Biochemistry (Moscow), Vol. 69, No. 4, 2004, pp. 445-454. Translated from Biokhimiya, Vol. 69, No. 4, 2004, pp. 549-559.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM03-165, February 9, 2004.

Reduction of Photosystem I Reaction Center in DrgA Mutant of the Cyanobacterium *Synechocystis* sp. PCC 6803 Lacking Soluble NAD(P)H:Quinone Oxidoreductase

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Received July 7, 2003
Revision received August 20, 2003

Abstract—Photoautotrophically grown cells of the cyanobacterium *Synechocystis* sp. PCC 6803 wild type and the Ins2 mutant carrying an insertion in the *drgA* gene encoding soluble NAD(P)H:quinone oxidoreductase (NQR) did not differ in the rate of light-induced oxygen evolution and Photosystem I reaction center ($P700^+$) reduction after its oxidation with a white light pulse. In the presence of DCMU, the rate of $P700^+$ reduction was lower in mutant cells than in wild type cells. Depletion of respiratory substrates after 24 h dark-starvation caused more potent decrease in the rate of $P700^+$ reduction in DrgA mutant cells than in wild type cells. The reduction of $P700^+$ by electrons derived from exogenous glucose was slower in photoautotrophically grown DrgA mutant than in wild type cells. The mutation in the *drgA* gene did not impair the ability of *Synechocystis* sp. PCC 6803 cells to oxidize glucose under heterotrophic conditions and did not impair the NDH-1-dependent, rotenone-inhibited electron transfer from NADPH to $P700^+$ in thylakoid membranes of the cyanobacterium. Under photoautotrophic growth conditions, NADPH-dehydrogenase activity in DrgA mutant cells was less than 30% from the level observed in wild type cells. The results suggest that NQR, encoded by the *drgA* gene, might participate in the regulation of cytoplasmic NADPH oxidation, supplying $NADP^+$ for glucose oxidation in the pentose phosphate cycle of cyanobacteria.

Key words: cyanobacterium *Synechocystis* sp. PCC 6803, *drgA* gene, NAD(P)H:quinone oxidoreductase, NAD(P)H-dehydrogenase, Photosystem I, EPR spectroscopy

Thylakoid membranes of cyanobacteria contain both photosynthetic and respiratory electron transport chains (ETC) [1]. Some intermediate electron carriers, including the plastoquinone (PQ) pool, cytochrome b_6f complex, and soluble cytochrome c_{553} (or plastocyanin) are involved in photosynthetic and respiratory electron transport [2]. Electrons from NADPH can enter the photosynthetic ETC via the b_6f complex; this process also involves NADH dehydrogenase-1 (NDH-1). The latter is a multi-subunit complex causing transmembrane proton translocation [3-5]. The role of NDH-1 in Photosystem I (PSI) involves electron cycling in the photoheterotrophic cyanobacterium *Synechocystis* sp. PCC 6803 (further *Synechocystis* 6803) [3-5] as demonstrated using a mutant lacking the NdhB subunit of the NDH-1 complex [6].

Succinate dehydrogenase (SDH) plays a central role in dark reduction of the PQ pool of *Synechocystis* 6803 cells [7]. Activity of SDH is mainly determined by the availability of succinate in cells. In *Synechocystis* 6803 mutants characterized by impairments in NDH-1 and NDH-2, succinate level is strongly reduced due to limits of $NADP^+$ or NAD^+, respectively. Based on these observations it was suggested that NDH-1 and NDH-2 are involved in regulation of the ratio between reduced and oxidized nicotinamide nucleotides, and this influences electron flow through SDH [7].

Synechocystis 6803 cells contain functionally active soluble NAD(P)H:quinone oxidoreductase (NQR) encoded by the *drgA* gene [8-10]. This oxidoreductase catalyzes reduction of exogenous quinones to hydroquinones [11]. Hydroquinones (e.g., menadiol) are involved in cyclic electron flow around PSI [12]; this can be measured by the increased rate of reduction of the PSI

Abbreviations: DCMU) 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ETC) electron transport chain; NQR) NAD(P)H:quinone oxidoreductase; PQ) plastoquinone; PS) photosystem; SDH) succinate dehydrogenase.

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reaction center ($P700^+$) [11]. Thus, NQR may influence electron transport in thylakoids of *Synechocystis* 6803.

