DOI: 10.1007/s11099-017-0762-8 PHOTOSYNTHETICA 56 (1): 244-253, 2018

Photooxidation and photoreduction of exogenous cytochrome *c* by photosystem II preparations after various modifications of the water-oxidizing complex

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Abstract

The redox interaction of exogenous cytochrome c_{550} (Cyt) with PSII isolated from spinach was studied. Illumination of PSII particles in the presence of Cyt led to: (*1*) Cyt photooxidation by PSII reaction center (demonstrated at the first time), (*2*) Cyt photoreduction *via* $O_2^{-\bullet}$ photoproduced on the acceptor side of PSII, and (*3*) Cyt photoreduction by reduced electron carriers of PSII. A step-by-step removal of components of water-oxidizing complex was accompanied by the appearance of Cyt photooxidation, an increase in the superoxide dismutase (SOD)-dependent Cyt photoreduction (related to $O_2^{-\bullet}$ formation), and a decrease in the SOD-independent Cyt photoreduction. Re-addition of PsbO protein diminished the Cyt-induced restoration of electron transfer in PSII. Addition of diuron led to inhibition of these photoprocesses, while exogenous Mn^{2+} inhibited only the Cyt *c* photooxidation. The results can be important for correct measurements of $O_2^{-\bullet}$ photoproduction in PSII and for elucidation of the role of cytochrome c_{550} in cyanobacterial PSII.

Additional key words: cytochrome *c*; photooxidation; photoreduction; photosystem II; superoxide anion radical.

Introduction

Cytochrome *c* is a small heme-containing protein capable of oxidation and reduction. It plays an important role in a variety of electron transfer processes. Cytochrome c_{550} (Cyt c_{550}) is a necessary structural unit of PSII in cyanobacteria and some eukaryotic algae but not in higher plants. In cyanobacterial PSII, the Cyt c_{550} is one of extrinsic proteins of the water-oxidizing complex (WOC) encoded by the *psbV* gene (Shen and Inoue 1993, Kerfeld and Krogmann1998, Shen *et al*. 1998, Roncel *et al*. 2012).

Photoreduction and photooxidation of exogenous Cyt *c* (isolated from horse heart) by chloroplasts from higher plants were described in a number of publications (Bishop *et al*. 1959, Nieman *et al*. 1959, Nieman and Vennesland 1959, Kok *et al*. 1964, Nelson *et al*. 1972, Asada *et al*. 1974). The photoreduction of Cyt *c* can occur either through electron transfer from the photosynthetic electron transport chain directly to Cyt c_{550} or by superoxide anion radicals ($O_2^{-\bullet}$) formed as a result of the univalent photoreduction of O_2. This conclusion was drawn from the fact that SOD suppressed the photoreduction of Cyt *c* although the SOD-induced inhibition was not complete (it could be from 50 to 80%) (Nelson *et al*. 1972, Asada *et al*. 1974, Khorobrykh and Ivanov 2002). The rate constant for the reaction of $O_2^{-\bullet}$ with ferricytochrome *c* resulting in ferrocytochrome *c* production is rather high (at pH 4.7–6.7 is 1.4 10^6 M^{-1} s^{-1}) (Butler *et al*. 1975), so that Cyt *c* effectively competes with the spontaneous dismutation of $O_2^{-\bullet}$ (Fridovich 1970) but not with its enzyme-induced dismutation (the rate constant determined for Cu, Zn-SOD is 6.4×10^9 M^{-1} s^{-1} (Gray and Carmichael 1992). That is why exogenous Cyt *c* is often used to estimate $O_2^{-\bullet}$ formation in various systems generating superoxide anion radicals. In particular, Cyt *c* is used to determine photoproduction of $O_2^{-\bullet}$ in chloroplasts and PSII preparations (Koppenol *et al*. 1976, Seki *et al*. 1976, Chen *et al*. 1992, Ananyev *et al*. 1994, Yanykin *et al*. 2015).

Received 29 April 2017, *accepted* 5 September 2017, *published as online-first* 3 January 2018.
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Abbreviations: Chl – chlorophyll; Cyt – cytochrome; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; P_{680} – the primary electron donor of PSII; Pheo – pheophytin (the primary electron acceptor of PSII); RC – reaction center**;** SOD – superoxide dismutase; TyrZ – redox active tyrosine residue 161 of D1 protein; WOC – water-oxidizing complex; ΔF – photoinduced changes of chlorophyll fluorescence yield of PSII.
Acknowledgements: The work was supported by the Russian Science Foundation (grant 14-14-00535). We thank T. Smolova for isolation, purification of PsbO protein from pea chloroplast and I. Proskuryakov for EPR measurements.

The photooxidation of ferrocytochrome *c* revealed in the detergent-treated chloroplasts from spinach was attributed to P_{700}^+, the oxidized primary electron donor of PSI (Nieman and Vennesland 1959, Kok *et al*. 1964). In this case, the reduced Cyt *c* can be oxidized directly by P_{700}^+ or *via* copper-containing protein (plastocyanin) involved in electron transfer between the cytochrome *f* of the cytochrome b_6f complex and P_{700}^+. Thus, the following reactions of the light-induced redox transformation of Cyt *c* in chloroplasts can be considered: (*1*) photoreduction by the photosynthetic electron transport chain and by $O_2^{-\bullet}$; (*2*) photooxidation related to the generation of P_{700}^+. Photooxidation of Cyt *c* by PSII was not described in spite of the facts that PSII generates the strongest biological oxidants $P_{680}^{+\bullet}$ (+1.2– +1.4 V) and $TyrZ^{\bullet}$ (+1.1–+1.2 V) (Klimov *et al*. 1979, Rappaport *et al*. 2002, Ishikita *et al*. 2005, Rappaport and Diner 2008, Allakhverdiev *et al*. 2010) and that there is a bound Cyt *c* in cyanobacterial PSII.

The paper is devoted to investigation of the light-induced redox transformations of exogenous Cyt *c* in PSII preparations after various modifications of the WOC.

