of the rCAH3 protein to the PSII preparations from *A. thaliana* 8-8 and 9-12 produced an unexpected result. During the first 15 min of incubation presence of rCAH3 caused slight suppression of the rate of O_2 evolution (by ~9% in comparison with the control), which disappeared after 30 min of incubation.

In the process of moderate thermal inactivation of PSII at pH 7.0, the PSII preparation from WT *C. reinhardtii* demonstrated slower reduction of the O_2-evolving activity in the first 15 min (by ~22% with ~9% initial inhibition at pH 7.0; Fig. 3c) and accelerated suppression during the following

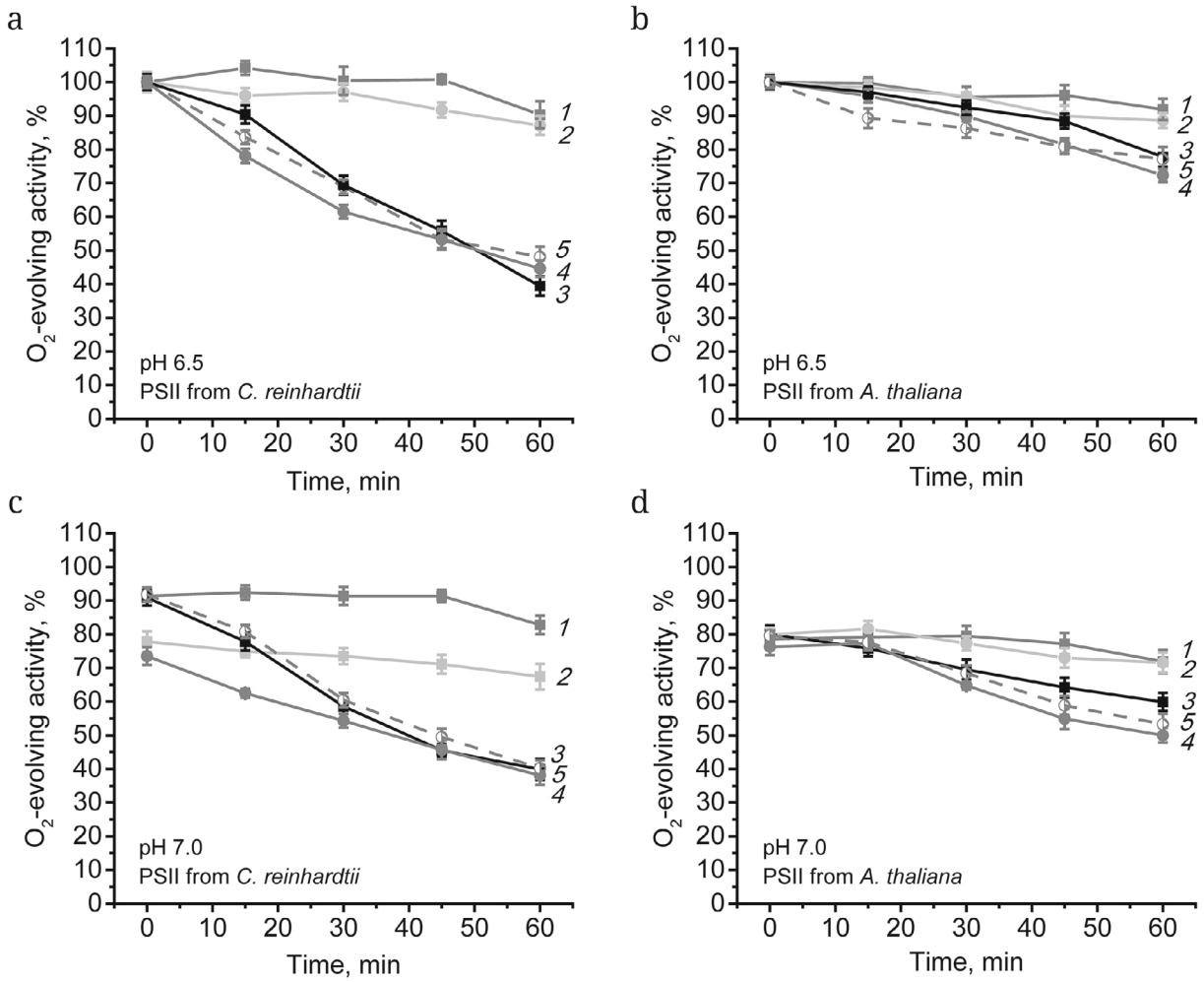

Fig. 3. Changes of the rate of O_2 evolution by the membrane preparations of PSII isolated from *C. reinhardtii* (a, c) and *A. thaliana* (b, d) during incubation for 1 h at 20°C (*1*, *2*) and at 33°C (*3*-*5*) in the dark. Measurements were carried out at pH 6.5 (a, b) and 7.0 (c, d). *1*, *3* – PSII preparations from WT (*C. reinhardtii* CC-503 and *A. thaliana* Col, respectively), *2*, *4*, *5* – PSII preparations from mutants *C. reinhardtii cia3* and *A. thaliana* 8-8, respectively; *5* – rate of O_2 evolution in the presence of 0.2 μl·ml^{-1} (142 ng·ml^{-1}) of rCAH3. Curves obtained for the PSII preparation from *A. thaliana* 9-12 were identical to the ones for 8-8 and are omitted for better visualization. Results are presented as a mean ± standard deviation of the mean (n = 3-5).

