Biochemistry (Moscow), Vol. 69, No. 8, 2004, pp. 926-933. Translated from Biokhimiya, Vol. 69, No. 8, 2004, pp. 1139-1148.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-023, May 23, 2004.

H_2O_2-Induced Inhibition of Photosynthetic O_2 Evolution by *Anabaena variabilis* Cells

V. D. Samuilov[1]*, K. N. Timofeev[2], S. V. Sinitsyn[1], and D. V. Bezryadnov[1]

[1]Department of Physiology of Microorganisms and [2]Department of Biophysics, Faculty of Biology, Lomonosov Moscow State University, Moscow 119992, Russia; fax: (7-095) 939-3807; E-mail: vds@8.cellimm.bio.msu.ru

Received February 2, 2004
Revision received March 17, 2004

Abstract—Hydrogen peroxide inhibits photosynthetic O_2 evolution. It has been shown that H_2O_2 destroys the function of the oxygen-evolving complex (OEC) in some chloroplast and Photosystem (PS) II preparations causing release of manganese from the OEC. In other preparations, H_2O_2 did not cause or caused only insignificant release of manganese. In this work, we tested the effect of H_2O_2 on the photosynthetic electron transfer and the state of OEC manganese in a native system (intact cells of the cyanobacterium *Anabaena variabilis*). According to EPR spectroscopy data, H_2O_2 caused an increase in the level of photooxidation of P700, the reaction centers of PS I, and decreased the rate of their subsequent reduction in the dark by a factor larger than four. Combined effect of H_2O_2, CN^-, and EDTA caused more than eight- to ninefold suppression of the dark reduction of $P700^+$. EPR spectroscopy revealed that the content of free (or loosely bound) Mn^{2+} in washed cyanobacterial cells was ~20% of the total manganese pool. This content remained unchanged upon the addition of CN^- and increased to 25-30% after addition of H_2O_2. The content of the total manganese decreased to 35% after the treatment of the cells with EDTA. The level of the H_2O_2-induced release of manganese increased after the treatment of the cells with EDTA. Incubation of cells with H_2O_2 for 2 h had no effect on the absorption spectra of the photosynthetic pigments. More prolonged incubation with H_2O_2 (20 h) brought about degradation of phycobilins and chlorophyll *a* and lysis of cells. Thus, H_2O_2 causes extraction of manganese from cyanobacterial cells, inhibits the OEC activity and photosynthetic electron transfer, and leads to the destruction of the photosynthetic apparatus. H_2O_2 is unable to serve as a physiological electron donor in photosynthesis.

Key words: photosynthesis, photosynthetic oxygen, oxygen-evolving complex, Photosystem II, Photosystem I, hydrogen peroxide, manganese, cyanobacteria, *Anabaena variabilis*

In oxygenic photosynthesis, water is oxidized by the oxygen-evolving complex (OEC), whose catalytic center contains a number of inorganic components denoted collectively as $Mn_4O_xCa_1Cl_y$ (see [1, 2] for review). An oxygen molecule evolves as a result of the light-dependent four-electron oxidation of two H_2O molecules. Bicarbonate was suggested to play a key role in the process of formation of the catalytic center of the OEC [2]. According to the results of thermodynamic calculations, in oxygenic photosynthesis HCO_3^- is a more effective electron donor than water [3, 4]: values of free energy of oxidation of H_2O and HCO_3^- with evolution of 0.5 mol O_2 are 37.3 and 24.8 kcal/mol, respectively [4]. Cells of algae and cyanobacteria, isolated chloroplasts, and chloroplast thylakoids incubated in water containing ^{18}O-labeled bicarbonate under exposure to light evolve oxygen enriched with ^{18}O [3]. Enrichment with $^{18}O_2$ was observed in experiments with cells of cyanobacteria and isolated thylakoids of chloroplasts depleted of carboanhydrase activity [3]. Carboanhydrase catalyzes the reaction $CO_2 + H_2O \rightleftarrows HCO_3^- + H^+$, which gives rise to oxygen exchange between CO_2 and H_2O ($C^{18}O_2 + H_2O \rightleftarrows CO_2 + H_2{}^{18}O$). As a result, exogenous $HC^{18}O_3^-$ is converted into HCO_3^- and $H_2{}^{18}O$. The resulting $H_2{}^{18}O$, whose amount is limited by the amount of $HC^{18}O_3^-$ added to the system, is diluted in the ocean of H_2O. Therefore, it is impossible to demonstrate evolution of $^{18}O_2$ from $HC^{18}O_3^-$ using preparations with active carboanhydrase. Because this could be the case in experiments reported in [5], these data should not be regarded as a counterargument against the hypothesis of the role of bicarbonate.

Abbreviations: BQ) *p*-benzoquinone; DCMU) 3(3′,4′-dichlorophenyl)-1,1-dimethylurea; EDTA) ethylenediaminetetraacetate; FeCy) potassium ferricyanide; OEC) oxygen-evolving complex; PS) photosystem; P680 and P700) reaction centers of PS II and PS I, respectively.

* To whom correspondence should be addressed.

0006-2979/04/6908-0926 ©2004 MAIK "Nauka / Interperiodica"

Hydrogen peroxide is an intermediate product of water oxidation regardless of the nature of the electron donor:

$$2H_2O \xrightarrow[-2H^+]{-2e^-} 2OH^{\bullet} \longrightarrow H_2O_2 \longrightarrow O_2 + 2e^- + 2H^+, \quad (1)$$

$$2H_2O + 2CO_2 \xrightarrow{-2H^+} 2HCO_3^- \xrightarrow{-2e^-} 2HCO_3^{\bullet} \longrightarrow$$

$$\longrightarrow HOOC{-}O{-}O{-}COOH \xrightarrow{-2CO_2}$$

$$\longrightarrow H_2O_2 \longrightarrow O_2 + 2e^- + 2H^+, \quad (2)$$

where $OH^{\bullet}$, H_2O_2, $HCO_3^{\bullet}$, and HOOC–O–O–COOH are the bound forms of the product of intermediate oxidation. Values E_0' of $OH^{\bullet}/H_2O$ and $HCO_3^{\bullet}/HCO_3^-$ (at pH 7 bicarbonate radical is in the form $CO_3^{\bullet-}$ [6]) are 2.31 [7] and 1.59 V [8], respectively.

