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Effect of K223E and K226E Amino Acid Substitutions in PsbO Protein of Photosystem 2 on Stability and Functional Activity of the Water-Oxidizing Complex in *Chlamydomonas reinhardtii*

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Abstract—Site-directed mutations were introduced into PsbO protein of photosystem 2 to study the role of two lysine residues, 223 and 226 (LGAKPPK), in the green alga *Chlamydomonas reinhardtii*. Lysines 223 and 226 homologous to His228 and His231 from cyanobacteria are located on the protein side facing the lumen and can participate in formation of a channel connecting the Mn cluster with the intrathylakoid space. The K223E and K226E mutants were generated on the basis of the *ΔpsbO* strain of *C. reinhardtii* with the substitution of glutamic acid for the lysine residues. The K226E mutation leads to a decrease in stability of the protein and development of the *ΔpsbO* phenotype (the absence of both photosynthetic activity of photosystem 2 and photoautotrophic growth), with substantially decreased PsbO content in the cells. In the case of K223E, the mutant strain accumulated the normal level of PsbO protein and was able to grow photoautotrophically and to evolve oxygen. However, the rate of oxygen evolution and the F_v/F_m ratio were reduced by 15-20% compared to the control. Also, the time of the dark decay of F_v in the presence of DCMU in the cells of the K223E mutant was increased, indicating impairment in the water-oxidizing complex. In general, our study shows the importance of amino acids K223 and K226 located at the lumenal surface of PsbO protein for the activity of the water-oxidizing complex.

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Key words: PsbO protein, site-directed mutagenesis, photosystem 2, water-oxidizing complex, *Chlamydomonas reinhardtii*

Manganese-stabilizing protein (MSP) is a necessary component of the water-oxidizing complex (WOC) of all oxygenic photosynthetic organisms [1, 2], though its role in the process of water oxidation is still unclear. Together with other external proteins of the WOC, MSP is needed for stabilization of the inorganic core of the WOC and for providing the optimal rates of oxygen release. Removal of MSP from photosystem 2 (PS-2) preparations reduces the rate of oxygen evolution and results in gradual withdrawal of two out of the four manganese atoms comprising the WOC [3, 4]. However, the ability of PS-2 to evolve oxygen can be retained (~25%) by increasing Cl^- and Ca^{2+} concentrations in the medium [4, 5]. Genetic studies have shown that inactivation of MSP *in vivo* results in development of a phenotype depending on the evolutionary position of the organism. Inactivation of the MSP-encoding gene in the higher plants and algae results in the loss of PS-2 complex and the absence of growth under photoautotrophic conditions [6, 7]. In contrast to eukaryotic organisms, cyanobacteria can form PS-2 and even oxidize water in the absence of MSP. The Δ*psbO* mutant of *Synechocystis* sp. PCC 6803 can grow photoautotrophically, though the oxygen-evolving activity of PS-2 is unstable and lower than that in wild type cells and requires the presence of another external WOC protein, cytochrome c_{550} (PsbV), which is not a component of the WOC in higher plants and green algae [8]. PS-2 formed in the Δ*psbO* mutant of cyanobacteria can oxidize water, though enhanced content of Cl^-, Ca^{2+}, or high pH of the medium (10.0) is required to maintain its activity [8, 9]. Hence, it may be supposed that the state of the Mn cluster in the absence of MSP will depend on the composition of the medium where it is present. Thus, the function of the protein in stabilization of the Mn_4Ca cluster may be

Abbreviations: DCBQ, 2,6-dichloro-*p*-benzoquinone; ETC, electron transport chain; F_v/F_m, ratio of variable chlorophyll fluorescence (F_v) to maximum fluorescence level (F_m); MSP, manganese-stabilizing protein (PsbO); PCR, polymerase chain reaction; PS-2, photosystem 2; RC, reaction center; WOC, water-oxidizing complex.

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regulation of the interaction between the WOC and the medium of the lumenal space of chloroplasts.

The manganese-stabilizing protein is located at the donor side of PS-2 so that it shields the inorganic core of the WOC and separates it from the intrathylakoid space [10]. Due to its position, it can regulate the access of cofactors, e.g. chloride, calcium, and bicarbonate ions, to the WOC and provide their retention near the Mn cluster. In addition, MSP can participate in withdrawal of protons from the reaction zone by forming a channel for H^+ removal. It was supposed that the proton channel could include the following amino acid residues: D61, E65, E312, K317 of D1 and D2 proteins (components of the "core" complex of PS-2), and D158, D222, D223, D224, H228, E229 of PsbO protein [11].