30 min (by ~41%) reaching values observed for the PSII from *cia3*. The decrease of the rate of O_2 evolution during thermal inactivation of the PSII from *cia3* was more linear – by ~10% at each tested time point (with initial ~26% inhibition at pH 7.0). Unlike in the case of results obtained at pH 6.5, addition of rCAH3 to the PSII preparation from *cia3* resulted in noticeable and complete restoration of the O_2-evolving activity of WOC to the levels observed for the preparations from WT even despite the more pronounced differences between the curves obtained for the PSII from WT and PSII from *cia3*, in comparison with the curve obtained at pH 6.5 (Fig. 3c).

The PSII preparations from *A. thaliana* also demonstrated strong and rapid suppression of the O_2-evolving activity at pH 7.0 in the course of moderate thermal inactivation in comparison with the data obtained at pH 6.5. The rate of O_2 evolution was maintained at the level of control samples for first 15 min, by it decreased significantly after that; the decrease was more pronounced in the PSII preparations from the 8-8 and 9-12 mutants (Fig. 3d). After one hour of incubation the O_2-evolving activity (with initial ~20% inhibition at pH 7.0) decreased by ~40% in the PSII preparation from Col and by ~50% – in the PSII from the mutants 8-8 and 9-12. It is worth to mention that this decrease was the highest observed suppression of the rate of O_2 evolution in the preparations from *A. thaliana* used in the study. Furthermore, differences observed between the PSII preparations from Col and from 8-8 and 9-12 mutants were most significant.

Investigation of the effect of conditions of photoinhibition and thermal inactivation of the PSII