Materials and methods

Isolation and treatments of PSII preparations: Oxygen-evolving PSII preparations were isolated from spinach leaves according to Ford and Evans (1983). The samples were suspended in the medium containing 50 mM MES-NaOH (pH 6.5), 35 mM NaCl, 0.33 M sucrose, and 10% glycerol and stored at –76°C. The concentration of chlorophyll (Chl) was determined as described previously (Lichtenthaler 1987).

In order to obtain PSII preparations with different degree of disassembly of the WOC, the oxygen-evolving PSII preparations were treated by 1 M NaCl (Miyao and Murata 1983), 1 M $CaCl_2$ (Ono and Inoue 1983) or 5 mM NH_2OH (Tamura and Cheniae 1987). According the literature data, the first treatment resulted in the removal of two extrinsic proteins (PsbP and PsbQ) of the WOC keeping the Mn-cluster intact (Miyao and Murata 1983, Nakatani 1984, Ghanotakis *et al*. 1984). Incubation of the oxygen-evolving PSII preparations in the presence of 1 M $CaCl_2$ led to removal of all the extrinsic proteins (PsbP, PsbQ, and PsbO) of the WOC without the extraction of manganese ions from the WOC suggesting that Mn-cluster was relatively unaffected (Ono and Inoue 1983, Cole *et al.* 1987, Seibert *et al.* 1988). NH_2OH-treatment induced essentially depletion of PsbP and PsbQ proteins and the extraction of manganese ions from the WOC but retained part of PsbO protein (Tamura and Cheniae 1987, Yamamoto and Akasaka 1995, Yamamoto *et al.* 1998) that resulted in a complete inhibition of photosynthetic oxygen evolution and drastic decrease in the yield of photoinduced changes of Chl fluorescence yield (ΔF).

Isolation and purification of PsbO from pea PSII preparations were done as described earlier (Pobeguts *et al*. 2010).

Ratio between oxidized and reduced Cyt *c*: Cyt *c* used in these experiments was purchased from *Sigma Aldrich*. To determine the proportion of Cyt c^{red} and Cyt c^{ox} in the reagent the batch of the Cyt *c* was dissolved in the buffer medium containing 50 mM Mes-NaOH (pH 6.5) and 35 mM NaCl (buffer medium A). The freshly prepared solution of the Cyt *c* was divided into two parts and placed into the sample and reference cells. After baseline correction, some amount of dithionite was added to the sample cuvette and differential absorption spectrum (spectrophotometer *Agilent 8453,* USA)(500–650 nm) was recorded. The absorbance change at 550 nm (ΔA_{550}) corresponded to the part of Cyt c^{ox} in the initial sample. The value of ΔA_{550} obtained after subsequent addition of ferricyanide to the reference cuvette showed the total amount of Cyt *c*. The difference between the two values of ΔA_{550} indicated the proportion of Cyt c^{red} in the sample (Fig. 1S, *supplement available online*). The measurement showed that the content of Cyt c^{ox} in the fresh prepared solution was about 98%.

In order to obtain reduced Cyt *c*, the oxidized Cyt *c* was dissolved in the buffer medium A and reduced by the addition of dithionite. To separate the Cyt *c* from dithionite the solution of Cyt *c* was applied to *Sephadex G25* column (2.5 × 40 cm) and eluted with the same buffer. The addition of methyl viologen to the solution of Cyt *c* passed through the column did not reveal the presence of dithionite. Percentage of Cyt c^{red} after this procedure was about 99%.

Photooxidation and photoreduction of Cyt *c*: Kinetics of photoinduced absorbance changes at 550 nm related to both oxidation and reduction of Cyt *c* induced by illumination of PSII preparations with red light [λ > 600 nm, 3,000 μmol(photon) m^{-2} s^{-1}] were measured in a 10-mm cuvette at 20°C using a spectrophotometer *Agilent 8453* (USA). The rate of photooxidation and photoreduction of Cyt *c* was calculated by monitoring the concentration of oxidized (Cyt c^{ox}) or reduced (Cyt c^{red}) Cyt *c* for 30 s after the start of illumination of PSII preparations. The amount of Cyt c^{ox} or Cyt c^{red} was calculated by using the differential extinction coefficient between ferrocytochrome *c* and ferricytochrome *c* at 550 nm (21.1 mM^{-1}). Dark incubation of Cyt *c* in the presence of the PSII preparations led to neither the reduction of Cyt *c* nor its oxidation.

Electron paramagnetic resonance (EPR) and Chl fluorescence: The content of PSI in the PSII preparations was determined by EPR spectroscopy. The EPR signals of the PSII preparations were measured by *EMX-6 ESR*

spectrometer (*Bruker*, Germany) using a flat cell in a standard TM102 cavity at room temperature. EPR measurements were done in the dark and during illumination of the samples by strong red light [λ > 600 nm, 15,000 μmol (photon) m^{-2} s^{-1}]. EPR parameters were: microwave frequency = 9.74 GHz, microwave power = 2mW, modulation amplitude = 4 G. The EPR spectra consisted of signals related to $Y_D^{\bullet}$ and $P_{700}^{+\bullet}$. In order to calculate of $P_{700}^{+\bullet}$ amount, the EPR spectrum recorded in the dark was subtracted from the spectrum measured during illumination of the samples. The concentration of $P_{700}^{+\bullet}$ was quantified from this difference spectrum (spin probe at concentration of 10^{-5} M was used as a calibration). The measurements showed that the ratio of PSI to PSII in the PSII preparations was approximately 1:9.

Kinetics of photoinduced ΔF were measured in a 10-mm cuvette at room temperature using a *MULTI-COLOR PAM* fluorometer (*Waltz*, Germany).