For elucidation of NQR function in *Synechocystis* 6803 cells we have investigated kinetics of $P700^+$ reduction after its oxidation with a white light pulse and some other characteristics of wild type cells and the mutant Ins2 lacking functionally active protein product of the *drgA* gene.

MATERIALS AND METHODS

Strains and cultivation conditions. A wild strain of photoheterotrophic cyanobacterium *Synechocystis* sp. PCC 6803 and the Ins2 mutant carrying insertion of kanamycin resistance gene in *BamHI*-site of the *drgA* gene [8] were from the collection of the Department of Genetics (Lomonosov Moscow State University). Cells were photoautotrophically grown at 30°C and constant illumination of 50 $\mu E \cdot m^{-2} \cdot sec^{-1}$ using the liquid mineral medium BG-11 [13] under aeration enriched with 2% CO_2. In some experiments, cells were grown without aeration at reduced illumination (20 $\mu E \cdot m^{-2} \cdot sec^{-1}$). The mutant cells were grown in the presence of 20 μg/ml kanamycin (Sigma, USA). For depletion of respiratory substrates, three-day cultures, grown under photoautotrophic conditions, were exposed to darkness at 30°C for 24 h. Glucose (final concentration 5 mM) was added where indicated.

Isolation of membrane fraction. Cells at late logarithmic stage of growth were sedimented by centrifugation (4000*g*, 4°C). The sediment was washed twice with solution A [5] containing 25% (v/v) glycerol, 10 mM $MgCl_2$, 10 mM NaCl, and 20 mM Na-phosphate buffer, pH 7.5. The resulting pellet was suspended in the same solution; chlorophyll concentration in the suspension was 50 μg/ml. Cell suspension incubated on ice for 1 h was sonicated using Soniprep-150 disintegrator (MSE, England). There were four ultrasonic treatments for 30 sec followed by 1 min intervals at 0°C. Unbroken cells were removed by centrifugation at 4000*g* for 10 min. For subsequent separation of membrane and soluble fractions, the cell-free preparations were centrifuged for 140,000*g* for 1 h. The pellet (membrane fraction) was resuspended in solution A, immediately placed on ice, and used for analysis.

EPR spectroscopy. Cells were sedimented by centrifugation and suspended in BG-11 medium containing 10 mM Hepes-NaOH, pH 7.5. Reduction of $P700^+$ was registered at room temperature using a PE-1304 3 cm range EPR-spectrometer (Chernogolovka, Russia). The cell contained 0.1 ml of cell suspension with chlorophyll concentration of 50 μg/ml. EPR-signal I was generated by pulse illumination (0.1 sec) of cell suspension with white light (2000 $\mu E \cdot m^{-2} \cdot sec^{-1}$). The EPR parameters were: power, 20 mW; modulation amplitude, 0.3 mT; modulation frequency, 100 kHz; time constant, 10 μsec. DCMU (20 μM), glucose (5 mM), NaCN (2 mM), NADPH (800 μM), and rotenone (40 μM) were added to the cell suspension just before registration of EPR spectra.

Rates of light induced oxygen evolution were measured polarographically using an LP-7e polarograph (Laboratorni Pristroje, Czech Republic), Clark type electrode, and the thermostatic amperometric cell (at 25°C). The assay medium contained 2 mM $K_3Fe(CN)_6$ and 1 mM phenyl-*p*-benzoquinone. Cells were sedimented by centrifugation at 5000*g* for 20 min and resuspended in