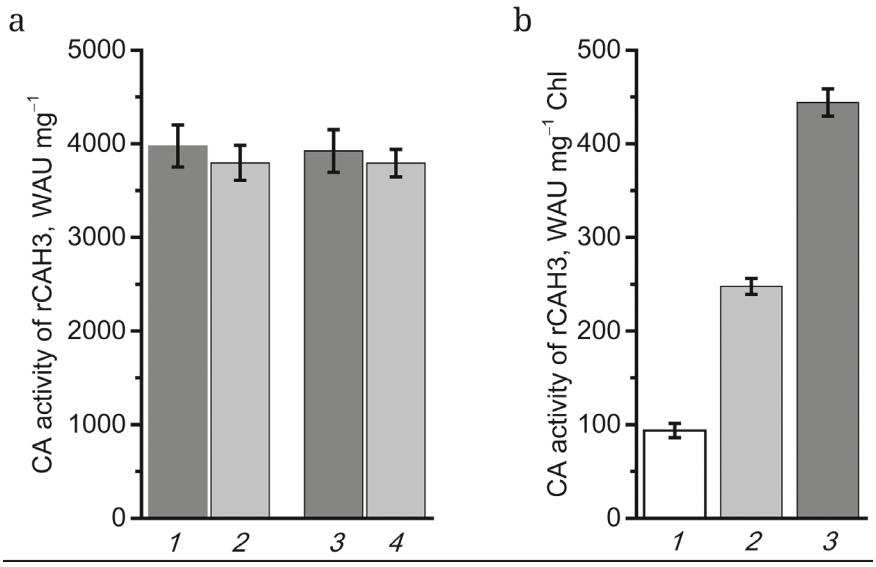

Fig. 4. CA activity of rCAH3. a) Activity of the enzyme per mg of protein after its addition to membrane preparations of PSII isolated from the mutant *C. reinhardtii cia3* at a ratio 0.2 μl·ml^{-1} (142 ng·ml^{-1}), measured before (*1*, *3*) and after 1-h photoinhibition (*2*) or thermal inactivation (*4*). b) Activity of rCAH3 per mg of chlorophyll (Chl) during addition of the enzyme protein to PSII preparations at a ratio 0.2 (*1*), 0.5 (*2*), and 1 (*3*) μl·ml^{-1}, respectively. Results are presented as a mean ± standard deviation of the mean (n = 3).

preparations on CA activity of the rCAH3 demonstrated that in both cases the enzyme maintained its full activity during incubation (Fig. 4a). Addition of rCAH3 at higher ratio to the PSII preparations, which resulted in binding of a larger amount of the enzyme protein to the membrane fraction, as was shown previously using Western blotting (Fig. 2a), also practically did not affect the rCAH3 activity (Fig. 4b). Hence, the observed changes in the O_2-evolving activity of the PSII membrane preparations are not associated with the change of CA activity of the rCAH3.

DISCUSSION

The molecule of CA CAH3 monomer from *C. reinhardtii* without two transport peptides (1-73) has molecular weight of ~29.5 kDa (based on the PAGE data), has a single S-S bond (Cys90–Cys258), which is critically important for the enzyme activity, and structure of the catalytic center conserved for α-CAs containing three histidine residues (His160, -162, -179) and zinc ion (Fig. 5a) [43]. At the edge of a broad active center cavity there is a histidine residue (His134), which plays a role of proton shuttle [43]. Presence of a hydrophobic region at one side of the CAH3 molecule has been assumed to facilitate its binding with the membrane fraction and correct orientation relative PSII [64].

An immature CA4 protein from *A. thaliana*, unlike CAH3, contains, as suggested, only one short signal peptide (1-26; https://www.uniprot.org/uniprotkb/F4JIK2/entry), and, therefore, despite the lower number of amino acids in the protein sequence (267 versus 310), the mature CA4 protein has molecular weight close to molecular weight of CAH3.

Spatial structure of the CA4 molecule calculated based of the amino acid sequence resembles the structure of CAH3 protein and also has a single S-S-bond (Cys59-Cys214), three histidine in active center (His125, -127, -144), and His99 residue playing a role of proton shuttle (Fig. 5b).

Hence, spatial structures of α-CAs CAH3 from *C. reinhardtii* and CA4 from *A. thaliana* demonstrate close similarity both in monomer structure, and in the presence of a single S-S-bond connecting first part of the β-sheet forming broad cavity of the enzyme active center and N-terminal domain of the molecule (Fig. 5).

According to the previously published results, both CAH3 [4, 13, 42] and CA4 [49] are found in the membrane preparations enriched with PSII and isolated from *C. reinhardtii* and *A. thaliana*, respectively. It was shown in the process that CAH3 maintains high photosynthetic activity of PSII at suboptimal for WOC pH levels due to stimulation of H^+ removal from the active center [4, 13, 42], as well as affects structural organization of PSII [44]. The suggested role of CA4 involves inducing qE through stimulation of protonation of the luminal amino acids in the PsbS protein [48], as well as participation in other adaptive reactions of *A. thaliana* PSII [12].

The results of photoinhibition and thermal inactivation of the membrane preparations of PSII from *C. reinhardtii* and *A. thaliana* demonstrated much