Analysis of the structure of PsbO reveals a region of the protein that can form a hydrophilic channel connecting the lumen and WOC (Fig. 1). The supposed channel is located in a "crevice" formed by the fold between the two protein domains and consists of the conservative regions of the PsbO sequence. On the side facing the Mn cluster, a channel is formed by the R152-R162 sequence. At the other side facing the lumen, it may be formed by the SDDDMGAHEPH loop (for the cyanobacterium *Thermosynechococcus elongatus*) located between the β13- and β14-layers. The R152(151) and R162(161) residues closest to Mn cluster were shown to regulate the Cl^- requirement of the WOC [12]. However, substitutions for these amino acids influence the binding of PsbO to PS-2 [12, 13]. When making such substitutions *in vivo*, e.g. in *Chlamydomonas reinhardtii*, one may expect complete degradation of the entire WOC, which eliminates the possibility of determining the functional role of these amino acids. Therefore, more suitable candidates for site-directed mutagenesis are amino acids on the other side of the channel facing the lumen, e.g. residues Lys223 and Lys226. In cyanobacteria, these are residues His228 and His231, but comparison of the *C*-terminal sequences of MSP (Fig. 2) shows that in higher plants and algae they are conservatively replaced by lysines. In addition, Lys223 and Lys226 are the only charged amino acids in the part of the loop bordering the lumen: LGA<u>K</u>PP<u>K</u>. These amino acids are located between the binding site of PsbP protein (one of its functions is retention of Cl^- for maintaining WOC activity) and residues R152 and R162 of PsbO protein, which are probable candidates for Cl^-