It follows from Eq. (2) that oxidation of bicarbonate, which is produced from CO_2 and H_2O, gives rise to formation of H_2O_2 and regeneration of CO_2. In other words, in this case, like in the case described by Eq. (1), water is an actual electron donor for PS II. However, this water is bound to CO_2, and CO_2 plays a catalytic role in water oxidation.

Although the OEC mediates light-induced generation of H_2O_2 both in free and bound forms [9, 10], the rate of accumulation of H_2O_2 is insignificant [11]. Isolated thylakoids of chloroplasts depleted of Cl^- are able to catalyze DCMU-sensitive oxidation of H_2O_2 but not H_2O [12]. It was suggested in the literature that at the initial stages of evolution of photosynthesis hydrogen peroxide played the role of electron donor for PS II. In other words, H_2O_2 was suggested to be a functional precursor of H_2O ([13], see [14, 15] for review). Values E_0' for O_2/H_2O_2 and O_2/H_2O are 0.27 and 0.82 V, respectively.

Oxidation of H_2O proceeds in four one-electron stages and gives rise to successive accumulation of oxidative equivalents in the Mn-cluster of the OEC [14, 15]. Therefore, there are five redox states of the Mn-cluster of OEC, which are denoted as S_0-S_4. States S_0 and S_1 are stable in the dark. The S_1/S_0 ratio in samples preincubated in the dark is 3-4. Therefore, under conditions of pulse photoactivation, when each light flash induces single turnover of P680, the maximum yield of the O_2 evolution from H_2O is observed per each third light pulse.

The maximum yield of the O_2 evolution induced by exposure of chloroplasts preincubated in the dark with H_2O_2 to pulse photoactivation is per the fifth rather than the third light pulse [16, 17]. This result was interpreted as an indication of the dark transition of Mn-cluster from state S_1 to a specific state S_{-1}. State S_{-1} is formed as a result of two-electron reduction of the Mn-cluster in state S_1 by hydrogen peroxide in a reaction associated with O_2 evolution (see Eq. (3) below).

A single light pulse induces the transition of the Mn-cluster of the OEC from state S_1 to state S_2, which relaxes to state S_0 in the presence of H_2O_2 [16]. Transitions $S_2 \rightarrow S_0$ and $S_1 \rightarrow S_{-1}$ are coupled to the dark reaction $H_2O_2 \rightarrow O_2 + 2e^- + 2H^+$. It was demonstrated in experiments with $H_2{}^{18}O_2$ that the whole body of O_2 evolved from H_2O_2 [17].

The presence of heme catalase associated with PS II makes it methodologically difficult to study the processes of production and degradation of H_2O_2 in photosynthesis [18]. Therefore, O_2 evolution by preparations incubated with H_2O_2 can largely be attributed to the catalase reaction: $H_2O_2 \rightarrow O_2 + 2H_2O$. This suggestion was confirmed by the results reported in [11]. Particles of PS II depleted of heme catalase demonstrated high rate of O_2 evolution but only 0.02% of the initial rate of O_2 evolution from H_2O_2. Therefore, the role of the OEC reactions $S_1 \rightarrow S_{-1}$ and $S_2 \rightarrow S_0$ in oxidation of H_2O_2 in PS II is insignificant, and degradation of H_2O_2 is mainly catalyzed by catalase.

It was suggested in [11] that light-induced oxidation of H_2O_2 in catalase-free subchloroplast particles was due to Mn-dependent (Eqs. (3) and (4)) and Mn-independent (Eqs. (5) and (6)) reactions:

$$H_2O_2 + S_1 \rightarrow O_2 + S_{-1} + 2H^+, \quad (3)$$

$$S_{-1} + 2h\nu \rightarrow S_1 + 2e^-, \quad (4)$$

$$H_2O_2 + Y_z^{\bullet} \rightarrow O_2^{\bullet-} + 2H^+ + Y_z, \quad (5)$$

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2, \quad (6)$$

where Y_z is the tyrosine-161 residue in polypeptide D1 of PS II, which becomes accessible to H_2O_2 after removal of Mn; $Y_z^{\bullet}$ is the photooxidized form of Y_z.

An insignificant rate of H_2O_2 degradation in the dark with formation of O_2 in catalase-free subchloroplast particles can be explained by catalytic activity of admixtures of transition metals (Fe, Cu, Mn) or nonheme iron of the electron acceptor complex of PS II [11].

Within the framework of the concept of the role of H_2O_2 as an electron donor, it is safe to suggest that photosynthetic objects are resistant to H_2O_2 and even can be stimulated by H_2O_2. However, the addition of H_2O_2 to catalase-free subchloroplast particles caused irreversible inhibition of O_2 evolution, the extent of the inhibition being larger than 50% at H_2O_2 concentration 0.25 mM [11]. The inhibition effect was exerted by endogenous H_2O_2 generated both in the electron donor and in the electron acceptor (as a result of reduction of O_2 by the secondary plastoquinone Q_B) sides of PS II in isolated thylakoids [19]. Removal of Cl^- or addition of CN^- caused an increase, whereas the addition of catalase caused a decrease in the inhibition effect of endogenous H_2O_2.