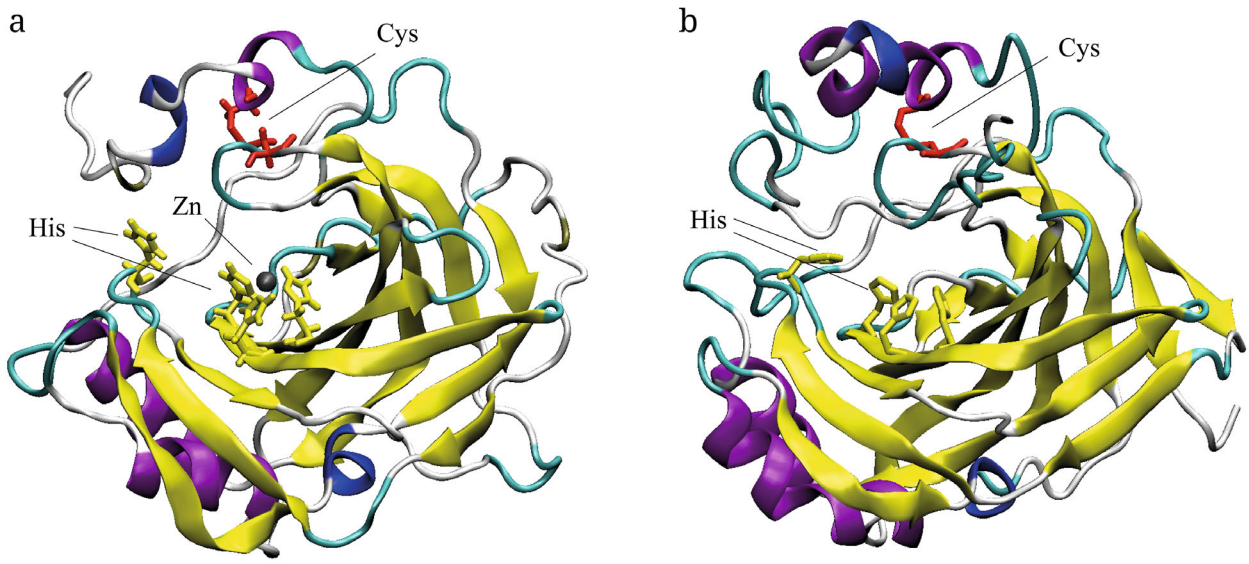

Fig. 5. Structures of CAs CAH3 (a) and CA4 (b) with shown locations of three histidines (His) of active center that bind Zn ion, and of H^+-transporting His at the edge of active center cavity, as well as of two cysteines (Cys) forming a S–S-bond. CAH3 structure is obtained based of the data of X-ray diffraction analysis of rCAH3 (PDB ID: 4xiw, https://www.wwpdb.org/pdb?id=pdb_00004xiw). CA4 structure is calculated using AlphaFold service (https://alphafold.ebi.ac.uk/) based on the data from UniProt (https://www.uniprot.org/) (F4JIK2 ATCA4_ARATH; https://www.uniprot.org/uniprotkb/F4JIK2/entry). Structures are visualized with the help of V.M.D. 1.9.2.

lower resistance of the PSII from *C. reinhardtii* to these factors even in the case of WT (Fig. 1 and 3). Absence of CAH3 (PSII from *cia3*) or its inhibition (addition of EA) resulted in the enhancement of the negative effect of these factors, and use of highly active rCAH3 protein, which fully maintained its activity in the course of incubation (Fig. 4a), allowed to investigate participation of the enzyme in these processes from the other point of view, including in the PSII preparations from *A. thaliana*. It must be mentioned that although the higher rate of O_2 evolution in the PSII from *C. reinhardtii* due to CA activity of CAH3 was maintained under continuous illumination for the entire period of the sample illumination (1 h), in the case of thermal inactivation stimulating effect decreased significantly after 30 min, and was completely absent after 45 min of incubation. This indicated existence of additional mechanisms of WOC inactivation at elevated temperature, which were not compensated fully by the removal of H^+ from the active center. These could include, for example, thermoinduced conformational rearrangements of proteins in WOC or even disorganization of their structure. On the one hand, minor removal of WOC proteins has been demonstrated previously during incubation of the PSII preparations at temperatures above 30°C [36]. On the other hand, conformational changes of the proteins in PSII caused by shift of pH to 7.0 [13] indeed caused loss of stimulation of O_2-evolving activity with the help of rCAH3 after 30-min incubation (Fig. 1c).

High functional stability of *A. thaliana* PSII both from Col, and from 8-8 and 9-12 mutants under used conditions of photoinhibition indicated lack of need of CA4 participation in maintenance of WOC activity. However, taking into account the possibility of conformational changes in the WOC proteins at pH 7.0 and elevated temperature mentioned above, it could be suggested that the PSII from mutants 8-8 and 9-12 were subjected to WOC disorganization, which was manifested by greater loss of O_2-evolving activity after 30 min of incubation in comparison with the PSII from Col (Fig. 1d and 3b). Addition of rCAH3 did not affect the curve of decrease of the rate of O_2 evolution by the PSII from 8-8 and 9-12 mutants during photoinhibition (Fig. 1, b and d) indicating either non-specificity of its interaction with PSII from *A. thaliana*, or lack of need in participation of CA in maintenance of WOC activity of the *A. thaliana* PSII.