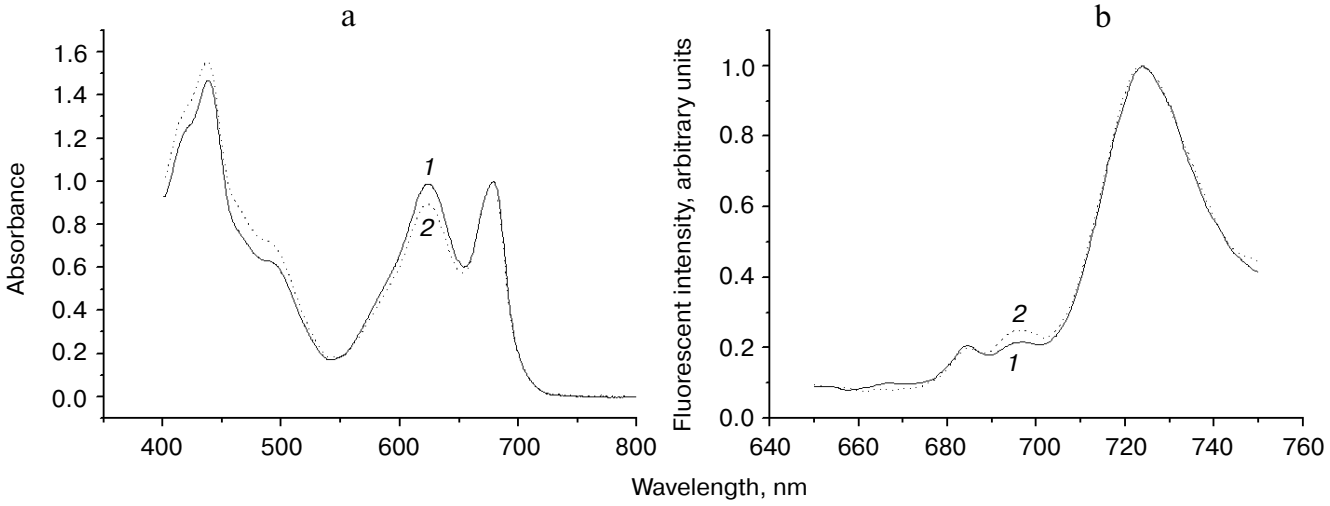

Fig. 1. Absorbance (a) and low temperature (77 K) fluorescence (b) spectra of wild type (*1*) and Ins2 mutant (*2*) *Synechocystis* 6803 cells grown photoautotrophically.