A detrimental effect of H_2O_2 was also observed in native systems: H_2O_2 inhibited growth of cyanobacteria under conditions of dialysis cultivation even in concentrations as low as 10^{-5}-10^{-4} M [20]. The effect of H_2O_2 on the processes of photosynthetic evolution of O_2 and photosynthetic electron transport was studied in *Anabaena variabilis* and *Anacystis nidulans* cells [21]. H_2O_2 inhibited evolution of O_2 in the Hill reaction with *p*-benzoquinone (BQ) and ferricyanide (FeCy) as electron acceptors. However, the fact of inhibition of O_2 evolution itself does not mean that oxidation of H_2O_2 is terminated. The process may take place without evolution of O_2, being stopped at an intermediate stage of formation of H_2O_2 [22]. For example, the amount of 2,6-dichlorophenolindophenol, phenyl-BQ, or phenyl-BQ + FeCy photoreduced in Hill reaction (as calculated per electron equivalent) was more than 1.5 times larger than the amount of O_2 evolved by the membrane particles of PS II [22]. However, this difference was eliminated after addition of catalase. Therefore, a significant fraction of the electron flow through PS II was due to formation of H_2O_2 rather than evolution of O_2.

It was found in our experiments [21] that H_2O_2 inhibited evolution of O_2 and the associated process of BQ + FeCy photoreduction in the Hill reaction. These data indicate that the inhibition effect of H_2O_2 is targeted at the initial stages leading to formation of H_2O_2. If H_2O_2 were unable to inhibit these stages, the process of BQ + FeCy photoreduction would not be inhibited. The value I_{50} for H_2O_2 was ~0.75 mM.

Treatment of Cl^--deficient thylakoids with H_2O_2 induced release of Mn from the OEC [23]. However, in catalase-free Cl^--containing preparations, the rate of the process of the H_2O_2 induced release of Mn from OEC was insignificant even in the presence of 120 mM H_2O_2 [11]. H_2O_2 in concentration 130 mM was unable to extract Mn from PS II particles devoid of water-soluble polypeptides with molecular weight 17 and 24 kD [24]. These data reported in [11, 24] are inconsistent with the results obtained either in PS II particles devoid of water-soluble polypeptides with molecular weight 17 and 24 kD [25] or in Cl^--deficient thylakoid membranes [23]. It was shown in [23, 25] that 20 mM H_2O_2 and some other agents serving as electron donors for PS II (NH_2OH, NH_2–NH_2, Fe^{2+}, benzidine, and hydroquinone) were able to reduce Mn incorporated in OEC, thereby causing its extraction. Perhaps, removal of PS II polypeptides of 17 and 24 kD modifies the OEC structure and makes it accessible for H_2O_2 added in low concentration [26].

It is fairly difficult to draw adequate conclusions on the basis of the results obtained in defective preparations. For example, specific treatment of PS II membrane particles intended to remove catalase and suppress its activity [11] may also eliminate components sensitive to H_2O_2. Concentrations of H_2O_2 tested in [11] were excessively high and could induce conformational changes and denaturation of proteins, preventing thereby Mn from being released from OEC. This is evidenced by a significant decrease in the sensitivity of PS II to DCMU [11].

Thus, the mechanisms of the inhibition effect of H_2O_2 either on O_2 evolution or on electron transport in oxygenic photosynthesis cannot be deduced from the results obtained in isolated preparations treated by different methods. The goal of this work was to test the effects induced by H_2O_2 on photosynthetic electron transport and state of manganese in the OEC in intact systems (cells of cyanobacteria). In addition to H_2O_2, we also tested the effects of CN^-, an inhibitor of such enzymes as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) [27], peroxidases (including ascorbate peroxidase), Cu,Zn-superoxide dismutase [28], cytochrome *c* oxidase, and heme catalases. The effects of EDTA, a chelator of divalent metal cations (including Mn^{2+}), were also tested.

MATERIALS AND METHODS

Cells of *Anabaena variabilis* Kütz No. 458 were grown under continuous light (~1000 lx) as a batch culture on mineral medium BG-11 [29] as described elsewhere [30]. Cells of three- to five-day-old cultures in the exponential stage of growth were used. Cells were sedimented by centrifugation at 2000*g*, washed three times with 10 mM Hepes-NaOH-buffer solution (pH 8.0) containing 25 mM KCl, and suspended in the same buffer solution. In experiments with EDTA treatment, after washed cells had been incubated for 10 min in buffer solution containing 5 mM Na_2EDTA, cells were again washed three times with buffer solution to remove EDTA.

Concentration of chlorophyll *a* in cells of cyanobacteria was measured using extinction coefficient at 663 nm in 80% acetone (75.05 $mM^{-1}\cdot cm^{-1}$) [31]. Concentration of H_2O_2 was measured the using extinction coefficient at 240 nm in aqueous solution (43.6 $M^{-1}\cdot cm^{-1}$) [32]. Optical spectra of cell suspensions were measured using a Hitachi-2000 spectrophotometer (Japan).

EPR spectra were measured at room temperature using an RE-1307 spectrometer. Cell suspension containing 1 mg/ml chlorophyll *a* in 10 mM Hepes-NaOH-buffer (pH 8.0) with 25 mM KCl was filled in a flat quartz cuvette (volume, 100 μl; optical path, 0.02 cm). EPR signal of reaction centers P700 was induced by rectangular pulses of light (duration, 0.2 sec) applied directly to the cavity of the EPR spectrometer from an incandescent lamp (power, 300 W; light flux, 2000 $\mu E/m^2$ per sec). The following settings of the EPR signal detection were used: microwave power, 20 mW; modulation amplitude, 0.3 mT; modulation frequency, 100 kHz; and detection time constant, 10 μsec. H_2O_2, NaCN, and other reagents were added to cell suspension just before EPR signal detection. All EPR records were run in no less than triplicate.

RESULTS

Effect of H_2O_2 and other agents on the EPR signal of reaction centers of PS I in *A. variabilis* cells. The light-induced EPR signal with *g*-factor 2.0026 attributed to photooxidation of P700, the primary electron donor of PS I, in *A. variabilis* cells is shown in Fig. 1a. A steady-state level of $P700^+$ is established as a result of photoactivation. In the dark $P700^+$ is reduced, and the EPR signal decays with half-time ($t_{1/2}$) 30 msec (Table 1). H_2O_2 induced an increase in the amplitude of the light-induced EPR signal and slowed down its further relaxation (Fig. 1b and Table 1). Because the results shown in Fig. 1 and Table 1 were obtained in a thick suspension of cells (chlorophyll concentration, 1 mg/ml, which was 100-150 times higher than chlorophyll concentration in spectrophotometric and oximetric experiments), concentrations of H_2O_2 in EPR measurements were increased correspondingly. Although CN^- caused a decrease in the amplitude of the light-induced EPR signal of $P700^+$, it had no effect on the kinetics of its dark decay (Fig. 1c and Table 1). The addition of EDTA had no effect on the kinetics of the redox changes of P700. The effect exerted by H_2O_2 + CN^- was similar to the effect of H_2O_2.

