

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* O₂^{•−} 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 O₂^{•−} 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 Mn²⁺ inhibited only the Cyt *c* photooxidation. The results can be important for correct measurements of O₂^{•−} 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 Krogmann 1998, 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 (O₂^{•−}) formed as a result of the univalent

photoreduction of O₂. 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 O₂^{•−} with ferricytochrome *c* resulting in ferrocyclochrome *c* production is rather high (at pH 4.7–6.7 is 1.4 · 10⁶ M^{−1} s^{−1}) (Butler *et al.* 1975), so that Cyt *c* effectively competes with the spontaneous dismutation of O₂^{•−} (Fridovich 1970) but not with its enzyme-induced dismutation (the rate constant determined for Cu, Zn-SOD is 6.4 · 10⁹ M^{−1} s^{−1} (Gray and Carmichael 1992). That is why exogenous Cyt *c* is often used to estimate O₂^{•−} formation in various systems generating superoxide anion radicals. In particular, Cyt *c* is used to determine photoproduction of O₂^{•−} 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).

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Abbreviations: Chl – chlorophyll; Cyt – cytochrome; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; P₆₈₀ – 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.

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The photooxidation of ferrocyanochrome *c* revealed in the detergent-treated chloroplasts from spinach was attributed to P_{700}^{+} , the oxidized primary electron donor of PSII (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 *b₆f* 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 $O_2^{-\bullet}$;

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 to 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

(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}^{++} (+1.2–+1.4 V) and $\text{TyrZ}^{\bullet}$ (+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.

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 [$\lambda > 600$ nm, $3,000 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$] 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 ferrocyanochrome *c* and ferricyanochrome *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 [$\lambda > 600$ nm, $15,000 \mu\text{mol (photon)} \text{m}^{-2} \text{s}^{-1}$]. 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}^{++} . In order to calculate of P_{700}^{++} amount, the EPR spectrum recorded in the dark

was subtracted from the spectrum measured during illumination of the samples. The concentration of P_{700}^{++} 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. 1A. 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 photo-reduced Cyt *c* after a 90-s illumination of the samples was

equal to $1.85 \mu\text{M}$. 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).