Results

Kinetics of photoreduction and photooxidation of Cyt *c* by NH_2OH-treated PSII preparations at different ratio of oxidized Cyt c^{ox} to its reduced form, Cyt c^{red}, are shown in Fig. 1*A*. The positive and negative ΔA_{550} corresponded to photoreduction of Cyt c^{ox} and photooxidation of Cyt c^{red}, respectively. When the ratio of concentrations (μM) between Cyt c^{ox} and Cyt c^{red} was 9.8/0.2 (curve 1) the illumination of the samples induced fast reduction of Cyt c^{ox} in the first 10 s, then the rate of Cyt c^{ox} photoreduction gradually slowed down. The concentration of photoreduced Cyt *c* after a 90-s illumination of the samples was equal to 1.85 μM. With change of the Cyt c^{ox}/Cyt c^{red} ratio towards the increase in concentration of Cyt c^{red}, the rate of photoproduction of Cyt c^{red} decreased (curves 2 and 3). If the Cyt c^{ox}/Cyt c^{red} ratio became equal to 5/5, no photoinduced change at 550 nm was observed (curve 4) (it may indicate that at this ratio the photoreduction and photooxidation of Cyt *c* occur with equal efficiency). The shift of the Cyt c^{ox}/Cyt c^{red} ratio in favor of Cyt c^{red} led to the appearance of negative values of the photoinduced ΔA_{550} showing that the photooxidation of Cyt *c* became dominant (curves 5 and 6).

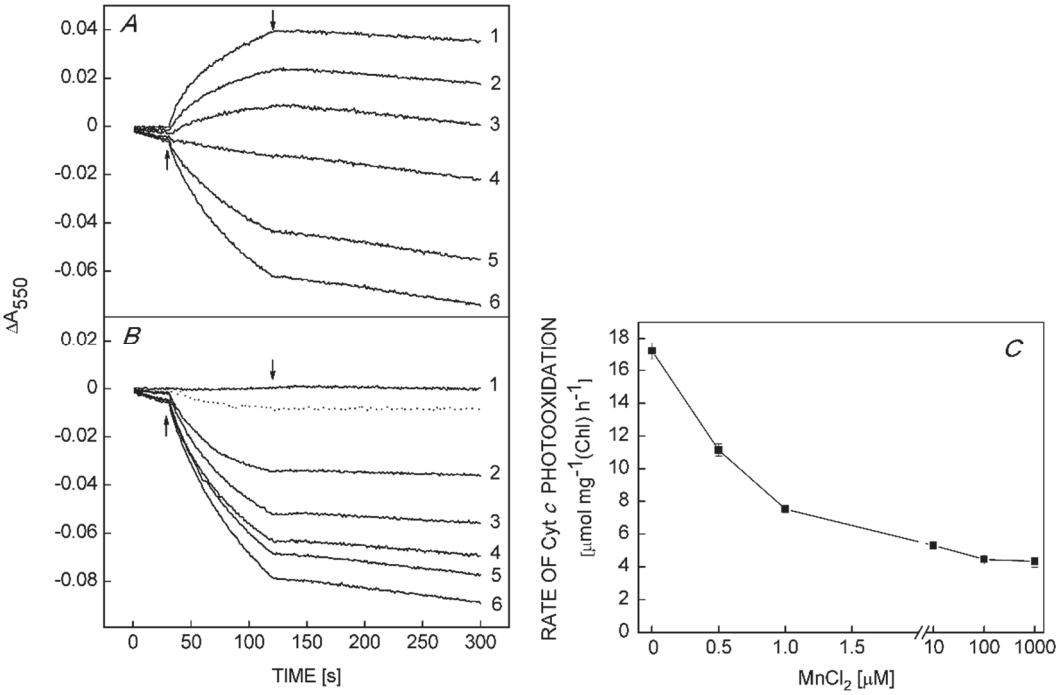

Fig. 1. (*A*,*B*) Kinetics of light-induced redox conversions of exogenous cytochrome *c* by NH_2OH-treated PSII preparations at different ratio of oxidized cytochrome *c* (Cyt c^{ox}) to its reduced form (Cyt c^{red}). The ratio of concentrations (μM) between Cyt c^{ox} and Cyt c^{red} before illumination of the samples was as follows: 9.8/0.2 (*curve 1*), 8/2 (*curve 2*), 6/4 (*curve 3*), 5/5 (*curve 4*), 2/8 (*curve 5*), and 0.1/9.9 (*curve 6*). (*C*) Dependence of the rate of Cyt *c* photooxidation by NH_2OH-treated PSII preparations on concentration of $MnCl_2$. Total concentration of Cyt *c* was 10 μM at Cyt c^{red}/Cyt c^{ox} ratio equal to 9.9/0.1. Reaction medium contained 50 mM MES-NaOH (pH 6.5), 35 mM NaCl and NH_2OH-treated PSII preparations at chlorophyll concentration of 10 μg ml^{-1}. The measurements were done in the absence (*A*,*C*) and in the presence (*B*) of SOD (50 U ml^{-1}). Up and down *arrows* indicate light [λ > 600 nm, 3,000 μmol(photon) m^{-2} s^{-1}] on and off, respectively. The data are the means of three measurements and the standard error of the mean was within 5%.

Table 1. The effect various additions on the rate of cytochrome *c* (Cyt *c*) photooxidation or photoreduction by PSII preparations at 10 μg(Chl) ml^{-1}. The illumination with the light [λ > 600 nm, 3,000 μmol(photon) m^{-2} s^{-1}] of the samples was carried out in the medium containing 50 mM Mes–NaOH (pH 6.5), 35 mM NaCl, and 10 μM Cyt *c*. The ratio between reduced and oxidized Cyt *c* was 9.9/0.1 or 0.2/9.8 when measuring the rate of photooxidation or photoreduction of Cyt *c*, respectively. The rate was measured for 30 s (10 s for the value in parentheses) after the start of illumination of the PSII preparations. Activity of added SOD and catalase was 50 U ml^{-1} and 200 U ml^{-1}, respectively. The data are the means of three measurements and the standard error of the mean was within 5%. *– The minus sign indicates photooxidation of Cyt *c*. This was due to the presence of reduced form of Cyt *c* in the solution of oxidized Cyt *c*.