Table 1. Amplitude of EPR signal of reaction center P700 and halftime $t_{1/2}$ of its post-illumination reduction in the dark in *A. variabilis* cells before and after treatment with EDTA

Additive	Concentration, mM	Amplitude of EPR signal, %	Time $t_{1/2}$ of dark relaxation of EPR signal, msec
Before EDTA treatment			
Control	—	100	30
H_2O_2	25	120	130
NaCN	1	70	30
EDTA	10	100	30
H_2O_2 + NaCN	100 + 1	120	150
H_2O_2 + NaCN + + EDTA	100 + 1 + 10	110	270
After EDTA treatment			
Control	—	100	50
H_2O_2	25	100	125
NaCN	1	70	50
H_2O_2 + NaCN + + EDTA	100 + 1 + 10	100	400

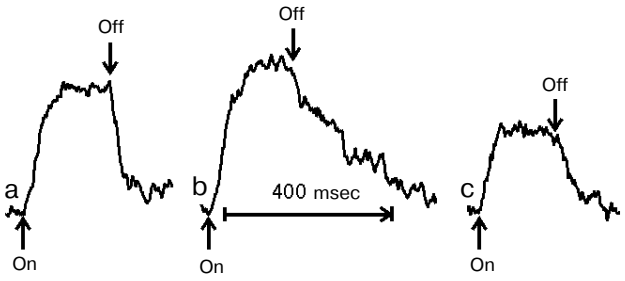

Fig. 1. Effect of H_2O_2 and NaCN on the photoinduced EPR signal of reaction center P700 in *A. variabilis* cells: a) control without addition; b) 25 mM H_2O_2; c) 1 mM NaCN. "On" and "Off" show actinic light on and off, respectively.

Combination H_2O_2 + CN^- + EDTA caused a significant increase in the characteristic time of the dark reduction of the photoinduced $P700^+$ (Table 1).

In *A. variabilis* cells treated with EDTA, the amplitude of the EPR signal was maintained at an invariable level, whereas the rate of dark reduction of $P700^+$ decreased (Table 1, compare controls before and after treatment with EDTA). Although H_2O_2 had no effect on the amplitude of the photoinduced signal of $P700^+$, it decreased the rate of its dark reduction. The effects induced by CN^- before and after treatment with EDTA were similar to each other. Cells treated with EDTA combination H_2O_2 + CN^- + EDTA showed an even larger decrease in the rate of the dark relaxation of the EPR signal than without EDTA treatment (Table 1).

Effect of H_2O_2 and other agents on the state of manganese in *A. variabilis* cells. The EPR spectrum of Mn^{2+} ions in aqueous solutions contains six bands. The EPR signal disappeared in the presence of EDTA, a complex-producing agent, and its amplitude decreased in the presence of CN^- (not shown). Bound Mn gives no such typical six-band EPR spectrum. Washed cells of *A. variabilis* contain unbound (or, perhaps, loosely bound) Mn^{2+} (Fig. 2a). Bound manganese is extracted as a result of treatment of thylakoids with HCl [23, 33]. The addition of HCl to cell suspension caused a significant increase in the EPR signal amplitude, which was due to release of bound Mn (Fig. 2c).

All of the released manganese was in divalent form: it is well known from the chemistry of transition elements that tri-, quadro-, hexa-, and heptavalent manganese is readily reduced by HCl to Mn^{2+}. For example, pyrolusite, the most widespread in the nature water-insoluble manganese ore (MnO_2), in the presence of HCl is converted into a soluble form being reduced to Mn^{2+}:

$$MnO_2 + 4HCl \rightarrow MnCl_2 + Cl_2 + 2H_2O. \quad (7)$$

It should be noted that Mn^{2+} is stable in acid solutions.

Unbound Mn^{2+} in *A. variabilis* cells accounted for 20% of the total amount of cell manganese (Figs. 2a and

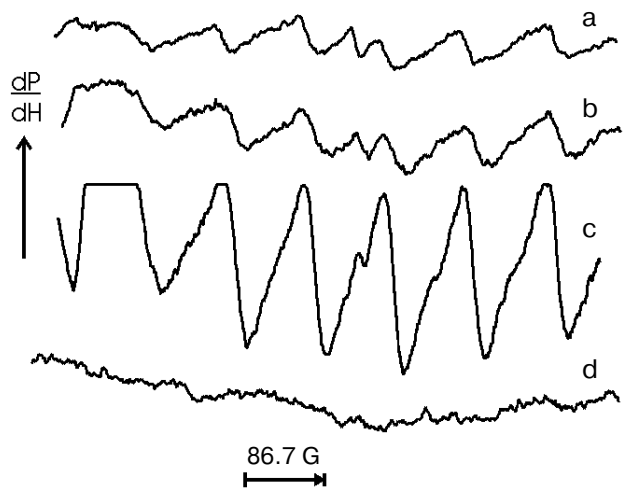

Fig. 2. EPR spectra of Mn^{2+} in *A. variabilis* cell suspension: a) control; b) 40 mM H_2O_2; c) 100 mM HCl; d) cells preincubated with 5 mM EDTA and washed three times to remove EDTA.

2c, Table 2). The content of free Mn^{2+} in cells remained unchanged after addition of CN^- (Table 2) and increased to 25% or even 30% of total cell manganese after addition of 40 or 200 mM H_2O_2, respectively (Fig. 2b and Table 2), and significantly decreased after cell treatment with EDTA (Fig. 2d and Table 2, last column). Treatment of cells with EDTA caused an increase in the rate of the H_2O_2-induced release of manganese. The level of released Mn^{2+} decreased after addition of H_2O_2 + CN^- to EDTA-treated cells (Table 2).