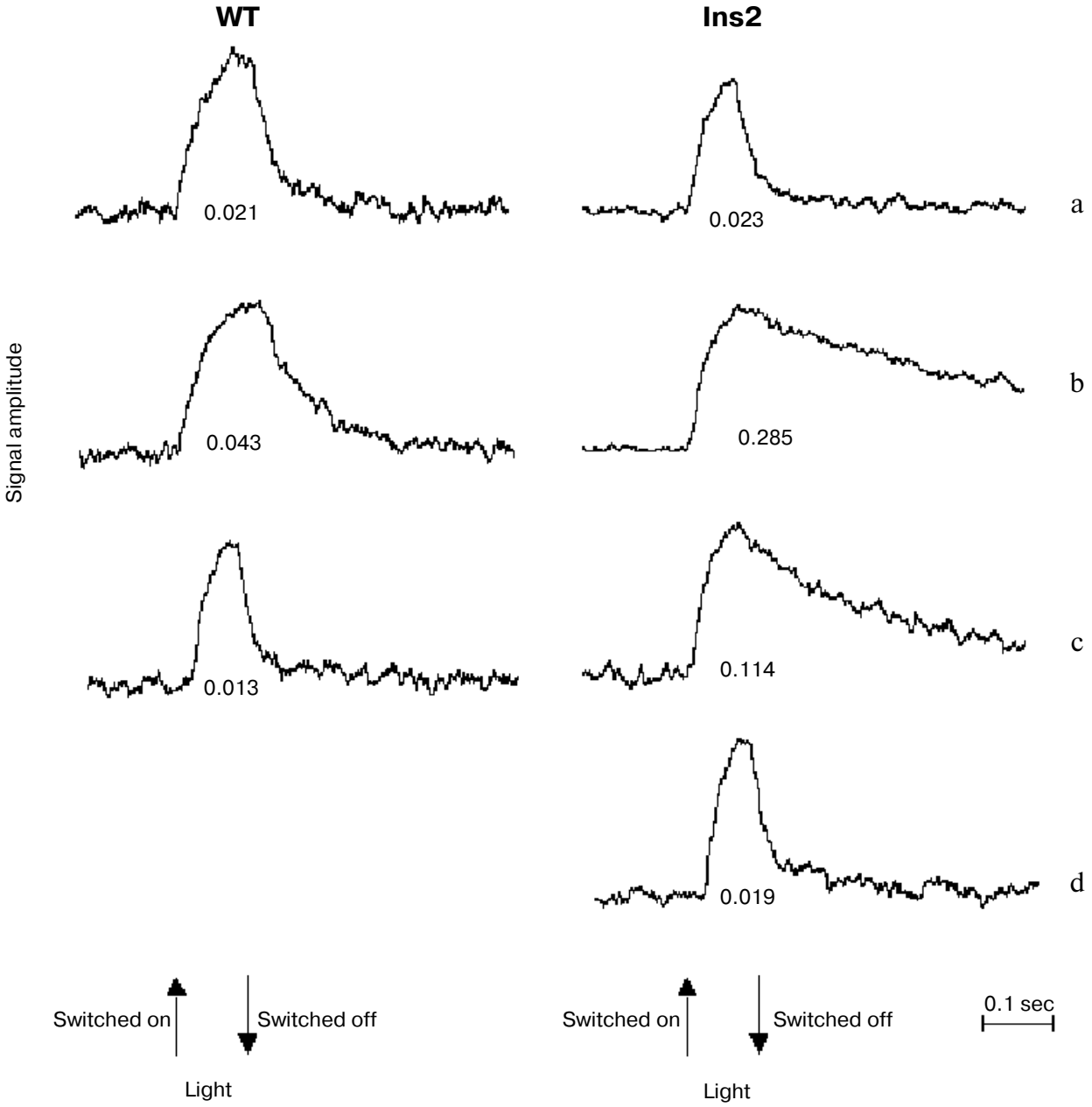

Fig. 2. Oxidation–reduction of P700 registered by EPR spectroscopy in photoautotrophically grown wild type (WT) and Ins2 mutant *Synechocystis* 6803 cells: a) without DCMU; b) 20 μM DCMU; c) 20 μM DCMU, 5 mM glucose; d) 20 μM DCMU, incubation with 5 mM glucose for 10 min. Numbers show time of half-reduction of signal I ($t_{1/2}$, sec).

fresh BG-11 medium containing 10 mM Hepes-NaOH, pH 7.5, at chlorophyll concentration 10-15 μg/ml. All experiments were done in triplicate.

Absorbance spectra and low temperature fluorescent spectra were registered directly in suspensions of growing cells. Absorbance spectra were recorded at room temperature using a Hitachi-557 spectrophotometer (Hitachi LTD, Japan). Spectra were normalized at the maximum absorbance at 680 nm. Low temperature fluorescence spectra were measured at 77 K with a Hitachi-850 spectrofluorimeter (Hitachi LTD) equipped with a cryostat. The excitation wavelength was 435 nm, spectral width of excitation and spectral width of registration of emission were 5 and 2 nm, respectively. Each sample contained 500-μl cell suspension in BG-11 medium with 50% glycerol. Spectra were normalized at the fluorescent maximum at 725 nm.

Measurement of MTT-reductase activity. Cell suspension in the growth medium (4.5 ml) was mixed with 0.5 ml of 10 mM phosphate buffer, pH 8.0, containing

5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). The mixture was incubated in darkness and aliquots of 0.8 ml were taken each 30 min. Each aliquot was added to 0.8 ml of the mixture of 96% isopropanol and formic acid (95 : 5 v/v). Samples were incubated in the darkness for 1 h and absorbance was measured at 580 nm using an Ultrospec II spectrophotometer (LKB, Sweden). NAD(P)H:MTT reductase activity in sonicated cells was registered in 10 mM sodium phosphate buffer, pH 8.0, at 580 nm.

RESULTS

Activity of PSII and spectral characteristics of wild type and Ins2 mutant cells. Activity of PSII measured as