Rate of Cyt *c* photooxidation [μmol mg^{-1}(Chl) h^{-1}]				
Additions	Untreated PSII	NaCl-treated PSII	$CaCl_2$-treated PSII	NH_2OH-treated PSII
no	1.35 ± 0.05	2.54 ± 0.1	3.7 ± 0.1	16.4 ± 0.3
SOD	1.81 ± 0.06	3.27 ± 0.09	4.5 ± 0.12	20.7 ± 0.9
20 μM DCMU	2.6 ± 0.05	4.7 ± 0.11	5.3 ± 0.16	4.38 ± 0.14
SOD and 20 μM DCMU	2.6 ± 0.06	4.7 ± 0.08	6.2 ± 0.3	4.38 ± 0.12
SOD plus catalase				20 ± 0.9
10 μM $MnCl_2$				5.3 ± 0.25
Rate of Cyt *c* photoreduction [μmol mg^{-1}(Chl) h^{-1}]				
Additions	Untreated PSII	NaCl-treated PSII	$CaCl_2$-treated PSII	NH_2OH-treated PSII
no	6.72 ± 0.31	7.98 ± 0.23	8.75 ± 0.31	12.5 (19.6) ± 0.5
SOD	3.4 ± 0.14	3.2 ± 0.12	2.45 ± 0.1	0.77 ± 0.04
20 μM DCMU	1.42 ± 0.07	0.92 ± 0.02	0.87 ± 0.04	0.43 ± 0.02
SOD and 20 μM DCMU	–0.21* ± 0.01	–0.6* ± 0.03	–0.63* ± 0.02	–0.62* ± 0.02

The addition of SOD (50 U ml^{-1}) drastically changed the kinetics of Cyt *c* photoconversion: (*1*) a complete disappearance of the Cyt c^{ox} photoreduction at the ratio of Cyt c^{ox}/Cyt c^{red} equal to 9.8/0.2 (Fig. 1*B*, curve 1); (*2*) when Cyt c^{ox}/Cyt c^{red} ratios were 8/2 or 6/4 (Fig. 1*A,B*, curves 2,3) or even 9.5/0.5 (Fig. 1*B*, dot curve), the photoreduction of Cyt *c* was replaced by its photooxidation; (*3*) at equal Cyt c^{ox}/Cyt c^{red} ratio only the Cyt c^{ox} photoreduction was observed; (*4*) the enhancement of Cyt *c* photooxidation at Cyt c^{ox}/Cyt c^{red} ratio equal to 2/8 and 0.1/9.9 (Fig. 1*B,* curves 5,6). These SOD-induced effects may indicate that SOD suppresses the redox interaction of Cyt c^{ox} with $O_2^{-\bullet}$ photoproduced on the acceptor side of PSII. Injection of catalase jointly with SOD had no additional effect on the Cyt c^{red} photooxidation (Table 1). This can indicate that H_2O_2 produced as a result of the enzyme-catalyzed dismutation of $O_2^{-\bullet}$ is not involved in the Cyt c^{red} photooxidation in the PSII preparations [although, as has been shown by Vandewalle and Petersen (1987); exogenous H_2O_2 can oxidize Cyt c^{red} at rates comparable to those at which Cyt c^{ox} c is reduced by superoxide].

In order to elucidate the efficiency of the redox interaction of Cyt c^{red} on the donor side of the NH_2OH-treated PSII preparations, we investigated the effect of added Mn^{2+} (which is an efficient and specific electron donor for PSII) on the rate of photooxidation of Cyt c^{red} (Fig. 1*C*). In contrast to Mn^{2+}, other artificial electron donors for PSII (such as diphenylcarbazide, ferrocyanide, NH_2OH) reduced Cyt c^{ox} in the darkness (Fig. 2S, *supplement available online*). As their redox interaction with oxidized Cyt *c* would complicate the interpretation of the obtained results, we did not use them in further experiments. As it can be seen in Fig. 1*C*, the rate of Cyt c^{red} photooxidation by NH_2OH-treated PSII preparations was considerably suppressed with the increase of concentration of added Mn^{2+}, so that the addition of 1 μM $MnCl_2$ resulted in a 60% inhibition of the rate of Cyt c^{red} photooxidation. Although the Mn^{2+}-induced suppression of Cyt *c* photooxidation was significant it was not possible to achieve a complete inhibition of the Cyt *c* photooxidation by Mn^{2+}. Even at a high (1 mM) concentration of added Mn^{2+}, the rate of Cyt *c* photooxidation was nearly 25% of that measured in the absence of Mn^{2+}. The Mn^{2+}-induced suppression of Cyt *c* photooxidation indicates that Mn^{2+} effectively competed with Cyt c^{red} for the site of photooxidation and that at least 75% of Cyt c^{red} photooxidation occurred on the donor side of PSII.

The conclusion on photooxidation of Cyt c^{red} on the donor side of PSII was confirmed by the data presented in Fig. 2. According to previous publications (Klimov *et al*. 1982, 1995), removal of manganese from the WOC results in a drastic decrease of photoinduced ΔF due to the loss of electron donation from the Mn-cluster to the reaction center of PSII. Addition of artificial electron donors for PSII leads to restoration of photoinduced ΔF. As it can be seen in Fig. 2*A*, the addition of either Mn^{2+} or Cyt c^{red} to NH_2OH-treated PSII preparations led to the reactivation of photoinduced ΔF. The capability of Cyt c^{red} to reactivate the photoinduced ΔF in the NH_2OH-treated PSII preparations was lower in comparison with exogenous Mn^{2+} [0.6 μM Mn^{2+}or 6 μM Cyt c^{red} was required to achieve a 50%-reactivation of ΔF (Fig. 2*B*)].