Effect of H_2O_2 and CN^- on absorption spectra of *A. variabilis* cells. Optical absorption spectra of *A. variabilis* cell suspension are shown in Fig. 3. These spectra contained bands of chlorophyll *a* and carotenoids within the spectral range 400-550 nm, bands of phycobilins with maximums at 580 (phycoerythrin) and 630 nm (phycocyanin), and chlorophyll *a* at 680 nm. Dark incubation of cells with H_2O_2 and CN^- (this agent was added to inhibit catalase and peroxidase, H_2O_2-degrading heme-containing enzymes) for 2 h had no effect on the absorption spectra (Fig. 3). Prolonged incubation of cells (20 h) with H_2O_2 gave rise to a progressive decrease in the optical density of phycobilins, a lesser decrease in the optical density of chlorophyll *a*, and decrease in the intensity of the cell suspension light scattering (Table 3). Perhaps, these effects were due degradation of pigments and lysis of cells.

DISCUSSION

Both photosynthetic and respiratory electron transport is sustained in the thylakoid membranes of cyanobacteria (Fig. 4). Photosynthetic and respiratory electron transport chains interact with each other by sharing chain components [34-38]: the membrane pool of plastoquinone, cytochrome b_6f complex, and plastocyanin (or cytochrome c_6 in case of copper deficiency in the medium).

On one hand, plastoquinone is an electron acceptor for PS II, type I NAD(P)H-dehydrogenase (NDH-1), and succinate dehydrogenase. On the other hand, plastoquinone is an electron donor for PS I, cytochrome aa_3 oxidase, and alternative oxidases. Quinol oxidase resistant to CN^- and sensitive to benzylhydroxamate [38-41] and CN^--sensitive quinol oxidase *bd* [42] are alternative oxidases in cyanobacteria. PS I complexes mediate both linear (noncyclic and pseudocyclic transport, in which O_2 evolution by PS II is compensated by O_2 uptake in PS I associated with formation of H_2O (water–water cycle [43])) and cyclic electron transport, in which a fraction of linear chain is closed as a cycle through NDH-1. The existence of cyclic electron transport was demonstrated in

Table 2. Extraction of Mn^{2+} from *A. variabilis* cells before and after treatment with EDTA. Extracted Mn^{2+} was determined by the amplitude of the fifth band of the EPR spectrum (bands were numbered starting from the lowest value of the constant magnetic field strength)

Additive	Concentration, mM	Free Mn^{2+}, %	Free Mn^{2+} after treatment with EDTA relative to its content before treatment with EDTA, %
Before EDTA treatment			
Control	—	20	
H_2O_2	40	25	
NaCN	3	20	
H_2O_2 + NaCN	40 + 3	25	
H_2O_2	200	30	
H_2O_2 + NaCN	200 + 3	30	
HCl	100	100	
After EDTA treatment			
Control	—	5	
H_2O_2	40	35	
NaCN	3	5	
H_2O_2 + NaCN	40 + 3	25	
H_2O_2	200	45	
H_2O_2 + NaCN	200 + 3	25	
HCl	100	100	35

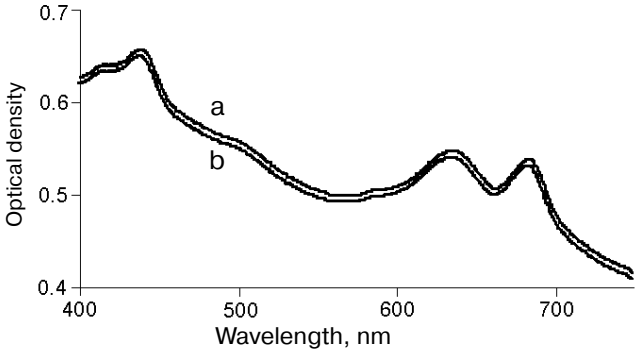

Fig. 3. Absorption spectra of *A. variabilis* cells: a) control; b) after 2 h of incubation in the dark with 1 mM NaCN and 10 mM H_2O_2.

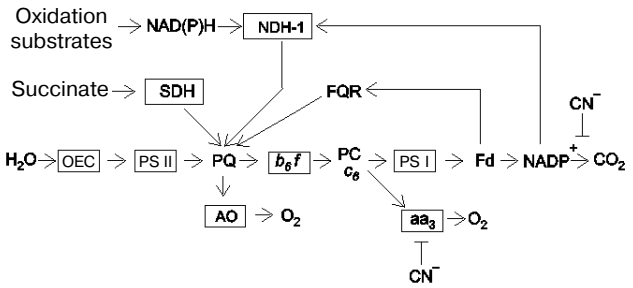

Fig. 4. Interaction between photosynthetic and respiratory electron transport chains in thylakoid membranes of cyanobacteria. Explanations are given in text. Symbols: AO) alternative oxidase; OEC) oxygen-evolving complex; PS I and PS II) photosystems I and II, respectively; aa_3) cytochrome oxidase aa_3; b_6f) cytochrome b_6f complex; Fd) ferredoxin; FQR) ferredoxin:plastoquinone reductase; NDH-1) type I NAD(P)H dehydrogenase; PQ) plastoquinone.

thylakoids of cyanobacteria [44, 45] and chloroplasts [46]. The electron transport chain can also be closed through ferredoxin:plastoquinone oxidoreductase. Perhaps there are other variants of cyclization of electron flow (see [47] for review).

