Biochemistry (Moscow), Vol. 66, No. 6, 2001, pp. 640-645. Translated from Biokhimiya, Vol. 66, No. 6, 2001, pp. 790-796.

Hydrogen Peroxide Inhibits Photosynthetic Electron Transport in Cells of Cyanobacteria

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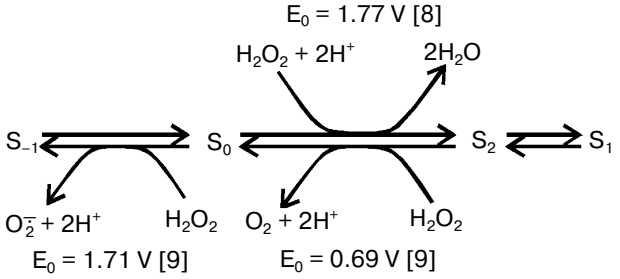
Received October 9, 2000

Abstract—The effect of H_2O_2 on photosynthetic O_2 evolution and photosynthetic electron transfer in cells of cyanobacteria *Anabaena variabilis* and *Anacystis nidulans* was studied. The following experiments were performed: 1) directly testing the effect of exogenous H_2O_2; 2) testing the effect of intracellular H_2O_2 generated with the use of methyl viologen (MV); 3) testing the effect of inhibiting intracellular H_2O_2 decomposition by salicylic acid (SA) and 3-amino-1,2,4-triazole (AT). H_2O_2 inhibited photosynthetic O_2 evolution and light-induced reduction of *p*-benzoquinone (BQ) + ferricyanide (FeCy) in the Hill reaction. The I_{50} value for H_2O_2 was ~0.75 mM. Photosynthetic electron transfer in the cells treated with H_2O_2 was not maintained by H_2O_2, NH_2OH, 1,5-diphenylcarbazide, tetraphenylboron, or butylated hydroxytoluene added as artificial electron donors for Photosystem (PS) II. The $H_2O \rightarrow CO_2$, $H_2O \rightarrow MV$ (involving PSII and PSI) and $H_2O \rightarrow BQ + FeCy$ (chiefly dependent on PSII) electron transfer reactions were inhibited upon incubation of the cells with MV, SA, or AT. The N,N,N′,N′-tetramethyl-*p*-phenylenediamine → MV (chiefly dependent on PSI) electron transfer was inhibited by SA and AT but was resistant to MV. The results show that H_2O_2 inhibits photosynthetic electron transfer. It is unlikely that H_2O_2 could be a physiological electron donor in oxygenic photosynthesis.

Key words: photosynthesis, photosynthetic oxygen, water-oxidizing complex, Photosystem II, Photosystem I, hydrogen peroxide, cyanobacteria, *Anabaena variabilis*, *Anacystis nidulans*

Photosynthetic O_2 evolution includes four stages resulting in a gradual accumulation of oxidizing equivalents in the Mn-containing water-oxidizing complex (WOC, reviewed in [1]). Based on these one-electron reactions, five distinct redox states of the WOC, designated S_0-S_4, are distinguished. The high E_0' value of P680, the reaction center of Photosystem (PS) II, estimated to be 1.12 V [2], enables the oxidation of a number of agents (in addition to H_2O) [1, 3] including (a) low molecular weight hydrophilic NH_2OH-like compounds that cause the formation of over-reduced states of the WOC (S_{-1} and S_{-2}); (b) hydrophobic tetraphenylboron-like compounds that efficiently compete with H_2O as electron donors; and (c) ADRY agents causing the deactivation of the WOC. Of particular interest among these compounds is hydrogen peroxide. Both chloroplasts [4] and cyanobacterial thylakoids [5] were shown to carry out light-dependent H_2O_2 oxidation and O_2 evolution in studies using $H_2{}^{18}O_2$. The interaction of H_2O_2 with the S states of the WOC is illustrated by the scheme below ([4, 6], see also review [7]):



It has been suggested that H_2O_2 was an evolutionary precursor of H_2O as the electron donor for PSII in cyanobacteria ([5], reviewed in [7, 10]). Moreover, it has been proposed that O_2 is produced from extra- and intracellular H_2O_2, not H_2O, during modern photosynthesis [11]. However, the H_2O_2 concentration in natural water bodies is low (10^{-6}-10^{-5} M) [12]. Therefore, plants were presumed to concentrate H_2O_2 by transpiration [11].