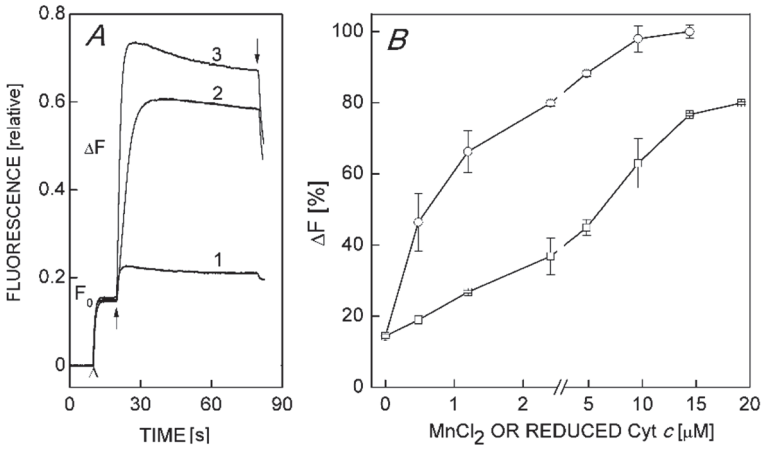

Fig. 2. Photoinduced changes of chlorophyll fluorescence yield (ΔF) related to photoreduction of the primary electron acceptor, Q_A, in NH_2OH-treated PSII preparations. (*A*) Kinetics of ΔF in the absence of additions (*1*) and in the presence of 19.2 μM reduced Cyt *c* (Cyt c^{red}) (*2*) and 9.2 μM $MnCl_2$ (*3*). (*B*) Dependence of ΔF amplitude on concentration of Cyt c^{red} (□) and $MnCl_2$ (○). The measurements were done in the medium containing 50 mM MES-NaOH (pH 6.5) and 35 mM NaCl at a concentration of 10 μg(Chl) ml^{-1}. The amplitude of ΔF measured in the NH_2OH-treated PSII preparations in the presence of 14.4 μM $MnCl_2$ was taken as 100%. Δ – switching the measuring light on; F_0 – the level of fluorescence induced by the measuring light; ↑ and ↓ – steady-state actinic light [λ = 625 nm; 1,000 μmol(photon) m^{-2} s^{-1}] on and off, respectively. Averages with standard error of 3–5 measurements are shown in panel *B*.

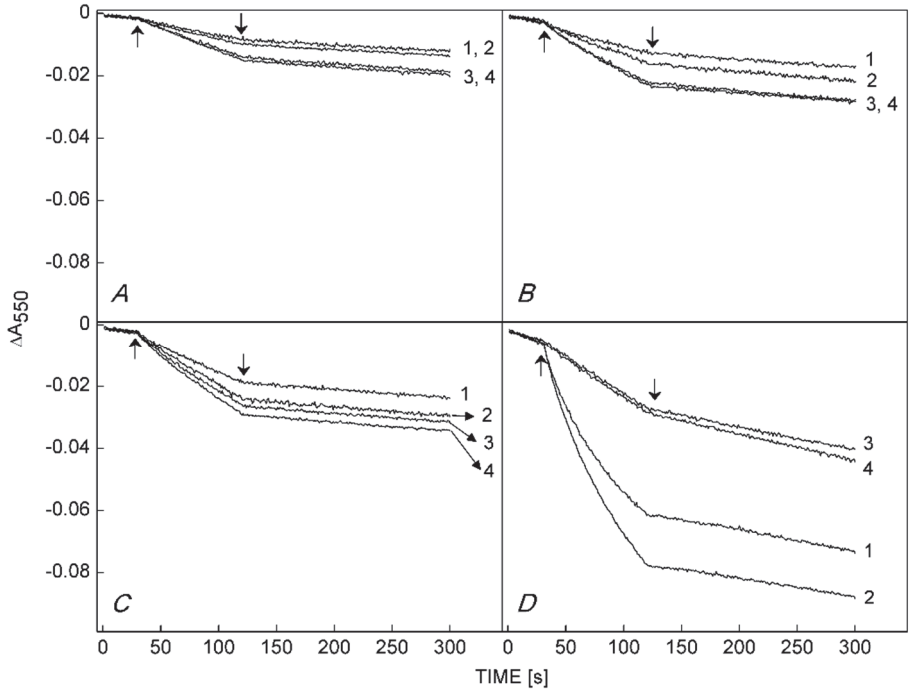

Fig. 3. Kinetics of cytochrome *c* photooxidation by PSII preparations before (*A*) and after modification of the water-oxidizing complex caused by treatments with NaCl (*B*), $CaCl_2$ (*C*), and NH_2OH (*D*). The measurements were done in the absence of additions (*1*) and after the addition of SOD (*2*), 20 μM DCMU (*3*), SOD added jointly with 20 μM DCMU (*4*). Total concentration of Cyt *c* was 10 μM at Cyt c^{red}/Cyt c^{ox} ratio equal to 9.9/0.1. Reaction medium contained 50 mM MES-NaOH (pH 6.5), 35 mM NaCl, and PSII preparations at concentration of 10 μg(Chl) ml^{-1}. Activity of added SOD was 50 U ml^{-1}. Up and down *arrows* indicate light [λ > 600 nm, 3,000 μmol(photon) m^{-2} s^{-1}] on and off, respectively.

The treatment of PSII preparations with 5 mM NH_2OH leading to the removal of manganese ions from the WOC can facilitate the redox interaction of exogenous electron donors with $P_{680}^{+\cdot}$ or $TyrZ^{\cdot}$. Therefore, we investigated the photooxidation and photoreduction of Cyt *c* in the oxygen-evolving PSII preparations (untreated PSII preparations) and after their treatment by either 1 M NaCl or 1 M $CaCl_2$ leading to different degrees of modification of the WOC

(for details see Materials and methods). In untreated PSII preparations, the rate of Cyt c^{red} photooxidation was significantly lower than that in NH_2OH-treated PSII preparations: 1.4 and 16.5 μmol(Cyt *c*) mg^{-1}(Chl) h^{-1}, respectively (Fig. 3*A*,*D*, curves 1). The Cyt c^{red} photooxidation slightly increased after the removal of extrinsic proteins of the WOC, so that its rate in NaCl-and $CaCl_2$-treated PSII preparations was 2.5 and 3.7 μmol(Cyt *c*) mg^{-1}(Chl) h^{-1}, respectively (Figs. 3*B*,*C*, curves 1; Table 1). The addition of SOD (curves 2) had no effect on the kinetic of the Cyt c^{red} photooxidation by untreated PSII preparations and it induced an enhancement of the Cyt c^{red} photooxidation in NaCl-, $CaCl_2$-, and (especially) in NH_2OH-treated PSII preparations. The addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) resulted in a 75%-inhibition of the Cyt c^{red} photooxidation in NH_2OH-treated preparations (Fig. 3*D*, curve 3), while in all other samples DCMU did not inhibited the Cyt c^{red} photooxidation (Fig. 3*A–C*, curves 3). The effect of joint addition of SOD and DCMU on the Cyt c^{red} photooxidation in the samples was similar to that obtained for DCMU alone. The rates of Cyt c^{red} photooxidation in these PSII preparations both in the absence and in the presence of additions are presented in Table 1.