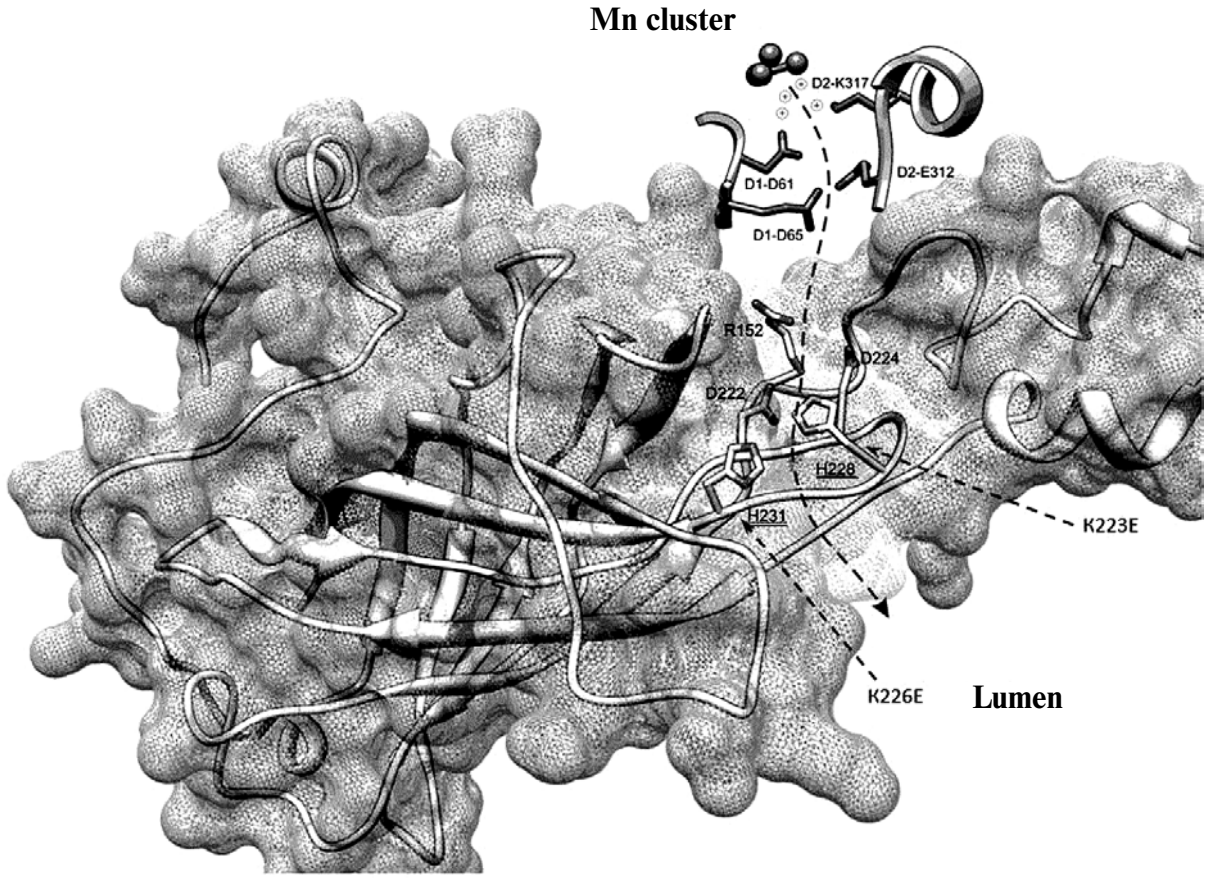

Fig. 1. Spatial organization of PsbO protein in the PS-2 complex (file 3BZ1.pdb from the Protein Data Bank). The dashed arrow shows the supposed pathway of proton transport from the Mn_4Ca cluster to the lumenal space of the thylakoid [11, 15]. The His228 and His231 residues located closer to the lumen are underlined.

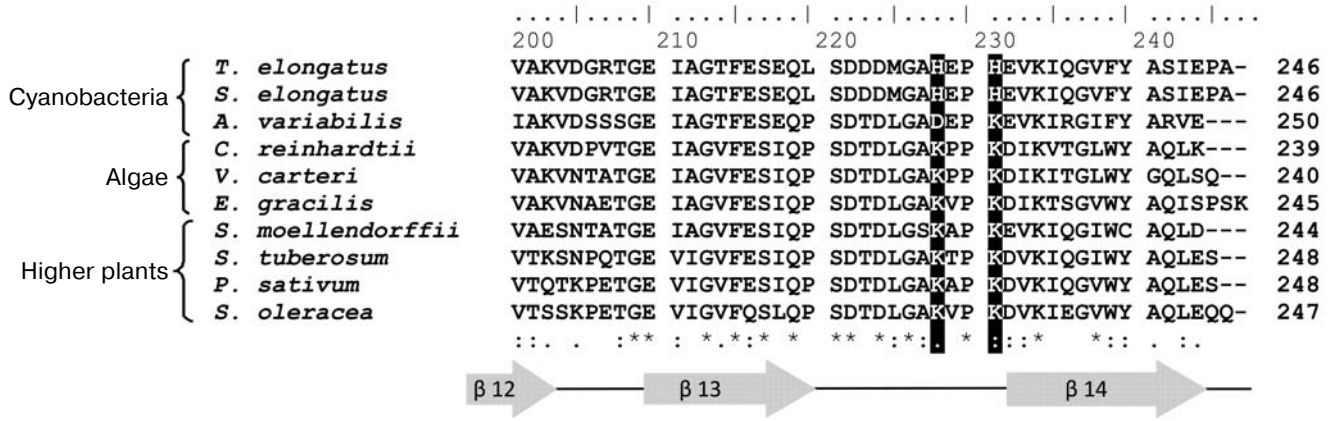

Fig. 2. Comparison of amino acid sequences of the *C*-terminal region of PsbO protein from different species of cyanobacteria, algae, and higher plants. Lysine amino acid residues in positions 223 and 226 in *C. reinhardtii* corresponding to histidine in positions 228 and 231 in the cyanobacterium *T. elongatus* are dark-colored. The sequences to be analyzed were taken from the UniProt site under the following IDs: *Thermosynechococcus elongatus* (P0A431), *Synechococcus elongatus* (P0A432), *Anabaena variabilis* (Q3MC21), *Chlamydomonas reinhardtii* (P12853), *Volvox carteri* (Q9SBN6), *Euglena gracilis* (P46483), *Selaginella moellendorffii* (D8TFZ6), *Solanum tuberosum* (P26320), *Pisum sativum* (P14226), and *Spinacia oleracea* (P12359).

binding near the Mn cluster [13, 14]. The D222 and D224 residues of PsbO protein located on the other side of the loop β13-β14 (SDDDMGAHEPH) can participate in formation of a hydrophilic channel but cannot bind the negatively charged chloride or bicarbonate ions due to the charge of aspartic acid. One should note the high conservation of these two lysine residues (in spite of the fact that they are components of the loop – the most variable element of protein structure).

Recent investigations of PS-2 structure show that cyanobacterial His231 homologous to the K226 of *C. reinhardtii* may be one of the ligands of the second calcium atom found in PS-2 but not involved in the reaction of water oxidation [10, 15]. Based on X-ray structure analysis, it was supposed for the other residue His228 (K223) that it could participate in the interaction with D2 protein via a hydrogen bond [11].