Abbreviations: AT) 3-amino-1,2,4-triazole; BQ) *p*-benzoquinone; DCMU) 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FeCy) potassium ferricyanide; MV) methyl viologen; PS) Photosystem; SA) salicylic acid; TB^-) sodium tetraphenylborate; TMPD) N,N,N′,N′-tetramethyl-*p*-phenylenediamine; WOC) water-oxidizing complex.

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0006-2979/01/6606-0640$25.00 ©2001 MAIK "Nauka / Interperiodica"

Based on the idea that H_2O_2 operates as an electron donor during oxygenic photosynthesis, photosynthetic organisms are expected to be H_2O_2-resistant. Moreover, H_2O_2 should exert a stimulatory influence on them, because its concentration in the environment is very low. However, our studies with cyanobacteria revealed an inhibitory effect of H_2O_2 [13]. The influence of H_2O_2 on the phototrophic growth of cyanobacteria was investigated. We tested (a) the direct action of exogenous H_2O_2; (b) the influence of the H_2O_2 generated inside the cell in the presence of methyl viologen, menadione, or phenazine methosulfate; and (c) the effect caused by suppressing the intracellular degradation of H_2O_2 by salicylic acid, a catalase inhibitor. The growth of cyanobacteria was suppressed in all these systems. H_2O_2 inhibited growth at concentrations as low as 10^{-5}-10^{-4} M under the conditions of a dialysis culture [13].

However, these data do not invalidate the suggestion that H_2O_2 functions as an electron donor during oxygenic photosynthesis, because the suppression of the growth of cyanobacteria may not be caused by the inhibition of the WOC. H_2O_2, an oxidant, can influence a large number of stages of cell metabolism including those involved in the induction of programmed cell death [14, 15]. In addition, the tested agents, apart from inducing H_2O_2 formation, can produce multiple effects on electron transport.

H_2O_2 was shown to inhibit light-dependent O_2 evolution in the Hill reaction with ferricyanide in studies with membrane preparations of spinach PSII with inactivated catalase that were incubated with the catalase inhibitor 3-amino-1,2,4-triazole [16]. The Mn cluster of the WOC in Cl^--depleted PSII preparations was earlier shown to lose manganese in the presence of H_2O_2 [17], but this loss proved insignificant even upon treating the membranes with 120 mM H_2O_2 [16]. Some objections can be raised concerning the results of work [16]: (a) the H_2O_2 effects should have been tested, if possible, in intact systems rather than in membranes prepared by a peculiar method that may lack the H_2O_2-sensitive components and (b) the tested H_2O_2 concentrations were too high, and they could cause conformational changes in, and even the denaturation of, the proteins contained in the system, thereby preventing the release of Mn from the WOC (this is consistent with data on a significant decrease in the sensitivity of PSII to the inhibitory effect of DCMU [16]).

H_2O_2 is generated by PSII in the light both in its electron-donating [18] and electron-accepting (see [19] and references therein) branches. There are data indicating that the H_2O_2 formed in both PSII branches causes the inhibition of light-dependent O_2 evolution by spinach thylakoids [20].

Hence, no unambiguous data on the role of H_2O_2 during oxygenic photosynthesis have been obtained, and no consensus has been reached on this point. The goal of this work was to investigate the effects of H_2O_2 on photosynthetic O_2 evolution and photosynthetic electron transfer in intact cells of cyanobacteria.

MATERIALS AND METHODS

Cells of *Anabaena variabilis* Kütz No. 458 and *Anacystis nidulans* Kütz No. 478 were grown under illumination in batch cultures on medium C [21] as described earlier [22]. Cells from 3-5 day old cultures undergoing exponential growth were used in the experiments.