There is no consensus about the ratio of linear and cyclic electron transport rates in thylakoid membranes. This is due to methodological problems in measurement of cyclic flow rate. Results obtained in three species of cyanobacteria, including *Anacystis nidulans* (*Synechococcus* sp. PCC 6301), which is metabolically close to *A. variabilis*, revealed that under physiological light intensities the fraction of cyclic electron transport was insignificant [48]. In *Synechococcus* sp. PCC 7002 the rate of cyclic electron transport was estimated to be ~3% of the rate of the linear transport [49]. However, results obtained in spinach leaves preincubated in the dark showed that within the first few seconds of illumination, the fractions of linear and cyclic electron transport were approximately equal to one another [50]. After that, the contribution of the cyclic branch of electron transport declines. It was suggested in [50], that the cyclic chain was involved in photosynthesis induction, providing synthesis of ATP required for the Calvin cycle.

The cyanobacterium *A. variabilis* is an obligate photolithoautotrophic organism. The rate of the CO_2-dependent linear photosynthetic electron transport in cells of this cyanobacterium is three to six times larger than the rate of dark respiration [38, 51]. Therefore, the amplitude of photooxidation of the reaction centers P700 and rate of their post-illumination reduction in *A. variabilis* cells are mainly determined by the processes in the photosynthetic chain.

The addition of H_2O_2 caused an increase in the amplitude of photooxidation of P700 and a more than fourfold decrease in the rate of the post-illumination reduction in the dark (Fig. 1, Table 1). This result is evidence that H_2O_2 inhibits the processes of reduction of the components of the electron-donor branch of PS I. This conclusion is consistent with inhibition of electron transfer from water to BQ + FeCy and concomitant O_2 evolution in the Hill reaction in cells treated with H_2O_2 [21]. The cyanide-induced inhibition of the flux of reducing equivalents to CO_2 through ribulose-1,5-bisphosphate carboxylase/oxygenase [27] gave rise to deficiency of $NADP^+$, the terminal electron acceptor in the linear electron transport chain, and decreased the amplitude of the photooxidation of P700 (Table 1). The value of $t_{1/2}$ of the process of dark reduction of $P700^+$ in this case was indistinguishable from the control. This indicated that CN^-, an inhibitor of the respiratory chain, had no effect on the redox balance of the components of electron donor segment of PS I in *A. variabilis* cells.

It is well known that being applied in high concentration, CN^- inhibits plastocyanin (30 mM CN^- [52]), induces extraction of manganese and copper from chloroplasts (50 mM CN^- [53]), and binds to the non-heme iron of the electron acceptor branch of PS II (50 mM CN^-, saturation at 240-360 mM CN^- [54]). It is

Table 3. H_2O_2-Induced degradation of phycocyanin ($\Delta A_{630\text{-}720\ nm}$) and chlorophyll *a* ($\Delta A_{680\text{-}720\ nm}$) and decrease in light scattering ($A_{720\ nm}$) in *A. variabilis* cell suspension (cells were incubated in the dark in the presence of H_2O_2 for 20 h)

H_2O_2, mM	$\Delta A_{630-720\ nm}$	$\Delta A_{680-720\ nm}$	$A_{720\ nm}$
0	0.18	0.17	0.67
1	0.11	0.12	0.38
10	0.08	0.12	0.29
100	0.03	0.08	0.10

obvious that none of these effects was observed in our experiments because the concentration of CN^- used in these experiments was 1 mM.

Neither the level of photooxidation of P700 nor the rate of the post-illumination reduction of $P700^+$ were changed in the presence of EDTA, a well-known complexing agent of divalent metal cations (Table 1). These findings are consistent with the results on two pools of Mn in *Synechococcus* sp. PCC 6803 cells: pool A, which was associated with the fraction of the outer membranes of this gram-negative bacterium, and pool B, which was associated with the fraction of the thylakoid and cytoplasmic membranes [55]. Pool A (~97.6% total Mn) is extracted with EDTA, whereas pool B (~2.4% total Mn) is conserved in cells in a bound state after their treatment with EDTA. Perhaps this pool provides activity of the OEC.

Nevertheless, EDTA added in combination with H_2O_2 and CN^- caused a significant decrease (by a factor of nine relative to control) in the half-time of $P700^+$ reduction (Table 1). Under conditions in which catalase- and peroxidase-catalyzed degradation of added H_2O_2 is suppressed by CN^-, the OEC manganese is thought to be reduced by hydrogen peroxide to Mn^{2+}, which is more loosely bound to OEC than Mn^{3+} and Mn^{4+} [25]. Under these conditions equilibrium between pools A and B is shifted toward manganese transition from pool B to pool A and OEC activity was reduced causing a significant decrease in the rate of reduction of $P700^+$.

EDTA itself causes a decrease in the rate of dark reduction of $P700^+$ (Table 1, compare values measured before and after treatment of cells with EDTA). In cells treated with EDTA, H_2O_2 caused a 2.5-fold (125 : 50 = 2.5) decrease in the rate of reduction of $P700^+$. However, the total decrease was equal to (125 : 50)·(50 : 30), i.e., 4.2 times, which corresponded to an approximately 4.3-fold H_2O_2-induced decrease (130 : 30 ≈ 4.3) in the rate of the process in cells untreated with EDTA (Table 1). A significant decrease in the rate of reduction of $P700^+$ (decrease in $t_{1/2}$ to 400 msec) was observed in cells treated with EDTA and exposed to a combination of H_2O_2, CN^-, and EDTA.

Treatment of cells with EDTA caused a decrease in the amount of manganese extractable with 100 mM HCl to 35% (Table 2). The content of free (or loosely bound) Mn^{2+} in cyanobacterial cells before and after treatment with EDTA was 20 and 5% of the total manganese pool, respectively. These values increased to 25 and 35%, respectively, after exposure to 40 mM H_2O_2 or to 30 and 45%, respectively, after exposure to 200 mM H_2O_2. The addition of CN^- (3 mM) had no effect on the efficiency of the manganese extraction by hydrogen peroxide.