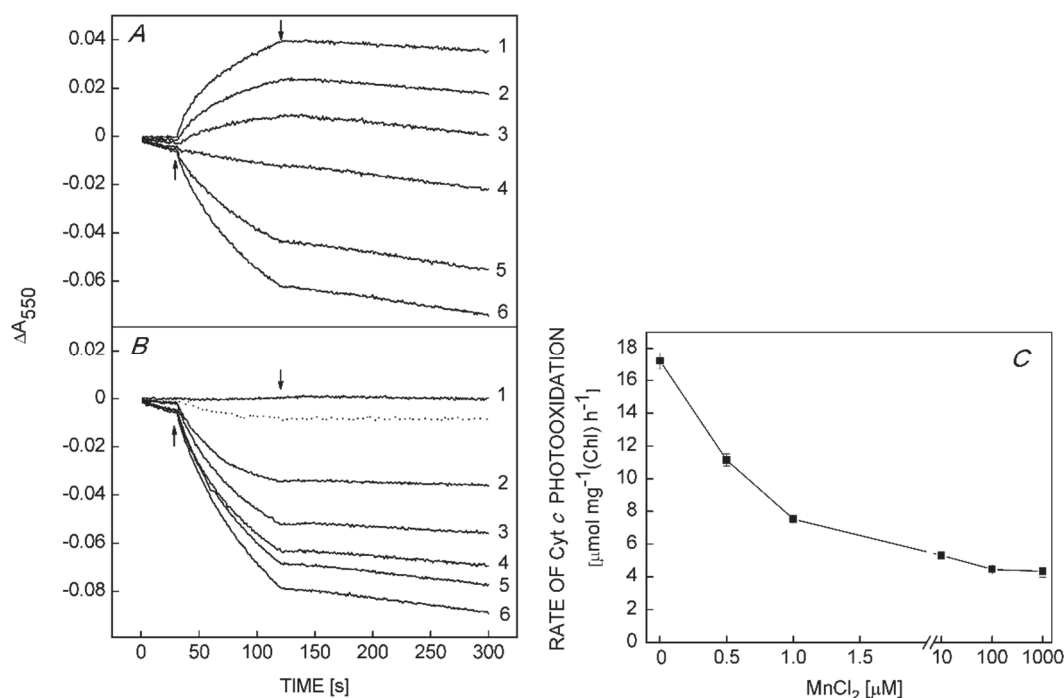


Fig. 1. (A,B) Kinetics of light-induced redox conversions of exogenous cytochrome *c* by NH_2OH -treated PSII preparations at different ratio of oxidized cytochrome *c* (Cyt c^{ox}) to its reduced form (Cyt c^{red}). The ratio of concentrations (μM) between Cyt c^{ox} and Cyt c^{red} before illumination 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 $10 \mu\text{M}$ 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 $10 \mu\text{g ml}^{-1}$. 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 [$\lambda > 600$ nm, $3,000 \mu\text{mol(photon)} \text{m}^{-2} \text{s}^{-1}$] 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 $\mu\text{g}(\text{Chl})\text{ml}^{-1}$. The illumination with the light [$\lambda > 600\text{ nm}$, $3,000\text{ }\mu\text{mol}(\text{photon})\text{m}^{-2}\text{ s}^{-1}$] 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 <i>c</i> photooxidation [$\mu\text{mol mg}^{-1}(\text{Chl})\text{ h}^{-1}$]				
Additions	Untreated PSII	NaCl-treated PSII	CaCl ₂ -treated PSII	NH ₂ OH-treated PSII
no	1.35 $\pm$ 0.05	2.54 $\pm$ 0.1	3.7 $\pm$ 0.1	16.4 $\pm$ 0.3
SOD	1.81 $\pm$ 0.06	3.27 $\pm$ 0.09	4.5 $\pm$ 0.12	20.7 $\pm$ 0.9
20 μM DCMU	2.6 $\pm$ 0.05	4.7 $\pm$ 0.11	5.3 $\pm$ 0.16	4.38 $\pm$ 0.14
SOD and 20 μM DCMU	2.6 $\pm$ 0.06	4.7 $\pm$ 0.08	6.2 $\pm$ 0.3	4.38 $\pm$ 0.12
SOD plus catalase				20 $\pm$ 0.9
10 μM MnCl ₂				5.3 $\pm$ 0.25
Rate of Cyt <i>c</i> photoreduction [$\mu\text{mol mg}^{-1}(\text{Chl})\text{ h}^{-1}$]				
Additions	Untreated PSII	NaCl-treated PSII	CaCl ₂ -treated PSII	NH ₂ OH-treated PSII
no	6.72 $\pm$ 0.31	7.98 $\pm$ 0.23	8.75 $\pm$ 0.31	12.5 (19.6) $\pm$ 0.5
SOD	3.4 $\pm$ 0.14	3.2 $\pm$ 0.12	2.45 $\pm$ 0.1	0.77 $\pm$ 0.04
20 μM DCMU	1.42 $\pm$ 0.07	0.92 $\pm$ 0.02	0.87 $\pm$ 0.04	0.43 $\pm$ 0.02
SOD and 20 μM DCMU	–0.21* $\pm$ 0.01	–0.6* $\pm$ 0.03	–0.63* $\pm$ 0.02	–0.62* $\pm$ 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. 1B, curve 1); (2) when Cyt *c*^{ox}/Cyt *c*^{red} ratios were 8/2 or 6/4 (Fig. 1A,B, curves 2,3) or even 9.5/0.5 (Fig. 1B, 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. 1B, curves 5,6). These SOD-induced effects may indicate that SOD suppresses the redox interaction of Cyt *c*^{ox} with O₂^{•–} 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 H₂O₂ produced as a result of the enzyme-catalyzed dismutation of O₂^{•–} is not involved in the Cyt *c*^{red} photooxidation in the PSII preparations [although, as has been shown by Vandewalle and Petersen (1987); exogenous H₂O₂ can oxidize Cyt *c*^{red} at rates comparable to those at which Cyt *c*^{ox} is reduced by superoxide].

In order to elucidate the efficiency of the redox interaction of Cyt *c*^{red} on the donor side of the NH₂OH-treated PSII preparations, we investigated the effect of added Mn²⁺ (which is an efficient and specific electron donor for PSII) on the rate of photooxidation of Cyt *c*^{red} (Fig. 1C). In contrast to Mn²⁺, other artificial electron donors for PSII (such as diphenylcarbazide, ferrocyanide, NH₂OH) 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. 1C, the rate of Cyt *c*^{red} photooxidation by NH₂OH-treated PSII preparations was considerably suppressed with the increase of concentration of added Mn²⁺, so that the addition of 1 μM MnCl₂ resulted in a 60% inhibition of the rate of Cyt *c*^{red} photooxidation. Although the Mn²⁺-induced suppression of Cyt *c* photooxidation was significant it was not possible to achieve a complete inhibition of the Cyt *c* photooxidation by Mn²⁺. Even at a high (1 mM) concentration of added Mn²⁺, the rate of Cyt *c* photooxidation was nearly 25% of that measured in the absence of Mn²⁺. The Mn²⁺-induced suppression of Cyt *c* photooxidation indicates that Mn²⁺ 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. 2A, the addition of either Mn²⁺ or Cyt *c*^{red} to NH₂OH-treated PSII preparations led to the reactivation of photoinduced ΔF . The capability of Cyt *c*^{red} to reactivate the photoinduced ΔF in the NH₂OH-treated PSII preparations was lower in comparison with exogenous Mn²⁺ [0.6 μM Mn²⁺ or 6 μM Cyt *c*^{red} was required to achieve a 50%-reactivation of ΔF (Fig. 2B)].