The Cyt c^{red} photooxidation significantly increased after the destruction of Mn cluster accompanied by the release of manganese from the WOC that may be associated with the increase in accessibility of Cyt c^{red} to the oxidation site (Fig. 3). Despite the fact that PsbO protein remained after the treatment of PSII preparations by 5 mM NH_2OH, the rate of Cyt c^{red} photooxidation was 4.5 times higher than that in $CaCl_2$-treated PSII preparation. This can indicate that the main barrier for the Cyt c^{red} photooxidation on the donor side of PSII is the presence of the Mn cluster. Nevertheless, we tested the effect of PsbO protein added on the Cyt c^{red} photooxidation in the NH_2OH-treated PSII preparations. The capability of Cyt c^{red} to reactivate the photoinduced ΔF in the NH_2OH-treated PSII preparations remarkably decreased after a 1-h incubation of the PSII preparations at room temperature in the presence of exogenous PsbO (0.5 μM that corresponded to ten molecules per one PSII reaction center) in comparison with the samples incubated in the absence of this protein (the incubation procedure was required to facilitate the binding of PsbO protein to the donor side of PSII) (Fig. 5). The effect of PsbO was not observed if the measurements of photoinduced ΔF were done without preliminary incubation of the samples. The data testify that PsbO protein retards the redox interaction of exogenous Cyt c^{red} with the donor side of PSII that is manifested in a suppression of the Cyt c^{red}-induced reactivation of the photoinduced ΔF. The incubation of the NH_2OH-treated PSII preparations in the presence of bovine serum albumin (BSA) (added at the same concentration as PsbO) induced the effect observed after the incubation of the PSII preparations with PsbO protein that revealed a specificity of the PsbO action. A better restoration of photoinduced ΔF by Cyt *c* observed after incubation of the samples in the presence of BSA (Fig. 4) may be related to protective

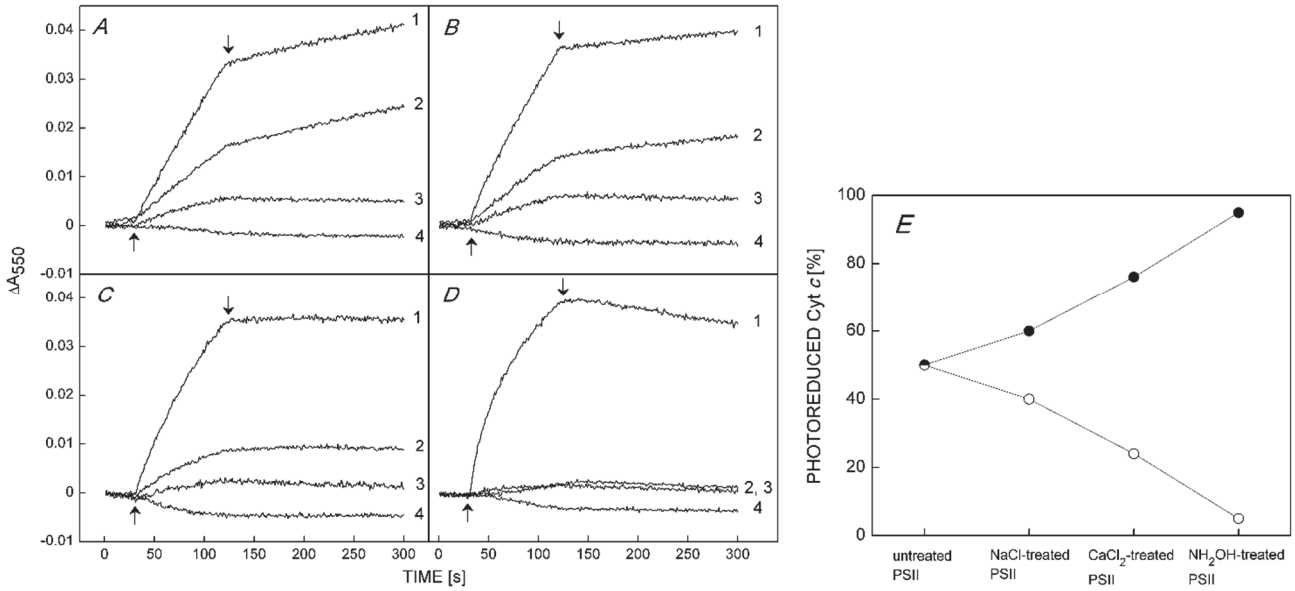

Fig. 4. Kinetics of Cyt c^{ox} photoreduction by PSII preparations before (*A*) and after modification of the water-oxidizing complex caused by treatments of NaCl (*B*), $CaCl_2$ (*C*), and NH_2OH (*D*). The measurements were done in the absence of additions (*1*) and after the addition of SOD (*2*), 20 μM DCMU (*3*), SOD added jointly with 20 μM DCMU (*4*). Total concentration of added Cyt *c* was 10 μM at Cyt c^{red}/Cyt c^{ox} ratio equal to 0.2/9.8. Reaction medium contained 50 mM MES-NaOH (pH 6.5), 35 mM NaCl, and PSII preparations at a concentration of 10 μg(Chl) ml^{-1}. Activity of added SOD was 50 U ml^{-1}. Up and down arrows indicate light [λ > 600 nm, 3,000 μmol(photon) m^{-2} s^{-1}] on and off, respectively. (*E*) Changes of Cyt c^{ox} photoreduction directly by the reduced electron acceptors of PSII (○) and by $O_2^{-\bullet}$ (●) upon the various destruction of the WOC. The amount of Cyt *c* reduced as a result of the sum of these two photoreactions is taken as 100%. The data are the means of three measurements and the standard error of the mean was within 5%.