Investigation of the function of PsbO protein *in vivo* is limited by sensitivity of PS-2 in the mutant cells to photoinactivation. Minor damages in PS-2 may accumulate over time, heavily distorting the original effect of mutation. In higher plants, this may result in the loss of the PS-2 complex. With cyanobacterial cells used as a model, it is also necessary to take into consideration the prime importance of PsbV protein for WOC activity in prokaryotic organisms: as a result, PsbO inactivation in prokaryotes (in contrast to higher plants and algae) does not result in cessation of oxygen evolution. Under certain conditions, the rate of O_2 evolution in the cells of the Δ*psbO* strain may be ≥50% of that in wild type cells, and oxygen evolution can be completely suppressed only in the case of double mutation: *ΔpsbO:ΔpsbV*. Therefore, we have used *C. reinhardtii* cells for site-directed mutagenesis of PsbO. The green alga *C. reinhardtii* is a eukaryote capable of heterotrophic growth in the dark, making it possible to avoid light-induced damage [16]. Previously we have shown the formation of a photochemically active PS-2 complex, though incapable of O_2 evolution, in dark culture cells of the *C. reinhardtii ΔpsbO* strain [17]. Thus, cultivation of *C. reinhardtii* cells in the dark allows the study of the influence of mutations even if they have a very strong effect on WOC activity.

The goal of this work was to study the effect of the K223E and K226E substitutions in PsbO protein on WOC stability and functional activity in *C. reinhardtii* cells.

MATERIALS AND METHODS

The following strains of *C. reinhardtii* were used: Δ*psbO* strain (FUD 44, CC 2892, Chlamydomonas Genetics Center), and its derivatives – pseudo wild type cells (pWT) and PsbO mutants K223E and K226E. The algae were grown on TAP medium (pH ~ 7.0) [18] during agitation in two modes: under continuous illumination at light intensity 80 μmol/(m^2·sec) (light culture) and heterotrophically at 0.05-0.1 μmol/(m^2·sec) (dark culture). The incubation temperature was 21 ± 2°C.

Transformation of the Δ*psbO* cells of *C. reinhardtii* with the MSP-encoding gene produced pseudo wild type (pWT) cells that were used as a control for the *psbO* mutants. The gene was derived from plasmid p190 containing an insert of *C. reinhardtii* genomic DNA (pUC19:*Eco*RI/*Kpn*I) [19]. The fragment with *psbO* was cut out at the *Eco*RI/*Sma*I restriction sites (~7940 bp) and cloned in plasmid pSI103 (ParR) — the vector for *C. reinhardtii* transformation (Fig. 3) [20]. The resulting construction pSI103:psbO was used for transformation of

the Δ*psbO* cells of *C. reinhardtii*. Transformants were selected by growth on selective medium with paromomycin. Amino acid substitutions in the *psbO* gene were made by PCR. For this purpose, the *Bst*1107I/*Eco*911 fragment of the *psbO* gene located in its *C*-terminal part was synthesized. Mutations were obtained using the primer for the region *Eco*911: Fw-*Bst*1107I, 5′-GAT**GTATAC**CAGCTCCGATCTCAC; Rv_1-*Eco*911-K223E, 5′-GCC**GGTGACC**TT-GATGTCCTTGGGGGGCTC-GGCGCCCAG, and Rv_2-*Eco*911-K226E, 5′-GCC**GGTGACC**TTGATGTCCTCGGGGGGCTT (restriction sites are in bold; base substitutions are underlined). The resulting fragments (~455 bp) were used for substituting the initial nucleotide sequence in plasmid p190. Then the modified *psbO* gene was cloned in the pSI103 vector (similar to the pWT cells). The correctness of mutations was checked by DNA sequencing at the Evrogen company (Russia).

DNA was isolated using Chelex-100 [21]. For this purpose, *C. reinhardtii* cells ($(1\text{-}2)\cdot10^6$) were precipitated and lysed in 50 μl of 5% Chelex-100 suspension at 100°C for 8-10 min. Then the mixture was centrifuged, and 1 μl of the supernatant was used for PCR analysis.

For immunoblot analysis of PsbO, *C. reinhardtii* cells in the exponential growth phase (5 ml) were precipitated and resuspended in 100 μl of lysing buffer for SDS electrophoresis [22]. Samples were denatured by heating to 95°C for 3 min and applied to the gel ($(1\text{-}1.5)\cdot10^6$ cells). The sample was separated in 12% SDS-polyacrylamide gel. After transfer to nitrocellulose membrane (Amersham, USA), PsbO content was determined using specific polyclonal antibodies (AntiProt, Germany). The signal was recorded with the ECL detection system (Amersham).