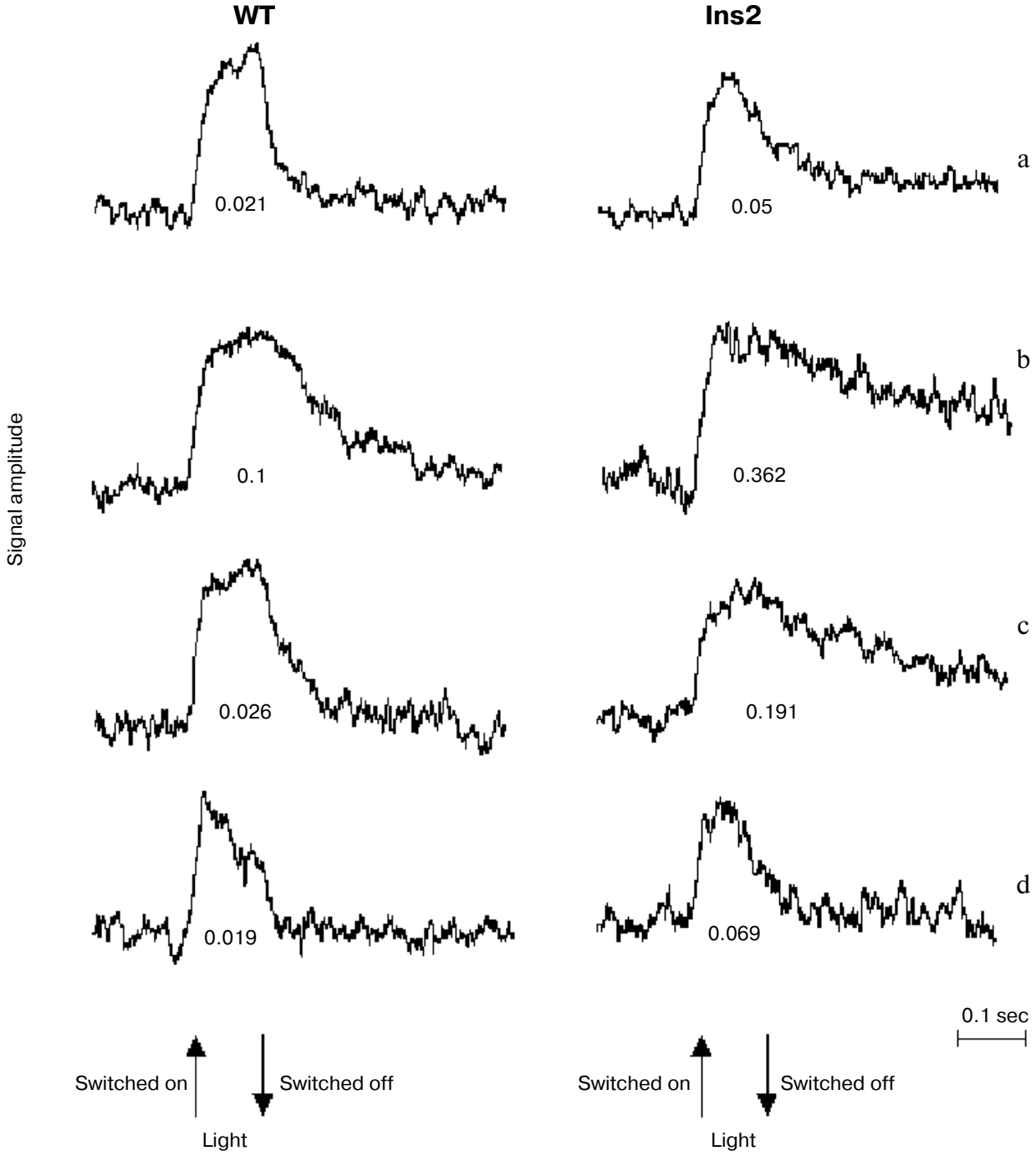

Fig. 3. Oxidation–reduction of P700 registered by EPR spectroscopy in photoautotrophically grown wild type (WT) and Ins2 mutant *Synechocystis* 6803 cells preincubated in the dark for 24 h. Designations are the same as for Fig. 2.