Interestingly enough, transfer of the PSII preparations from *A. thaliana* to the buffer with pH 7.0 decreased the rate of O_2 evolution by ~20% in these preparations in comparison with the one at pH 6.5 (Fig. 1d and 3d). In the case of *C. reinhardtii* similar decrease was observed only for the PSII from *cia3* not containing CAH3 that was completely eliminated by addition of rCAH3 to the preparations (Fig. 1a), which binds strongly to the membrane preparations (Fig. 2). Another unusual fact is that suppression of O_2-evolving activity observed in the preparations from the mutants without CAs was different in the PSII from

C. reinhardtii and *A. thaliana*. While in the first case inhibition appeared immediately and was maximal after 15 min of photoinhibition or thermal inactivation as a result of absence of protective effect mediated through CA activity of the enzyme, in the second case inhibition manifested itself only after 30 min of incubation, when conformational changes accumulated in the proteins (Figs. 1 and 3). All these facts could be considered as an additional confirmation of absence of participation of CA in functioning of the PSII from *A. thaliana*.

Surprisingly, addition of rCAH3 during thermal inactivation of the PSII preparations from the *A. thaliana* mutants 8-8 and 9-12 at pH 6.5, resulted in a slight suppression of the O_2-evolving activity of PSII after 15 min of incubation, which disappeared after 30 min of incubation (Fig. 3b). Comparison with the data on photoinhibition revealed that this effect was present there too, although was insignificant (Fig. 1b). Considering the Western blotting data on strong binding of rCAH3 with the membrane preparations of PSII from *A. thaliana* (Fig. 2), it could be suggested that this inhibiting effect indicated existence of albeit small, but functional interaction between rCAH3 and PSII from *A. thaliana*, i.e., between the highly active CA (Fig. 4) and WOC in PSII from *A. thaliana*. However, unlike in the case of PSII from *C. reinhardtii*, similar interaction is, likely, not natural for the PSII from *A. thaliana* thus providing an opposite effect. Addition of rCAH3 to the PSII preparations *A. thaliana* mutants 8-8 and 9-12 at pH 7.0, did not reveal the similar effect, which indicated loss of functional interaction between rCAH3 and PSII at this pH. At the same time, at pH 7.0 protective effect of CAH3 on the O_2-evolving activity of PSII from *C. reinhardtii* was most pronounced especially during the first 15 min of thermal inactivation both in the case of native protein (PSII from WT), and of recombinant protein (Fig. 3c).

Hence, based on the obtained data, the following conclusions could be made on the properties of α-CAs CAH3 and CA4 (in the membrane PSII preparations): 1) CAH3 exerts protective effect on the activity of PSII from *C. reinhardtii* under conditions of moderate photoinhibition and thermal inactivation; 2) CAH3 protein binds strongly with the membrane preparations of PSII, including those isolated from *A. thaliana*, while preserving full activity; 3) CAH3 does not provide noticeable protective effect under conditions of moderate photoinhibition and thermal inactivation of the PSII preparations from *A. thaliana* or even results in suppression of O_2-evolving activity; 4) CA4 does not participate in supporting activity of WOC in PSII similar to CAH3, however, absence of the CA4 protein results in larger disorganization of WOC under the action of stress factors.

Abbreviations. 8-8 and 9-12, mutant lines of *A. thaliana* with knockout of the gene encoding α-CA4; CA, carbonic anhydrase; *cia3*, *Chlamydomonas reinhardtii* mutant lacking CAH3 in thylakoid lumen; Col, *Arabidopsis thaliana* ecotype Columbia-0; PSII, photosystem II; rCAH3, recombinant CAH3 protein; ROS, reactive oxygen species; qE, energy-dependent component of non-photochemical chlorophyll quenching; WOC, water-oxidizing complex; WT, wild-type.

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Contributions. V. V. Terentyev – concept and supervision of the study, writing and editing text of the paper; V. V. Terentyev, L. I. Trubitsina, T. P. Khoroshaeva, and I. V. Trubitsin – conducting experiments; V. V. Terentyev, L. I. Trubitsina, and T. P. Khoroshaeva – discussing results of the study.

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Conflict of interest. The authors of this work declare that they have no conflicts of interest.

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