PS-2 activity was studied in cultures in the logarithmic growth phase at a density of $(1\text{-}2)\cdot10^6$ cells/ml. Cell number was counted in Goryaev chamber. The pigment content was determined by spectrophotometry in 80% acetone [23].

The rate of photosynthetic O_2 evolution was measured by polarography with a Clark electrode. Before the measurement, the sample was incubated in the dark for 5 min at 23°C. Oxygen evolution was measured in the presence of 7 mM $NaHCO_3$ or on addition of exogenous electron acceptors (100 μM 2,6-dichloro-*p*-benzoquinone (DCBQ) and 30 mM ferricyanide).

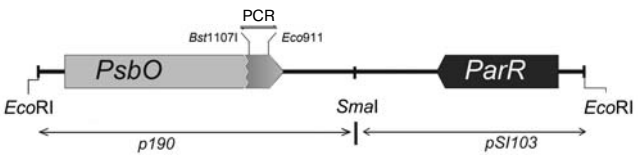

Fig. 3. Scheme of pSI103::psbO vector for transformation of *C. reinhardtii* Δ*psbO* cells.

Variable chlorophyll fluorescence of PS-2 (F_v) was measured with a XE-PAM fluorimeter (Walz, Germany) by recording the signal using PowerGraph Professional 3.3. The background level of fluorescence (F_0) of the cells adapted to darkness was determined under illumination with low intensity measuring light (λ = 490 nm, BG39, 64 Hz). The maximum fluorescence level (F_m) was determined during illumination of the samples with flashes at saturation actinic light intensity (≥4500 μmole/(m^2·sec)). Effective efficiency of energy transformation in PS-2 was calculated using the formula: $F_v/F_m = (F_m - F_0)/F_m$ [24, 25].

RESULTS AND DISCUSSION

The scheme of the vector for *C. reinhardtii* transformation is presented in Fig. 3. Since the pSI103 sequence contains several restriction sites for *Eco*911, the site-directed mutagenesis of *psbO* was performed in plasmid p190 and only after that the modified gene was transferred into the vector for transformation. Amino acid substitutions were confirmed by sequencing of the vector DNA. The primary screening of transformants was performed on selective medium with paromomycin (20 μg/ml). Transformation was confirmed by PCR. The cells of all transformed strains were shown to contain an insert of plasmid DNA with the gene of resistance to paromomycin, the transformation marker (Fig. 4). Western blot analysis confirmed PsbO expression in the cells.

The pWT-type cells obtained after transformation of Δ*psbO* cells by the native MSP gene were used as a control. After transformation, the pWT cells completely restored the phenotype corresponding to the wild type. The obtained pWT cells were capable of photosynthetic oxygen evolution and growth under photoautotrophic conditions. The degree of recovery of PS-2 activity estimated by the F_v/F_m ratio (characterizing the efficiency of light energy conversion in PS-2) in the pWT cells was 0.75-0.80, which was also typical of the cells of WT strain (137C+).

K226E mutant. The substitution of glutamic acid for Lys226 produced cells with the phenotype of the Δ*psbO* strain of *C. reinhardtii*. The consequence of the mutation was nearly complete absence of PS-2 photosynthetic activity (table). K226E mutant cells are incapable of O_2 evolution and growth under photoautotrophic conditions. The F_v/F_m value in the light culture is 0.1-0.2, which is typical of Δ*psbO* [17]. Thus, K226E cells are analogous in their functional characteristics to Δ*psbO* lacking PsbO protein. During the incubation of K226E in the dark, the F_v/F_m value increases to 0.25-0.35; however, this is also characteristic of the Δ*psbO* mutant under the same conditions. The expression of PsbO protein in the cells of the K226E mutant was confirmed by immunoblot analysis. As seen from Fig. 5, PsbO protein was detected in the K226E