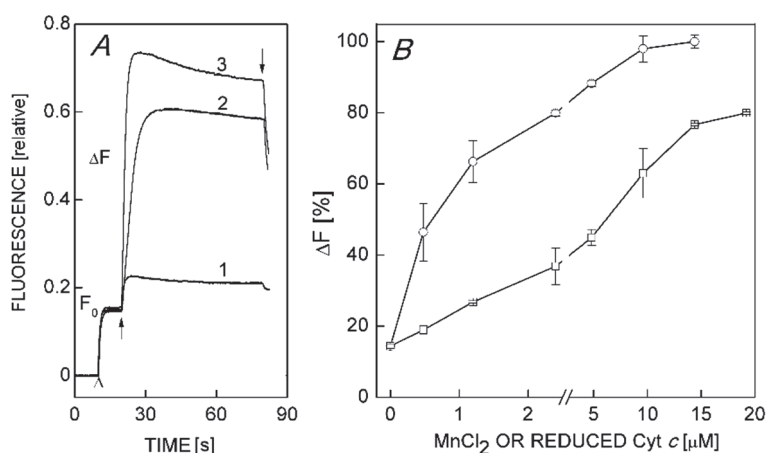


Fig. 2. Photoinduced changes of chlorophyll fluorescence yield (ΔF) related to photoreduction of the primary electron acceptor, Q_A , in NH_2OH -treated PSII preparations. (A) Kinetics of ΔF in the absence of additions (1) and in the presence of 19.2 μM reduced Cyt c^{red} (2) and 9.2 μM MnCl_2 (3). (B) Dependence of ΔF amplitude on concentration of Cyt c^{red} ($\square$) and MnCl_2 ($\circ$). The measurements were done in the medium containing 50 mM MES-NaOH (pH 6.5) and 35 mM NaCl at a concentration of 10 $\mu\text{g}(\text{Chl}) \text{ml}^{-1}$. 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; $\uparrow$ and $\downarrow$ – steady-state actinic light [$\lambda = 625 \text{ nm}$; 1,000 $\mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$] on and off, respectively. Averages with standard error of 3–5 measurements are shown in panel B.