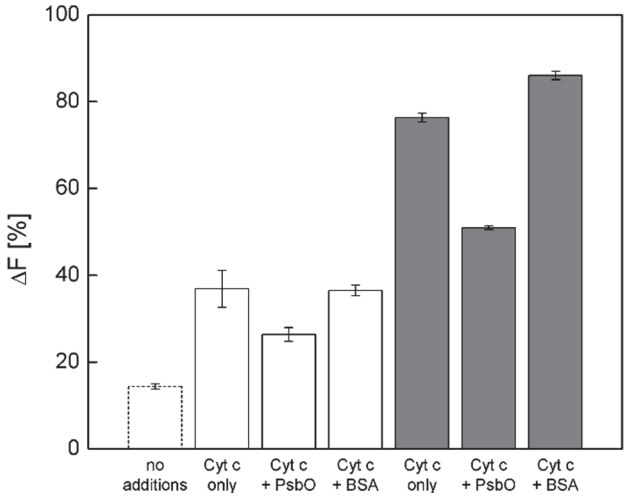

Fig. 5. Effect of PsbO and BSA on the amplitude of photoinduced ΔF measured in NH_2OH-treated PSII preparations in the presence of reduced Cyt *c* (Cyt c^{red}). Before the addition of Cyt c^{red} the preparations were incubated for 1 h in the medium containing 50 mM MES-NaOH (pH 6.5) and 35 mM NaCl at a concentration of 200 μg(Chl) ml^{-1} in the absence (*dashed column*) and in the presence (*other columns*) of either ten molecules of PsbO per one PSII reaction center or ten molecules of BSA per one PSII reaction center. The measurements were done in the medium containing 50 mM MES (pH 6.5), 35 mM NaCl, in the presence of 2.4 μM Cyt c^{red} (*white columns*) and 19.2 μM Cyt c^{red} (*gray columns*) at a concentration of 10 μg(Chl) ml^{-1}. The amplitude of ΔF measured in the NH_2OH-treated PSII preparations in the presence of 14.4 μM $MnCl_2$ was taken as 100% (*see* Fig. 2). Averages with standard error of 3–5 measurements are shown.

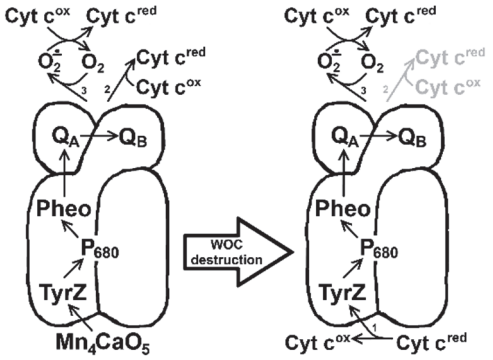

Fig. 6. A proposed scheme of possible ways of photooxidation and photoreduction of exogenous Cyt *c* by PSII preparations before and after removal of the WOC. *Arrows* indicate the routes of electron transfer. *Numbers* indicate the following redox transformations of Cyt *c*: (*1*) photooxidation of reduced Cyt *c* directly by PSII reaction center (rather than through Mn-containing cluster) which appears after the removal of the WOC, (*2*) photoreduction of oxidized Cyt *c* directly by reduced electron carriers of PSII, which is drastically decreased after the WOC destruction, and (*3*) photoreduction of oxidized Cyt *c via* superoxide anion radical photoproduced on the acceptor side of PSII (which is significantly increased after the WOC disassembly).

effect of BSA against inactivation which could take place during the 1-h incubation of the samples at room temperature.

Photoreduction of Cyt c^{ox} in untreated PSII preparations as well as in NaCl- and $CaCl_2$-treated PSII preparations (Fig. 4*A–C*, curves1) occurred at a similar rate. The NH_2OH-treated PSII preparations (Fig. 5*D*, curve 1) showed a much higher rate of Cyt c^{ox} photoreduction in comparison with other samples (especially, during the first 10 s of illumination). The SOD added to the samples suppressed the Cyt *c* photoreduction and its inhibitory effect depended on the degree of damage of the WOC (curves 2). Suppression of the Cyt c^{ox} photoreduction with SOD was equal to 50, 60, 76, and 95% in untreated, NaCl-treated, $CaCl_2$-treated, and NH_2OH-treated PSII preparations, respectively. These data indicate that the proportion of Cyt *c* reduced by $O_2^{-\bullet}$ photoproduced on the acceptor side of PSII increased with the increase in the degree of the WOC destruction. At the same time, Cyt c^{ox} photoreduction by the reduced electron carriers on the acceptor side of PSII decreased with the increase of disassembly of the WOC so that it became negligible in PSII deprived of the WOC (Fig. 4*E*). The addition of 20 μM DCMU led to a significant inhibition of the Cyt *c* photoreduction in all the samples (Fig. 4). The rates of Cyt c^{ox} photoreduction by PSII preparations are also presented in Table 1.

Discussion

The results demonstrate that PSII is capable of both photooxidation and photoreduction of exogenous Cyt *c* in PSII preparations with removed WOC. The relationship between these two photoprocesses is shown in Fig. 1*A*,*B*. The photoreduction of Cyt c^{ox} in the NH_2OH-treated PSII preparations occurs mainly *via* $O_2^{-\bullet}$ photoproduced on the acceptor side of PSII since it is almost completely inhibited by SOD or DCMU (Figs. 1*B*, curve 1 and 4*D*, curves 2, 3). The photooxidation of Cyt c^{red} can be manifested even at insignificant amount of Cyt c^{red} (when the ratio Cyt c^{red}/Cyt c^{ox} is 0.5/9.5) if the superoxide-induced photoreduction of Cyt c^{ox} is inhibited by SOD.