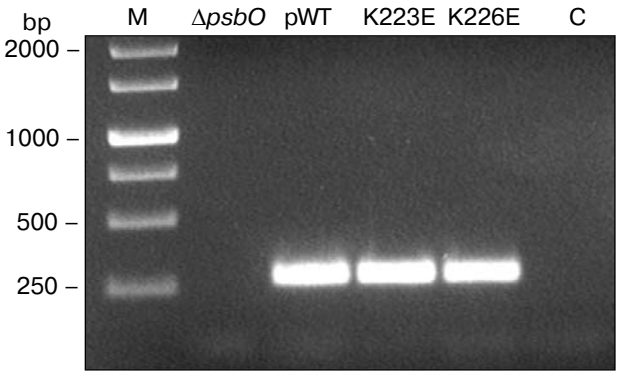

Fig. 4. PCR analysis of genomic DNA of *C. reinhardtii* cells for the presence of the marker gene of paromomycin resistance: aminoglycoside-3′-phosphotransferase from *Streptomyces rimosus*. The pWT-type cells, the cells of Δ*psbO* strain, and the K223E and K226E mutants were used for analysis. The pair of primers (5′-GGGGATTCCCGTACCTCGTGTTG and 3′-CCTCGTCCAGATCCTCCAAGTCG) was used to synthesize the *parR* gene fragment (270 bp). M, molecular weight marker; C, control: PCR without DNA.

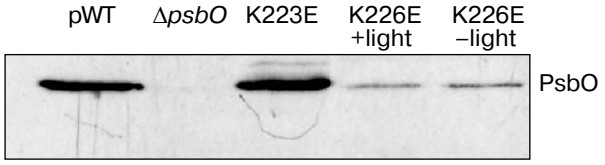

Fig. 5. Immunoblot analysis of PsbO protein content in cells of *C. reinhardtii* pWT-type, Δ*psbO*, K223E, and K226E (light and dark culture) strains grown on TAP medium. Sample quantity for the analysis of pWT-type and K223E cells corresponded to protein content of (1-1.5)·10^6 and (3-4)·10^6 cells for the Δ*psbO* and K226E cells, respectively.

cells, in contrast to the cells of the Δ*psbO* strain used as a control where the protein was not detected. However, protein content analysis shows that the mutant form of MSP K226E in *C. reinhardtii* is unstable and accumulates in the mutant cells at a low concentration (Fig. 5). For protein detection on the gel, it was necessary to increase the quantity of applied sample 3-fold compared to the pWT and K223E cells. It seems that the amino acid substitution in this position destabilizes the protein structure, which results in protein degradation. The fact that disturbance of MSP stability is not a consequence of random mutation was confirmed by sequencing the entire PCR fragment (*Bst*1107I/*Eco*911) and its immediate surroundings. The sequence results confirm the substitution of glutamic acid for lysine in position 226.

The analysis of PsbO structure suggests the cause of changes in the stability of the K226E variant of MSP and development of the Δ*psbO* phenotype. The Lys226 (His231 in cyanobacteria) is located directly before the *C*-terminal β-sheet: K*DIKVTGLWYAQLK (Fig. 2), which is necessary for the binding of MSP to the PS-2 complex as established previously [26]. It is known that the removal of 2-4 amino acids from the carboxyl terminus of MSP blocks its binding to PS-2. It seems that the K226E mutation affects also the structure of this region and influences the interaction between the protein and PS-2, disturbing its binding. In addition, the *C*-terminal region is a structural element of the major MSP domain, β-barrel, important for protein stability. As a consequence, the amino acid substitution in this region of MSP may prevent normal protein folding. As a result, the binding of the protein to PS-2 is disturbed and the protein is degraded. A similar situation is observed in cyanobacterial cells when replacement of a cysteine important for protein stability results in degradation of MSP [27].

The assumption that the observed disturbances in WOC activity are associated with the change in MSP structure and do not result from disturbance of protein function in the work of the PS-2 WOC is confirmed by the decrease in protein content in the dark culture of the K226E strain. If the amount of protein in the cell decreased due to photoinactivation of PS-2, then its content during incubation in the dark would correspond to that in the pWT-type cells. In addition, it is known that MSP synthesis in *C. reinhardtii* is not blocked during

Parameters characterizing functional activity of PS-2 in pseudo wild type (pWT) cells and in cells of K223E, K226E, and Δ*psbO* strains of *C. reinhardtii* measured in exponential culture growth phase at cell density of (0.7-2)·10^6 cells/ml

Strain	F_v/F_m	Photosynthetic O_2 evolution, μmol O_2/h per mg Chl
Light cultures		
pWT	0.79	252
K223E	0.70	210
K226E	0.17	0
Δ*psbO*	0.13	0
Dark cultures		
pWT	0.71	+
K223E	0.61	+
K226E	0.25 (0.33)*	0
Δ*psbO*	0,22 (0.3)*	0

* After weak pre-illumination of 10-20 μmol/(m^2·sec) for 15 min.