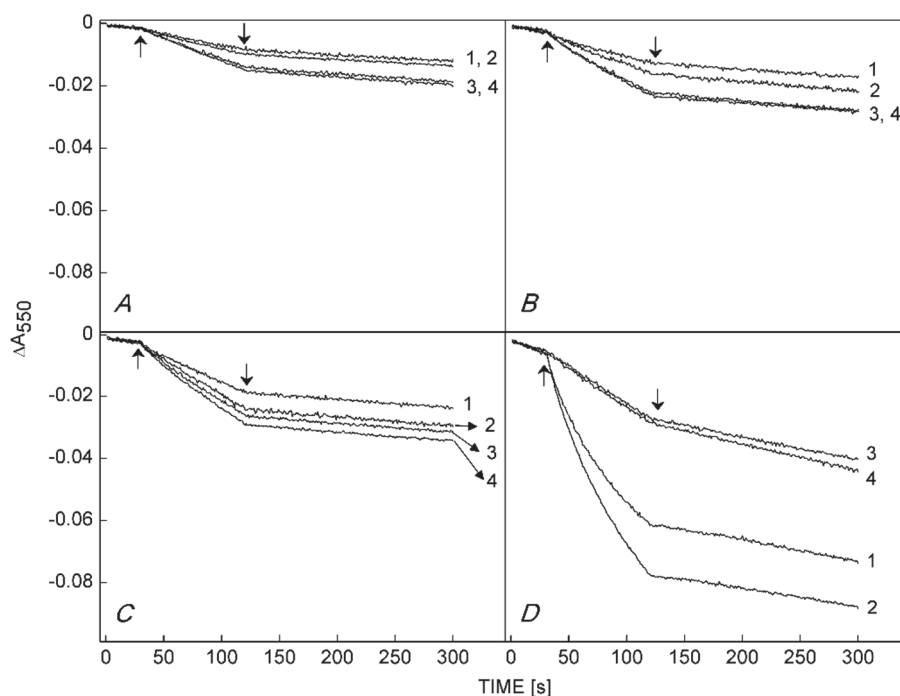


Fig. 3. Kinetics of cytochrome c photooxidation by PSII preparations before (A) and after modification of the water-oxidizing complex caused by treatments with NaCl (B), CaCl_2 (C), and NH_2OH (D). The measurements were done in the absence of additions (1) and after the addition of SOD (2), 20 μM DCMU (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 $\mu\text{g}(\text{Chl}) \text{ml}^{-1}$. Activity of added SOD was 50 U ml^{-1} . Up and down arrows indicate light [$\lambda > 600 \text{ nm}$, 3,000 $\mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$] 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}^{++} or $\text{TyrZ}^{\cdot}$. 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 NH₂OH-treated PSII preparations: 1.4 and 16.5 $\mu\text{mol}(\text{Cyt } c) \text{ mg}^{-1}(\text{Chl}) \text{ h}^{-1}$, 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 CaCl₂-treated PSII preparations was 2.5 and 3.7 $\mu\text{mol}(\text{Cyt } c) \text{ mg}^{-1}(\text{Chl}) \text{ h}^{-1}$, 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-, CaCl₂-, and (especially) in NH₂OH-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 NH₂OH-treated preparations (Fig. 3*D*, curve 3), while in all other samples DCMU did not inhibit 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 NH₂OH, the rate of Cyt *c*^{red} photooxidation was 4.5 times higher than that in CaCl₂-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 NH₂OH-treated PSII preparations. The capability of Cyt *c*^{red} to reactivate the photoinduced ΔF in the NH₂OH-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 NH₂OH-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

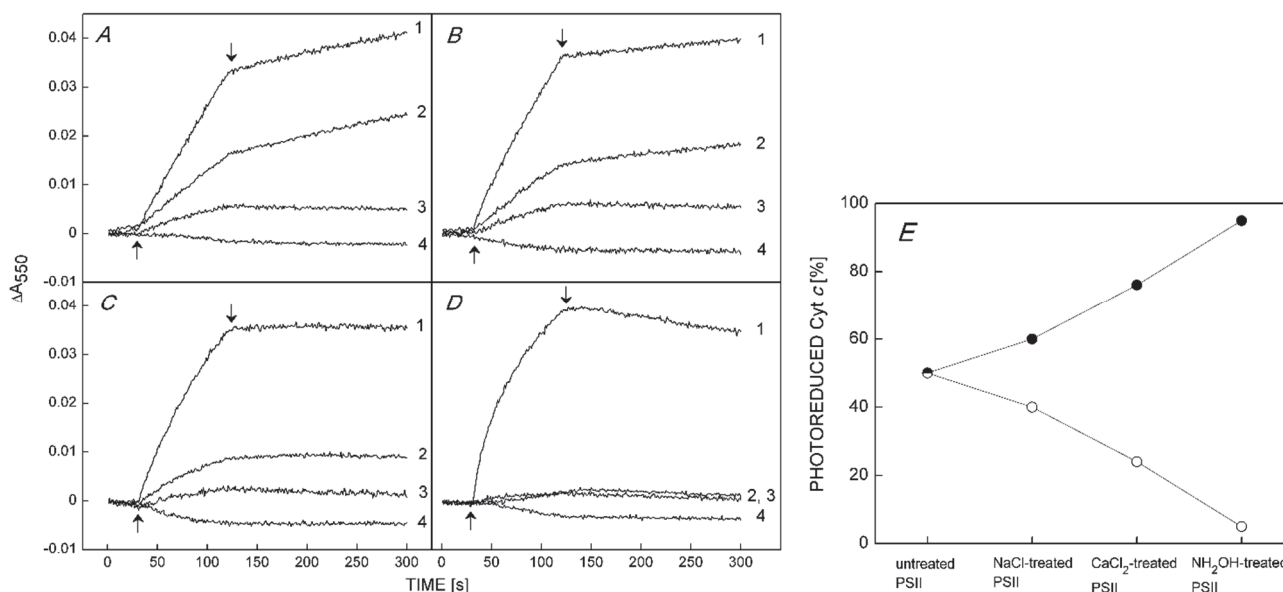


Fig. 4. Kinetics of Cyt *c*^{ox} photoreduction by PSII preparations before (*A*) and after modification of the water-oxidizing complex caused by treatments of NaCl (*B*), CaCl₂ (*C*), and NH₂OH (*D*). The measurements were done in the absence of additions (*I*) and after the addition of SOD (2), 20 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 $\mu\text{g}(\text{Chl}) \text{ ml}^{-1}$. Activity of added SOD was 50 U ml^{-1} . Up and down arrows indicate light [$\lambda > 600 \text{ nm}$, 3,000 $\mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$] on and off, respectively. (*E*) Changes of Cyt *c*^{ox} photoreduction directly by the reduced electron acceptors of PSII (○) and by O₂^{•-} (●) 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%.