O_2 evolution and uptake by illuminated *A. variabilis* cells were monitored using a closed Clark platinum electrode. The cells were illuminated with white light of saturating intensity (~0.1 W/cm^2). Photoreduction of *p*-benzoquinone (BQ) in the Hill reaction was followed employing standard sterile polystyrene 96-well plates used in immunological studies. The volume of the cell suspension in a well was 150 μl. The rate of BQ reduction was determined from ferrocyanide formation: photoreduced BQ + $K_3[Fe(CN)_6]$ → oxidized BQ + $K_4[Fe(CN)_6]$. The experiment was done in eight repeats. Ferricyanide reduction was monitored using a vertical Multiscan Plus 314 photometer (Labsystem, USA) with an interference filter with maximum transmission band of 405 nm.

RESULTS AND DISCUSSION

Figure 1a shows the CO_2-dependent evolution of O_2 by illuminated *A. variabilis* cells. The process is suppressed by NaCN, an inhibitor of ribulose-1,5-bisphosphate carboxylase (see [23] and references therein). The addition of potassium ferricyanide (FeCy), an electron acceptor in the Hill reaction, does not cause the reactivation of O_2 evolution, indicating that the cells of the cyanobacteria are intact: their cytoplasmic membrane is FeCy-impermeable. Upon the subsequent addition of the penetrating agent *p*-benzoquinone (BQ), illuminating the cells induces O_2 evolution, whose rate does not change upon the addition of catalase (catalase was added because it is contained in the incubation medium of the experiment in Fig. 1b; 1 mM NaCN only partially inhibits catalase activity). The process is blocked by DCMU, an inhibitor of electron transfer at the secondary plastoquinone level. Figure 1b demonstrates that *A. variabilis* cells that were preincubated with H_2O_2 and NaCN for 45 min in the dark and then with catalase for 5 min in the light to remove the added H_2O_2, completely lost their activity in the Hill reaction with BQ + FeCy. Similar data were obtained with *A. nidulans* cells.

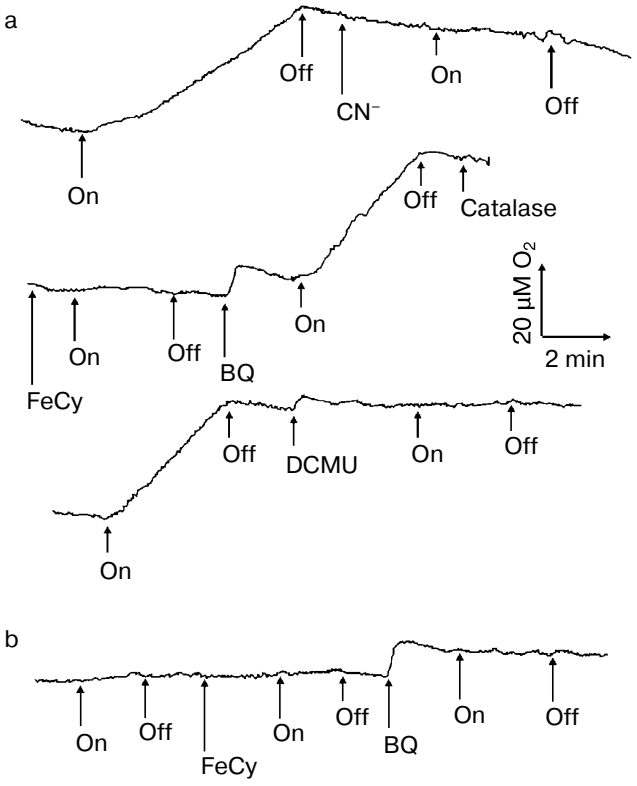

Fig. 1. Effect of H_2O_2 on photosynthetic O_2 evolution by *A. variabilis* cells: a) control system; O_2 evolution with CO_2 and BQ + FeCy as electron acceptors; the data were recorded continuously, and they are presented in a three-tier diagram because these data cannot be arranged in one tier; b) suppression of the Hill reaction by BQ + FeCy upon preincubation of the cells for 45 min in the dark with 1 mM NaCN and 3 mM H_2O_2 and then for 5 min with catalase (100 μg/ml). The cells were washed with 20 mM Tricine-NaOH buffer (pH 7.6) containing 10 mM NaCl and subsequently incubated in the same solution. The chlorophyll concentration was 7 μg/ml. Additions: 1 mM NaCN, 3 mM FeCy, 100 μM BQ, 100 μg/ml catalase, 5 μM DCMU. On and Off, switching on and off the light.