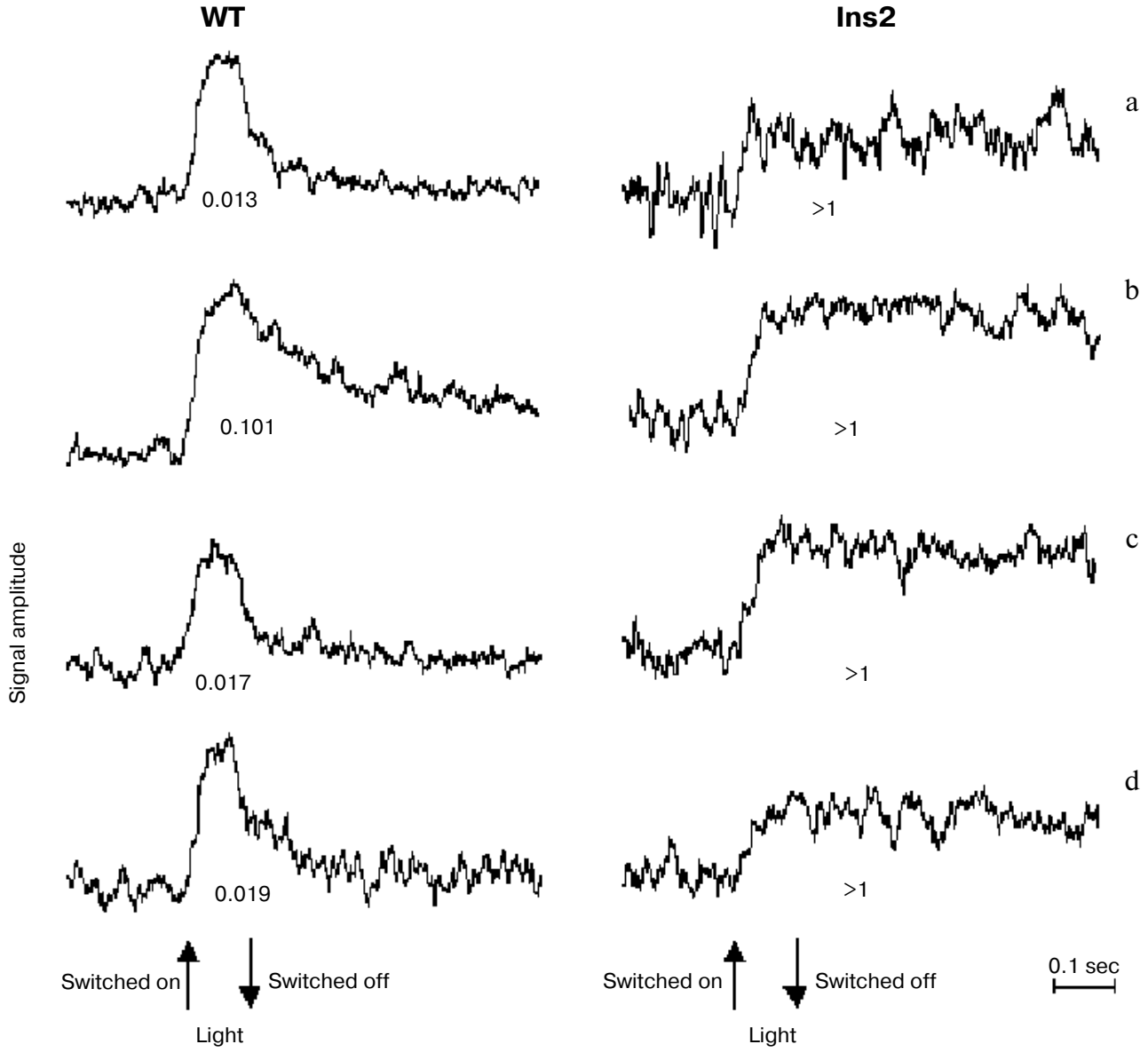

Fig. 4. Oxidation–reduction of P700 registered by EPR spectroscopy in wild type (WT) and Ins2 mutant *Synechocystis* 6803 cells grown photoautotrophically without aeration at illumination 20 $\mu E \cdot m^{-2} \cdot sec^{-1}$ and preincubated in the dark for 24 h. Designations are the same as for Fig. 2.

O_2 evolution by photoautotrophically grown wild type cells and Ins2 mutant lacking NQR was roughly the same; using phenyl-*p*-benzoquinone as the electron acceptor it was 270 ± 10 and 260 ± 9 μmol O_2/h per mg Chl in the case of wild type and Ins2 mutant, respectively. Absorbance (Fig. 1a) and low temperature fluorescence (Fig. 1b) spectra of photoautotrophically grown wild type and Ins2 mutant cells also insignificantly differed. Thus, the mutation in *drgA* gene did not cause significant changes in PSII functioning and structure of the photosynthetic machinery of *Synechocystis* 6803.

Reduction of P700$^+$ in wild type and Ins2 mutant cells. Kinetics of reduction of P700$^+$ after its oxidation in response to a white light pulse in photoautotrophically grown wild type and Ins2 mutant cells of *Synechocystis* 6803 was studied using EPR spectroscopy. In both strains, P700$^+$ was rapidly reduced (Fig. 2a). In the presence of DCMU blocking electron flow from PSII, the rate of P700$^+$ reduction was lower in the mutant cells than in the wild type cells (Fig. 2b). This suggests reduced effectiveness of cyclic electron transfer through PSI and/or reduced electron transfer from respiratory substrates located in the cytoplasm [4] of the NQR lacking mutant.