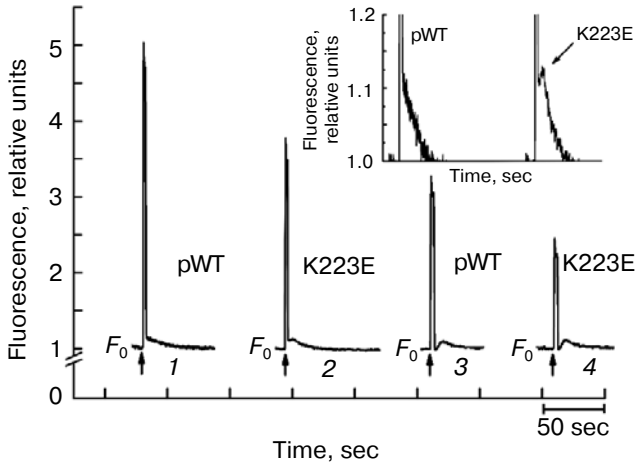

Fig. 6. Kinetics of variable fluorescence (F_v) measured in the pWT-type and K223E *C. reinhardtii* cells grown on TAP medium (*1*, *2* – light cultures; *3*, *4* – dark cultures). Fluorescence intensity corresponding to F_0 was taken as the unit. For F_m determination the cells were illuminated with 1-sec flashes of saturating light at intensity of 4500 μmol/(m²·sec). The inset shows an additional maximum on the curve of dark relaxation of variable fluorescence in cells of dark K223E culture.

degradation of PS-2, in contrast to most other protein constituents of the PS-2 core complex [28].

K223E mutant. The expression of modified MSP in K223E cells was confirmed by Western blot analysis (Fig. 5). As shown in the table, the cells of the K223E mutant are capable of photoautotrophic growth and photosynthetic oxygen evolution. The rate of photosynthetic oxygen evolution in the K223E mutant was 210 μmol O_2/h per mg Chl, i.e. lower by 15-20% than in the pWT-type cells. The addition of artificial electron acceptors to the measuring medium does not change this ratio (data not shown). It should be noted that no appreciable differences have been revealed in the pigment composition of the K226E mutant cells compared to the pWT-type cells.

The analysis of variable fluorescence demonstrates some changes in PS-2 activity in K223E mutant cells. Both light and dark cultures show noticeable decrease in the F_v/F_m ratio (Fig. 6). In the cells of the K223E mutant grown in the light, the efficiency of energy transformation in PS-2 (F_v/F_m) is 0.7 (85% of F_v/F_m for pWT cells (0.8)). After incubation in the dark, this ratio for K223E and pWT decreases to 0.61 and 0.71, respectively.

The cells of *C. reinhardtii* K223E mutant were shown to have changes in the dark relaxation kinetics of variable fluorescence. The slowing of F_v dark decay in the K223E mutant is accompanied by the appearance of a transitional maximum in the area of the slow component of F_v relaxation (inset in Fig. 6). The time of variable fluorescence relaxation to F_0 is 0.4 sec for both cultures, and the difference is noticeable only in the region of the additional maximum. Previously it has been noted [17] that the "additional" maximum in the region of F_v dark relaxation is probably associated with damage to the Mn_4Ca cluster of the WOC. Similar changes can be observed in wild type PS-2 preparations (chloroplasts and cells) where WOC has been damaged by heating [29] or after the treatment with hydroxylamine (data not shown). In addition, it is observed in the absence of MSP, as shown for cells of the Δ*psbO* strain of *C. reinhardtii* [17]. As a result, the F_v/F_m ratio decreases, and an additional peak depending on the degree of WOC damage emerges on the dark decay curve [17, 29]. It should be noted that in this case it is the light culture grown on the medium with acetate. For dark cultures, where Mn cluster and PS-2 reaction center are not fully formed, such kinetics is quite typical of wild type cells as well (Fig. 6).

The assumption that variations in the dark relaxation kinetics of fluorescence of the K223E mutant are associ-