The suppression of O_2 evolution by illuminated cells does not necessarily imply that H_2O oxidation is arrested. The reaction may proceed without O_2 evolution, coming to a stop at the intermediate stage of H_2O_2 formation (see [24] and review [7]). The 2,6-dichlorophenolindophenol, phenyl-*p*-benzoquinone, or phenyl-*p*-benzoquinone + FeCy amounts reduced in the Hill reaction (on the electron equivalent basis) exceeded more than 1.5-fold the O_2 amount evolved by PSII membrane particles [24]. Adding catalase eliminated this discrepancy. Hence, a significant part of the electrons transferred *via* PSII was used to form H_2O_2, not to evolve O_2 [24].

Figure 2 demonstrates that BQ photoreduction determined from the oxidation of reduced BQ by added FeCy proceeded in an almost linear fashion (vs. time) in *A. variabilis* cell suspension. The process was completed in 30-40 min. It was suppressed by treating cells with H_2O_2. The slight FeCy photoreduction occurring with H_2O_2 and H_2O_2 + DCMU is in all likelihood due to the operation of PSI, because DCMU did not cause any additional inhibitory effect. FeCy and other electron acceptors in the Hill reaction are reduced by both PSII and PSI [25]. The contributions of PSI and PSII to FeCy reduction increase with an increase in light intensity and FeCy concentration, respectively [25]. Thus, treating cells with H_2O_2 results in the suppression of both O_2 evolution and coupled BQ + FeCy reduction in the Hill reaction. It follows that the inhibitory action of H_2O_2 apparently affects the initial stages of H_2O oxidation, not the intermediate stages resulting in H_2O_2 formation.

Figure 3 shows that the I_{50} value for H_2O_2 (causing a 50% decrease in WOC activity) was ~0.75 mM for *A. nidulans* cells. *A. variabilis* cells were characterized by a similar I_{50} value (data not shown).

Apart from H_2O, PSII can oxidize a number of other compounds [1, 3]. The oxidation of some of them, including H_2O_2 and NH_2OH, involves the Mn cluster of the WOC [1]. Other compounds can be oxidized without the involvement of the Mn cluster, directly interacting with component Y_Z, the tyrosine-161 residue in the D1 subunit of the PSII complex. They include diphenylcar-

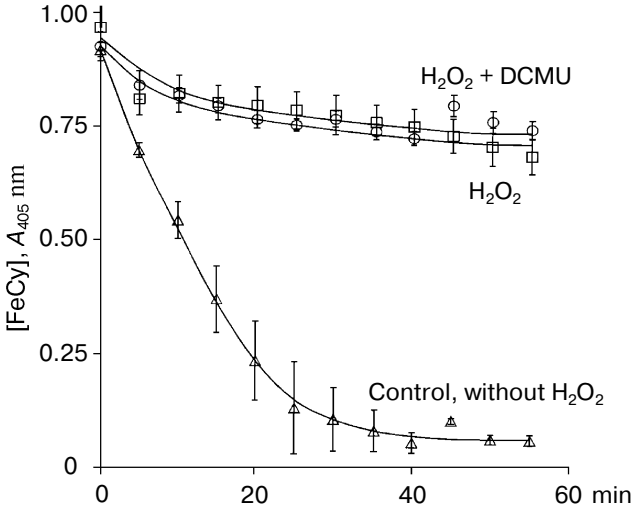

Fig. 2. Effect of H_2O_2 on light-induced electron transfer from H_2O to BQ + FeCy in *A. variabilis* cells. Cells with chlorophyll content of 7 μg/ml were preincubated in the culture medium with consecutive additions of 1 mM NaCN (10 min of incubation), 3 mM H_2O_2 (45 min), and 40 μg/ml catalase (5 min) in the dark. Then, 3 mM FeCy and 100 μM BQ were added and the system was incubated in the light. The DCMU concentration was 10 μM.