Synechocystis 6803 is a photoheterotrophic strain that can utilize glucose. Addition of 5 mM glucose to DCMU treated wild type cells increased the rate of P700$^+$

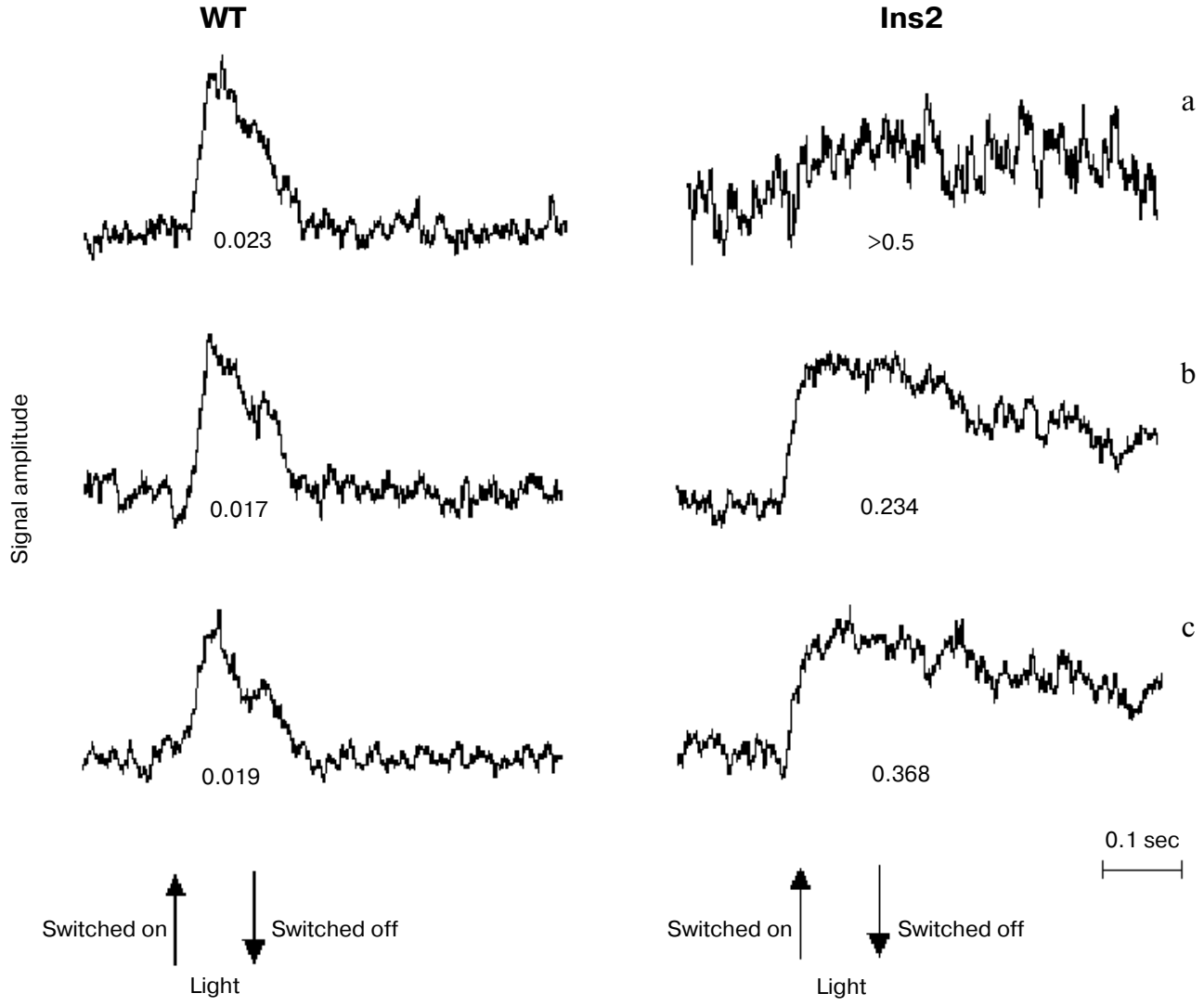

Fig. 5. Effect of 2 mM NaCN on oxidation–reduction of P700 in wild type (WT) and Ins2 mutant *Synechocystis* 6803 cells grown photoautotrophically without aeration at illumination 20 $\mu E \cdot m^{-2} \cdot sec^{-1}$ and preincubated in the dark for 24 h. Designations are the same as for Fig. 2.

reduction. In the case of the mutant cells (pretreated exactly as the wild type cells), the initial glucose-induced increase of $P700^+$ reduction rate was lower than in the wild type cells (Fig. 2c). In the mutant cells the rate of $P700^+$ reduction reached the level of the wild type cells only after incubation with glucose for 10 min (Fig. 2d).