The inhibition effect of H_2O_2 was not restricted to OEC alone. In addition to H_2O, PS II is able to oxidize some other compounds (see [21, 56] and references therein). Oxidation of some of these compounds (e.g., H_2O_2 and NH_2OH) is mediated by the Mn-cluster of the OEC. Other compounds are oxidized without involvement of the Mn-cluster of the OEC, for instance such compounds as diphenylcarbazide, tetraphenylboron, and ionol (butylhydroxytoluene) are able to interact directly with component Y_z. None of the compounds of the two types supported photoinduced reduction of BQ + FeCy in *A. variabilis* cells treated with H_2O_2 [21]. The light-dependent electron transfer from NH_2OH or diphenylcarbazide to methyl viologen was not supported either. These data are evidence of the H_2O_2-induced inhibition of Y_z. Perhaps, this inhibition is due to the H_2O_2-dependent oxidation of tyrosine residues by the mechanism similar to that observed in case of oxidation of phenylpropane derivatives during lignin biosynthesis [57] and further interaction of resulting tyrosine radicals giving rise to dityrosine cross-linking. Possible formation of dityrosine cross-links was described elsewhere [19]. Therefore, it seems fairly improbable that the mechanism of the photoinduced Mn-independent oxidation of H_2O_2 mediated by $Y_z^{\cdot}$ (Eqs. (5) and (6)) are actually found in fact (see [11] for more detail).

More remote aftereffects induced by H_2O_2 include photoinhibition of photosynthesis associated with functional insufficiency of the electron donor branch of PS II [19]. In a long run, this insufficiency results in degradation of photosynthetic pigments, cell lysis (Table 3), and cell death [20]. Phycocyanin capable of neutralizing radicals $HO^{\cdot}$, $HO_2^{\cdot}$, $O_2^{\cdot-}$, and peroxynitrite (see [58] and references therein) were subjected to considerable degradation (Table 3). It was shown in experiments with *Synechocystis* 6803 that H_2O_2 inhibited reparation of the photoinduced damage of PS II by suppressing protein biosynthesis at the level of translation (including biosynthesis of protein D1, which is a component of the reaction center complex of PS II [59]). Inhibition of reparation of photo-inactivated PS II in *Synechocystis* 6803 at the level of transcription and translation of genes *psbA*, which is responsible for biosynthesis of protein D1, can be induced by 0.5-1.0 M NaCl [60]. Salt-induced death of *A. variabilis* cells occurs by the mechanism of apoptosis: activity of proteases in cells increases, cytoplasm membrane integrity is disrupted, DNA is fragmented, cytoplasm undergoes vacuolization, and at the terminal stage of the process the cell is subjected to autolysis [61]. Cell death with all features inherent in apoptosis was demonstrated in the obligate photolithoautotrophic single-cell green alga *Dunaliella tertiolecta* exposed to conditions of light deficiency [62].

Thus, H_2O_2 induces extraction of manganese from cyanobacterial cells, degradation of the photosynthetic apparatus, and cell death. These results rule out the hypothesis that H_2O_2 is able to serve as a possible physiological electron donor in photosynthesis [13, 63].

This study was supported by the Russian Foundation for Basic Research (project No. 01-04-48356). We are grateful to T. A. Fedorenko for assistance in reinoculation of *A. variabilis* and preparation of pH buffer solutions.