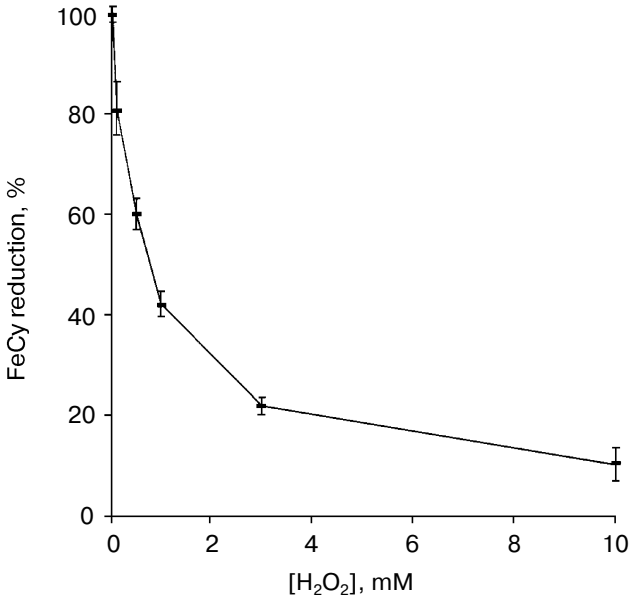

Fig. 3. Effect of various H_2O_2 concentrations on the photosynthetic electron transfer from H_2O to BQ + FeCy in *A. nidulans* cells. Cells with chlorophyll content of 7 μg/ml were preincubated in the culture medium with consecutive additions of 1 mM NaCN (10 min of incubation), 3 mM H_2O_2 (45 min), and 40 μg/ml catalase (5 min) in the dark. Then, 3 mM FeCy and 100 μM BQ were added and the system was incubated in the light. The 100% FeCy photoreduction rate was 524 μmol/mg of chlorophyll per hour.

bazide [1], TB^- [26, 27], and butylated hydroxytoluene [28-30]. The tested compounds of both types did not maintain BQ + FeCy photoreduction in H_2O_2-treated cells. The reaction rate with H_2O, H_2O_2, TB^-, and butylated hydroxytoluene as electron donors in H_2O_2-treated cells practically did not exceed that in cells with DCMU-blocked PSII (table). No light-dependent electron transfer from NH_2OH or diphenylcarbazide to methyl viologen (resulting in O_2 uptake) occurred in H_2O_2-treated *A. variabilis* cells (data not shown).

The final experiments dealt with the influence of intracellular H_2O_2 formation on the photosynthetic electron transport in *A. variabilis*. Methyl viologen (MV) was used as inducer of H_2O_2 generation. It is reduced by components of the photosynthetic chain (chiefly by FeS center F_B and, to a lesser extent, by FeS center F_A of the electron acceptor complex of PSI [31]) and by the dehydrogenases of the respiratory chain. A one-electron reaction between MV and O_2 results in the generation of $O_2^{\cdot-}$, which is converted to H_2O_2 in a superoxide dismutase-dependent reaction. Experiments have also been done with salicylic acid (SA), a catalase [32, 33] and ascorbate peroxidase [32] inhibitor. MV and SA were earlier shown by us to suppress the phototrophic growth of *A. variabilis* and *A. nidulans* [13]. SA, a phenolic compound, can cause a side effect, a dinoseb-like inhibition of the photosynthetic electron transport at the plastoquinone Q_B level. Therefore, we used, in addition to SA, 3-amino-1,2,4-triazole (AT), which also inhibits Fe-catalase activity. AT was earlier used in work [16]. The three tested compounds suppressed electron transfer with CO_2 or MV as terminal electron acceptor (Fig. 4), a process involving the complete set of the photosynthetic chain components (WOC, PSII, the cytochrome b_6f complex, and PSI). Unlike the $H_2O \rightarrow CO_2$ pathway, the $H_2O \rightarrow$ MV pathway does not involve ribulosebisphosphate carboxylase, which is blocked by CN^-. We also separately tested the links of the photosynthetic chain: the $H_2O \rightarrow$ BQ + FeCy (predominantly involving PSII) and the TMPD $\rightarrow$ MV (primarily involving PSI) electron transfer. The $H_2O \rightarrow$ BQ + FeCy electron transfer is inhibited by treating the cells of cyanobacteria with MV, SA, or AT (Fig. 4). The TMPD $\rightarrow$ MV electron transfer is retarded by SA and AT, but it is resistant to MV.