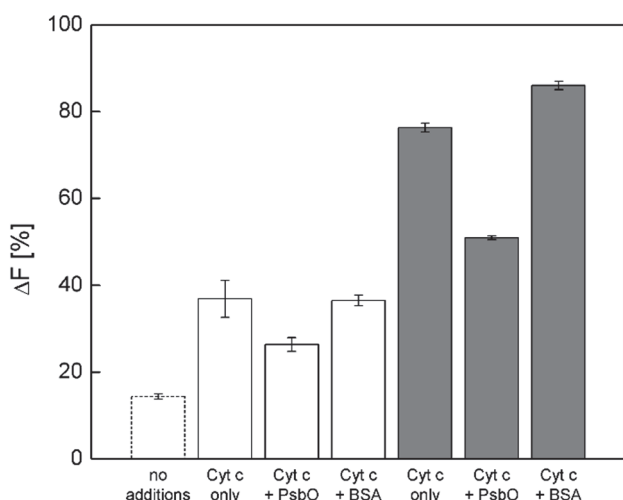


Fig. 5. Effect of PsbO and BSA on the amplitude of photoinduced ΔF measured in NH_2OH -treated PSII preparations in the presence of reduced Cyt *c* (Cyt *c*^{red}). Before the addition of Cyt *c*^{red} the preparations were incubated for 1 h in the medium containing 50 mM MES-NaOH (pH 6.5) and 35 mM NaCl at a concentration of 200 $\mu\text{g}(\text{Chl})\text{ ml}^{-1}$ 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 $\mu\text{g}(\text{Chl})\text{ ml}^{-1}$. 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.

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. 4A–C, curves 1) occurred at a similar rate. The NH_2OH -treated PSII preparations (Fig. 5D, 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

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. 1A,B. The photoreduction of Cyt *c*^{ox} in the NH_2OH -treated PSII preparations occurs mainly *via* $\text{O}_2^{\cdot-}$ photoproduced on the acceptor side of PSII since it is almost completely inhibited by SOD or DCMU (Figs. 1B, curve 1 and 4D, 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.

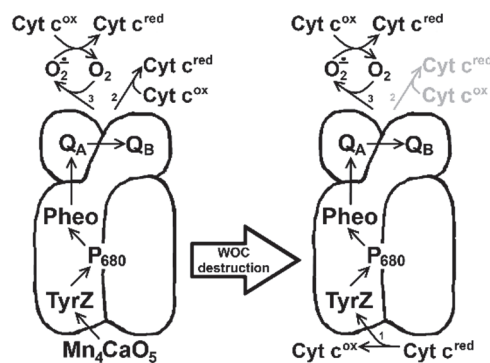


Fig. 6. A proposed scheme of possible ways of photooxidation and photoreduction of exogenous Cyt *c* by PSII preparations before and after removal of the WOC. *Arrows* indicate the routes of electron transfer. *Numbers* indicate the following redox transformations of Cyt *c*: (1) photooxidation of reduced 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 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 $\text{O}_2^{\cdot-}$ 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. 4E). 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.

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. 2B)] 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}^{++} 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}^{++} or TyrZ $^{\bullet}$. The Cyt c^{red} photooxidation not suppressed by Mn^{2+} or DCMU may be related 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}^{++} 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}^{++} 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 ($\text{O}_2^{\bullet-}$) 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 $\text{O}_2^{\bullet-}$ was activated despite of the loss of electron donation from the WOC (Fig. 4E). 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 $\text{O}_2/\text{O}_2^{\bullet-}$ 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 $\text{O}_2^{\bullet-}$ photoproduction in the CaCl_2 - and NH_2OH -treated PSII preparations. Besides, the increase in photoformation of $\text{O}_2^{\bullet-}$ 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}^{++} or to TyrZ $^{\bullet}$), (2) photoreduction of Cyt c^{ox} directly by reduced electron acceptors of PSII; (3) photoreduction of Cyt c^{ox} by $\text{O}_2^{\bullet-}$ 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 $\text{O}_2^{\bullet-}$ in PSII preparations.

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