REFERENCES

1. Debus, R. J. (1992) *Biochim. Biophys. Acta*, **1102**, 269-352.
2. Ananyev, G. M., Zaltsman, L., Vasko, C., and Dismukes, G. C. (2001) *Biochim. Biophys. Acta*, **1503**, 52-68.
3. Metzner, H. (1983) in *Bioelectrochem. Proc. Course*, Vol. 1, N. Y., pp. 51-113.
4. Dismukes, G. C., Klimov, V. V., Baranov, S. V., Kozlov, Yu. N., DasGupta, J., and Tyryshkin, A. (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 2170-2175.
5. Radmer, R., and Ollinger, O. (1980) *FEBS Lett.*, **110**, 57-61.
6. Bonini, M. G., Radi, R., Ferrer-Sueta, G., Ferreira, A. M. D. C., and Augusto, O. (1999) *J. Biol. Chem.*, **274**, 10802-10806.
7. Koppenol, W. H. (1994) in *Free Radical Damage and Its Control* (Rice-Evans, C. A., and Burdon, R. H., eds.) Elsevier Sci., pp. 3-24.
8. Koppenol, W. H., and Rush, J. D. (1987) *J. Phys. Chem.*, **91**, 4429-4430.
9. Ananyev, G., Wydrzynski, T., Renger, G., and Klimov, V. V. (1992) *Biochim. Biophys. Acta*, **1100**, 303-311.
10. Klimov, V. V., Ananyev, G., Zastryzhnaya, O., Wydryzynski, T., and Renger, G. (1993) *Photosynth. Res.*, **38**, 409-416.
11. Sheptovitsky, Y. G., and Brudvig, G. W. (1998) *Biochemistry*, **37**, 5052-5059.
12. Kelly, P., and Izawa, S. (1978) *Biochim. Biophys. Acta.*, **502**, 198-210.
13. Bader, K. P. (1994) *Biochim. Biophys. Acta*, **1188**, 213-219.
14. Samuilov, V. D. (1997) *Biochemistry (Moscow)*, **62**, 451-454.
15. Blankenship, R. E., and Hartman, H. (1998) *Trends Biochem. Sci.*, **23**, 94-97.
16. Velthuys, B., and Kok, B. (1978) *Biochim. Biophys. Acta*, **502**, 211-221.
17. Mano, J., Takahashi, M., and Asada, K. (1987) *Biochemistry*, **26**, 2495-2501.
18. Sheptovitsky, Y. G., and Brudvig, G. W. (1996) *Biochemistry*, **35**, 16255-16263.
19. Bradley, R. L., Long, K. M., and Frasch, W. D. (1991) *FEBS Lett.*, **286**, 209-213.
20. Samuilov, V. D., Bezryadnov, D. V., Gusev, M. V., Kitashov, A. V., and Fedorenko, T. A. (1999) *Biochemistry (Moscow)*, **64**, 47-53.
21. Samuilov, V. D., Bezryadnov, D. V., Gusev, M. V., Kitashov, A. V., and Fedorenko, T. A. (2001) *Biochemistry (Moscow)*, **66**, 640-645.
22. Wydrzynski, T., Ångstrom, J., and Vänngard, T. (1989) *Biochim. Biophys. Acta*, **973**, 23-28.
23. Sandusky, P. O., and Yocum, C. F. (1988) *Biochim. Biophys. Acta*, **936**, 149-156.
24. Frasch, W. D., Mei, R., and Sanders, M. A. (1988) *Biochemistry*, **27**, 3715-3719.
25. Ghanotakis, D. F., Topper, J. N., and Yocum, C. F. (1984) *Biochim. Biophys. Acta*, **767**, 524-531.
26. Schröder, W. P., and Åkerlund, H.-E. (1986) *Biochim. Biophys. Acta*, **848**, 359-363.
27. Ishida, H., Shimizu, S., Makino, A., and Mae, T. (1998) *Planta*, **204**, 305-309.
28. Asada, K., and Badger, M. R. (1984) *Plant Cell Physiol.*, **25**, 1169-1179.
29. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y. (1979) *J. Gen. Microbiol.*, **111**, 1-61.
30. Barsky, E. L., Gusev, M. V., Kondrashin, A. A., and Samuilov, V. D. (1982) *Biochim. Biophys. Acta*, **680**, 304-309.
31. Dawson, R., Elliott, D., Elliott, W., and Jones, K. (1991) *Handbook of Biochemist* [Russian translation], Mir, Moscow.
32. Bielski, B. H. J., and Allen, A. O. (1977) *J. Phys. Chem.*, **81**, 1048-1050.
33. Yocum, C., Yerkes, C. T., Blankenship, R., Sharp, R. R., and Babcock, G. T. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7507-7511.
34. Hirano, M., Satoh, K., and Katoh, S. (1980) *Photosynth. Res.*, **1**, 149-162.
35. Lockau, W. (1981) *Arch. Microbiol.*, **128**, 336-340.
36. Peschek, G. A., and Schmetterer, F. (1982) *Biochem. Biophys. Res. Commun.*, **108**, 1188-1195.
37. Aples, I., Stürzl, E., Scherer, S., and Böger, P. (1984) *Z. Naturforsch.*, **C 39**, 623-625.
38. Abdourashitova, F. D., Barsky, E. L., Gusev, M. V., and Samuilov, V. D. (1985) *Planta*, **166**, 182-186.
39. Rubin, P. M., Zetooney, E., and McGowan, R. E. (1977) *Plant Physiol.*, **60**, 407-411.
40. Peschek, G. A. (1980) *Arch. Microbiol.*, **125**, 123-131.
41. Finnegan, P. M., Umbach, A. L., and Wilce, J. A. (2003) *FEBS Lett.*, **555**, 425-430.
42. Berry, S., Schneider, D., Vermaas, W. F. J., and Rögner, M. (2002) *Biochemistry*, **41**, 3422-3429.
43. Asada, K. (2000) *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **355**, 1419-1431.
44. Mi, H., Endo, T., Schreiber, U., Ogawa, T., and Asada, K. (1992) *Plant Cell Physiol.*, **33**, 1233-1237.
45. Mi, H., Endo, T., Ogawa, T., and Asada, K. (1995) *Plant Cell Physiol.*, **36**, 661-668.
46. Joët, T., Cournac, L., Peltier, G., and Havaux, M. (2002) *Plant Physiol.*, **128**, 760-769.
47. Bendall, D. S., and Manasse, R. S. (1995) *Biochim. Biophys. Acta*, **1229**, 23-38.
48. Myers, J. (1987) *Photosynth. Res.*, **14**, 55-69.
49. Yu, L., Zhao, J., Mühlenhoff, U., Bryant, D. A., and Golbeck, G. H. (1993) *Plant Physiol.*, **103**, 171-180.
50. Joliot, P., and Joliot, A. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 10209-10214.
51. Samuilov, V. D., and Fedorenko, T. A. (1999) *Biochemistry (Moscow)*, **64**, 610-619.
52. Takano, M., Takahashi, M.-A., and Asada, K. (1982) *Arch. Biochem. Biophys.*, **218**, 369-375.
53. Takahashi, M.-A., and Asada, K. (1976) *Eur. J. Biochem.*, **64**, 445-452.
54. Koulougliotis, D., Kostopoulos, T., Petrouleas, V., and Diner, B. A. (1993) *Biochim. Biophys. Acta*, **1141**, 275-282.
55. Keren, N., Kidd, M. J., Penner-Hahn, J. E., and Pakrasi, H. (2002) *Biochemistry*, **41**, 15085-15092.
56. Samuilov, V. D., Renger, G., Paschenko, V. Z., Oleskin, A. V., Gusev, M. V., Gubanova, O. N., Vasil'ev, S. S., and Barsky, E. L. (1995) *Photosynth. Res.*, **46**, 455-465.
57. Önnerud, H., Zhang, L., Gellerstedt, G., and Henriksson, G. (2002) *Plant Cell*, **14**, 1953-1962.
58. Bhat, V. B., and Madyastha, K. M. (2001) *Biochem. Biophys. Res. Commun.*, **285**, 262-266.
59. Nishiyama, Y., Yamamoto, H., Allakhverdiev, S. I., Inaba, M., Yokota, A., and Murata, N. (2001) *EMBO J.*, **20**, 5587-5594.
60. Allakhverdiev, S. I., Nishiyama, Y., Miyairi, S., Yamamoto, H., Inagaki, N., Kanesaki, Y., and Murata, N. (2002) *Plant Physiol.*, **130**, 1443-1453.
61. Ning, S.-B., Guo, H.-L., Wang, L., and Song, Y.-C. (2002) *J. Appl. Microbiol.*, **93**, 15-28.
62. Segovia, M., Haramaty, L., Berges, J. A., and Falkowski, P. G. (2003) *Plant Physiol.*, **132**, 99-105.
63. Komissarov, G. G. (1995) *Khim. Fiz.*, **14**, 20-28.