Thus, extra- and intracellular H_2O_2 causes multiple effects: it inhibits PSII and, to some extent, PSI activities. These effects manifest themselves in native systems—intact cells of cyanobacteria. Therefore, they cannot be due to a lack of certain components (such as the enzymes eliminating a reactive oxygen species) in this system, in contrast to isolated membranes or membrane particles. Added H_2O_2 produces a half-maximum inhibitory effect on PSII activity at concentrations as low as ~0.75 mM (Fig. 3). The results indicate that H_2O_2 disrupts the operation of PSII. H_2O_2 and other reactive oxygen species

Effect of H_2O_2 on Photosystem II activity in *A. variabilis* cells with various electron donors. The experimental conditions were the same as in Fig. 2. The 100% FeCy photoreduction rate was 860 μmol/mg chlorophyll per hour

Electron transfer	Reaction rate, %
H_2O_2-untreated cells	
$H_2O \rightarrow$ BQ + FeCy	100
The same plus 10 μM DCMU	7
H_2O_2-treated cells	
$H_2O \rightarrow$ BQ + FeCy	7
$H_2O_2 \rightarrow$ BQ + FeCy	6
$TB^- \rightarrow$ BQ + FeCy	12
Butylated hydroxytoluene $\rightarrow$ BQ + FeCy	0