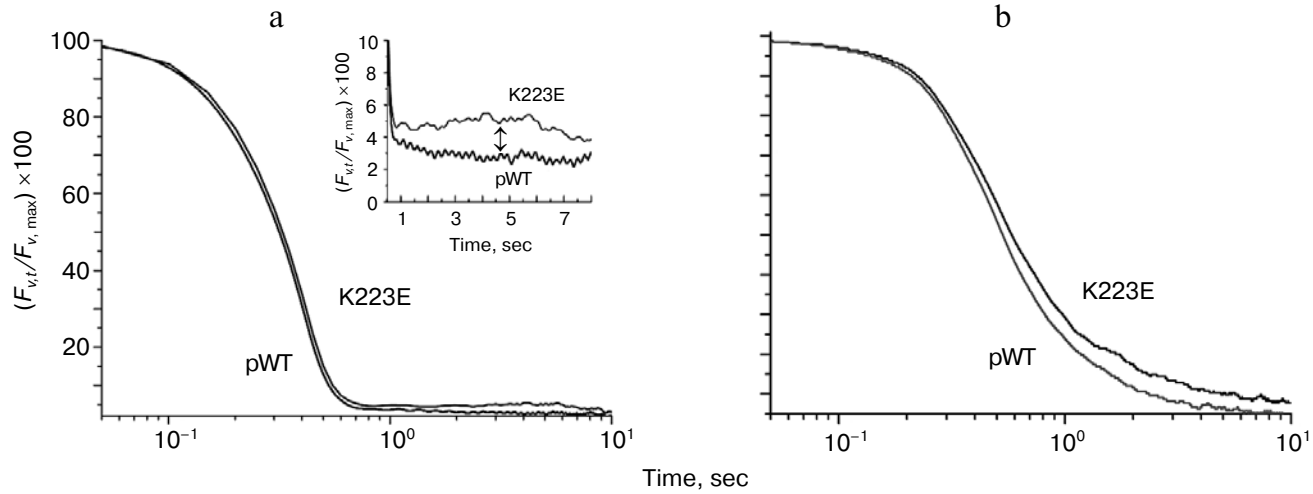

Fig. 7. Dark relaxation kinetics of variable fluorescence (F_v) in cells of *C. reinhardtii* pWT and K223E light cultures after 1-sec flash of actinic light with intensity of 4500 μmol/(m²·sec). The measurements were made in the absence (a) and presence (b) of DCMU (10 μM).

ated with disturbances on the donor side of PS-2 is confirmed by the analysis of fluorescence decay after a 1-sec saturating light flash in the presence of diuron blocking electron transfer between Q_A^- and the plastoquinone pool. The kinetics of F_v relaxation in this case will reflect recombination of charges between reduced Q_A and oxidized components of the donor side of PS-2. In the medium without diuron (Fig. 7), the rates of decay kinetics in the pWT and K223E strains were in fact equal, except for the region with the "additional" maximum (inset), and most of the fluorescence (90-95%) relaxed during the first 400 msec. The time of 50% F_v relaxation was 310 msec. In the presence of diuron, the rate of F_v dark decay slows and the time of reaching F_0 increases. However, the differences between the strains become more evident. In the mutant the decay kinetics slows and the time of relaxation to the 50% fluorescence level is 670 msec (compared to 529 msec for pWT). Especially noticeable differences are observed for the slow relaxation component of "residual" 20-30% of F_v (which reflects Q_A^- relaxation with the S_2-S_3 states of the WOC [30]). Since the rate of dark relaxation in the presence of diuron decreases, the additional maximum is not detected on the F_v dark decay curve.

Thus, the K223E substitution influences the state of the WOC. The mutant is characterized by lower rate of O_2 evolution, decrease in F_v/F_m, and probably increase in the lifetime of the S_2-S_3 states, indicating disturbances in WOC function. The decrease in PS-2 activity may be due to the fact that K223 is the most suitable candidate for Cl^- and/or HCO_3^- binding at the outer side of the channel connecting the lumen with the manganese cluster. The fact that charge substitution does not result in more intense changes in WOC functioning may be associated with the free spatial shift of the loop. As a result of electrostatic repulsion, the loop may shift to the lumen without affecting the access of Cl^- and/or HCO_3^- to the WOC. Another assumption concerning the decrease in WOC activity might be that it changes the conformation of the external part of the channel (it causes minor changes in the location of neighboring amino acids that form the channel). The study of PS-2 preparations isolated from this mutant under regulation of the Ca^{2+}, Cl^-, and HCO_3^- content and pH of the medium will give a more detailed answer to this question.

On the whole, our research has confirmed the importance of amino acids at the lumenal surface of PsbO protein for the activity of the WOC. It has been shown that the K226E substitution produces Δ*psbO*-phenotype (the absence of PS-2 photosynthetic activity). Such a strong effect may be due to the importance of the lysine residue in this position both for the binding to PS-2 and for the stability of the protein. For the second residue, it has been shown that, though K223 has no effect on association of the protein with PS-2 and despite it is located relatively far from the WOC inorganic core, its mutation influences the function of the WOC: the F_v/F_m index and oxygen evolution rate decrease.

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