Photooxidation of Cyt *c*: At least 75% of the Cyt c^{red} photooxidation in the PSII preparations occurs on the donor side of PSII. This follows from the data showing that the addition of Mn^{2+} [which is more effective electron donor to PSII than Cyt c^{red} (Fig.2*B*)] suppresses the Cyt c^{red} photooxidation. It should be noted that the Cyt c^{red} photooxidation on the donor side of PSII is efficient only after a complete removal of manganese from the WOC since other treatments disturbing the WOC due to a partial or complete extraction of the extrinsic proteins without removal of Mn (treatments with NaCl or $CaCl_2$) did not facilitate this photoreaction on the donor side of PSII. This

may indicate that the Mn-cluster within the WOC as well as the extrinsic proteins create a barrier for the redox interaction between the reduced form of Cyt *c* with $P_{680}^{+\bullet}$ or $TyrZ^{\bullet}$. That is why the rebinding of PsbO protein to the donor side of PSII diminished the restoration of photoinduced ΔF by Cyt c^{red} in the NH_2OH-treated PSII preparations (Fig. 5).

As mentioned above, the addition of either Mn^{2+} or DCMU did not completely suppress the Cyt c^{red} photooxidation by the NH_2OH-treated PSII preparations that may suggest the existence of an alternative mechanism for the Cyt c^{red} photooxidation, not related to $P_{680}^{+\bullet}$ or $TyrZ^{\bullet}$. The Cyt c^{red} photooxidation not suppressed by Mn^{2+} or DCMU may be related to to P_{700}^{+}. [The PSII preparations contained a fraction of PSI and the ratio of PSI to PSII was 1:9, besides, it was shown that Cyt c^{red} photooxidation in detergent-treated chloroplasts was associated with the redox activity of P_{700}^{+} (Nieman and Vennesland 1959, Kok *et al*. 1964)]. DCMU-induced inhibition of the Cyt c^{red} photooxidation in NH_2OH-treated PSII preparations can be attributed to the decrease of the photogeneration of the long-lived states of $P_{680}^{+\bullet}$ or $TyrZ^{\bullet}$ involved in the Cyt c^{red} oxidation. Thus**,** it can be summarized that the site of Cyt c^{red} photooxidation in PSII is located at its donor side and that the photooxidation of Cyt c^{red} takes place only after the damage of the WOC accompanied by the manganese removal that evidently opens access for redox interaction of Cyt *c* with $P_{680}^{+\bullet}$ or $TyrZ^{\bullet}$.

Photoreduction of Cyt *c*: As was shown earlier (Nelson *et al*. 1972, Asada *et al*. 1974), the photoreduction of Cyt *c* in chloroplasts can occur either through electron transfer from the photosynthetic electron transport chain directly to Cyt c_{550} or by superoxide anion radicals ($O_2^{-\bullet}$) formed as a result of the univalent reduction of O_2. In our study of the Cyt c^{ox} photoreduction in PSII preparations after various damage to the WOC, we found that the Cyt c^{ox} photoreduction probably related to the direct redox interaction of Cyt c^{ox} with the electron transport chain of PSII decreased upon the increase in destruction of the WOC while the Cyt c^{ox} photoreduction by $O_2^{-\bullet}$ was activated despite of the loss of electron donation from the WOC (Fig. 4*E*). The suppression of direct reduction of Cyt c^{ox} by PSII induced by the step by step disassembly of the WOC can occur due to inhibition of the electron transfer on the donor side of PSII or due to changes on the acceptor side leading to an enhancement in electron transfer to O_2 which competes with the direct redox interaction of Cyt c^{ox} with the reduced components of PSII acceptor side.

It was reported that the midpoint redox potential (E_m) for Q_A/Q_A^- in intact PSII preparations isolated from spinach had values of −84 mV (Krieger *et al*. 1995), about –162 mV (Shibamoto *et al*. 2010) or −146 mV (Allakhverdiev *et al*. 2011). The discrepancy of the values is attributed to the removal of bicarbonate from PSII during the measurement procedure of E_m (Q_A/Q_A^-) (Brinkert *et al*. 2016). When the removal of inorganic core of the WOC occurred, the E_m (Q_A/Q_A^-) was shifted approximately 150 mV towards positive values (Krieger and Weis 1992, Johnson *et al*. 1995, Allakhverdiev *et al*. 2011), indicating that destruction of the WOC severely affected the potential of Q_A. The shift E_m (Q_A/Q_A^-) towards the positive values is disadvantageous for O_2 reduction since E_0 for the pair O_2/O_2^- is –160 mV (Wood 1974). The manganese extraction from the WOC causes also the transition of the high-potential form of Cyt b_{559} (E_m = 400 mV, pH 7) to its low-potential form (E_m = 40–80 mV, pH 7) (Mizusawa *et al*. 1997) which can be oxidized by O_2 (Kruk and Strzałka 2001, Pospíšil *et al*. 2006). One can suggest that the increase in the fraction of the low-potential form of Cyt b_{559} contributes to the enhancement of $O_2^{-\bullet}$ photoproduction in the $CaCl_2$- and NH_2OH-treated PSII preparations. Besides, the increase in photoformation of $O_2^{-\bullet}$ by the treatments leading to disassembly of the WOC can be related to inhibition of the intrinsic SOD activity which probably comprises the HP-form of Cyt b_{559} (Ananyev *et al*. 1994).

Possible pathways of redox conversion of exogenous Cyt *c* in isolated PSII can be summarized by the following reactions (Fig. 6): (*1*) photooxidation of Cyt c^{red} on the donor side of PSII (which depends on the accessibility of the cytochrome to $P_{680}^{+\cdot}$ or to $TyrZ^{\cdot}$), (*2*) photoreduction of Cyt c^{ox} directly by reduced electron acceptors of PSII; (*3*) photoreduction of Cyt c^{ox} by $O_2^{-\bullet}$ produced on the acceptor side of PSII (which dominates after the treatments leading to the WOC destruction).

Thus, the paper demonstrated for the first time the conditions for revealing the photooxidation of exogenous Cyt *c* by isolated PSII. The results can be important for investigation of the role of PsbV protein in the functioning of cyanobacterial PSII. The results can be also important for investigation of the interrelation between reactions of photooxidation and photoreduction of Cyt *c* by PSII as well as for correct measurements of photoformation of $O_2^{-\bullet}$ in PSII preparations.

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