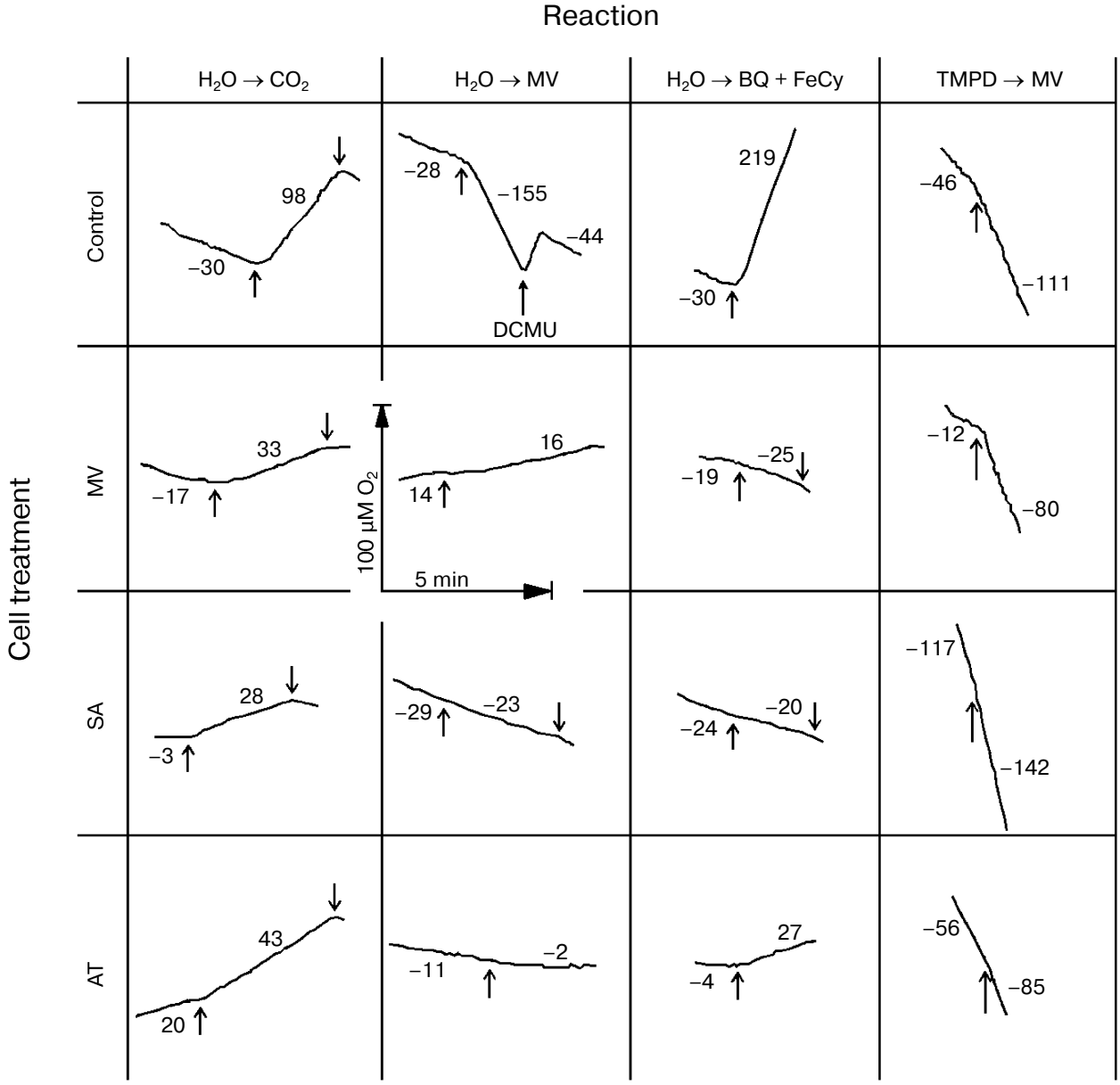

Fig. 4. Effects of methyl viologen (MV), salicylic acid (SA), and 3-amino-1,2,4-triazole (AT) on the electron transport in illuminated *A. variabilis* cells. Cells with chlorophyll content of 7 μg/ml were preincubated with 100 μM MV, 5 mM SA sodium salt, or 40 mM AT under illumination for 15 h in the culture medium; the electron transport in the $H_2O \rightarrow CO_2$ (based on light-dependent O_2 evolution), $H_2O \rightarrow$ MV (based on light-dependent O_2 evolution), and $H_2O \rightarrow$ BQ + FeCy (based on light-dependent O_2 uptake) reactions was monitored. Before measuring $H_2O \rightarrow$ MV activity, the cells were preincubated in the dark for 15 min with 1 mM NaCN and 1 mM MV. Before measuring $H_2O \rightarrow$ BQ + FeCy activity, the cells were preincubated in the dark for 15 min with 1 mM NaCN and 3 mM FeCy; 100 μM BQ was added into the oxymetric cell. Before measuring TMPD → MV activity, the cells were preincubated in the dark for 15 min with 1 mM NaCN, 1 mM MV, 5 mM ascorbate, and 10 μM DCMU; 100 μM TMPD was added into the oxymetric cell. Upward and downward arrows signify switching on and off the light, respectively. Numbers quantify the O_2 evolution or uptake (a minus in front of the number), O_2/mg chlorophyll per hour.

appear to be the main factors causing the photoinhibition of photosynthesis. H_2O_2 at a concentration of 10 μM inhibits CO_2 fixation [34] by inactivating the Calvin cycle enzymes containing SH groups [35, 36]. This effect could be due to the inhibition of the ascorbate peroxidase system of H_2O_2 detoxication [37] because the studies described in work [34] were conducted in the presence of KCN.

This work was supported by grant 98-04-48226 from the Russian Foundation for Basic Research.

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