For detailed evaluation of the contribution of glucose to feeding of the thylakoid membrane ETC, photoautotrophically grown *Synechocystis* 6803 cells were incubated for 24 h in the dark to deplete respiratory substrates [4]. Addition of glucose to DCMU-treated dark-starved Ins2 mutant cells increased the rate of $P700^+$ reduction less than in the wild type cells and in the mutant cells the rate of $P700^+$ reduction did not reach the level of corresponding control even after incubation with glucose for 10 min (Fig. 3). Thus, oxidation of exogenous glucose occurs more slowly in the Ins2 mutant than in the wild type cells.

The differences between wild type and Ins2 mutant cells were especially noticeable when the cells grown under lowered illumination (20 $\mu E \cdot m^{-2} \cdot sec^{-1}$) and without aeration were subjected to the dark starvation. During photoautotrophic growth under lowered illumination, the rate of O_2 evolution by wild type and Ins2 mutant cells with phenyl-*p*-benzoquinone was 74 ± 3 and 71 ± 3 μmol O_2/h per mg Chl, and the two strains insignificantly differed from the cells grown under conditions of high illumination and aeration in the rate of $P700^+$ reduction (data not shown). However, after dark starvation for 24 h, wild type cells effectively utilized exogenous glucose for $P700^+$ reduction, whereas $P700^+$ reduction in the mutant cells occurred slower and their prolonged incubation with

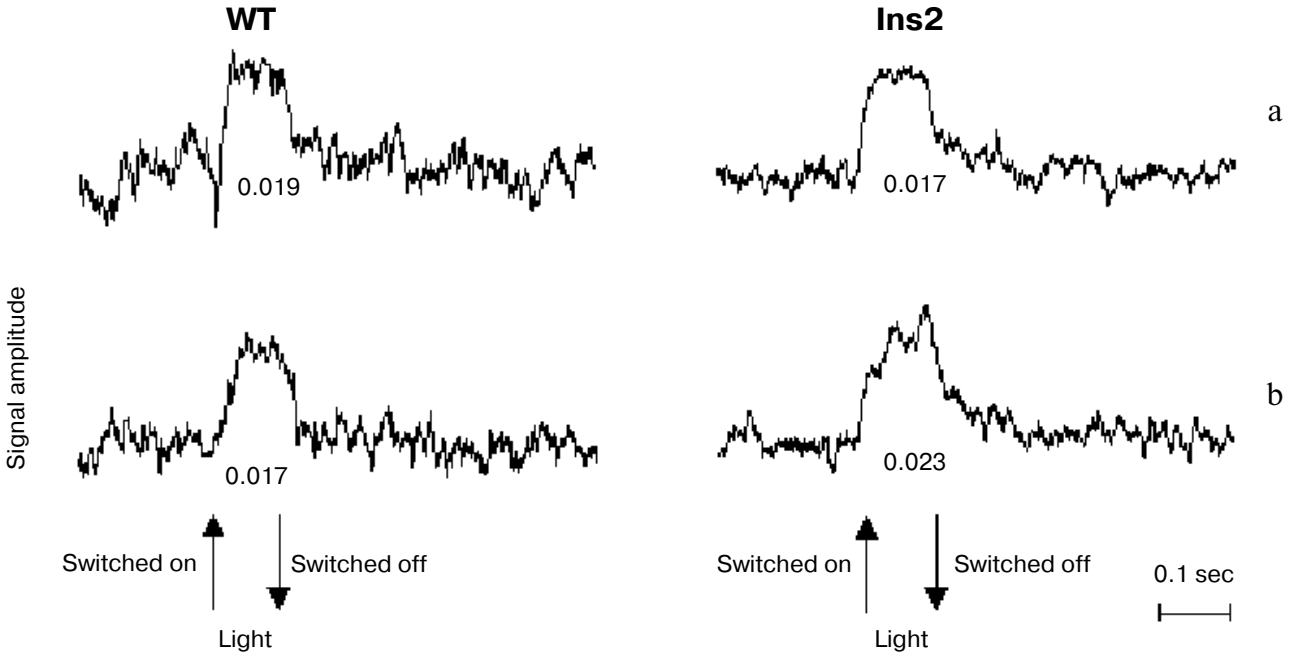

Fig. 6. Oxidation–reduction of P700 in wild type (WT) and Ins2 mutant *Synechocystis* 6803 cells grown photoautotrophically and preincubated in the dark for 24 h in the presence of 5 mM glucose: a) without DCMU; b) in the presence of 20 μM DCMU. Conditions are the same as for Fig. 2.

glucose did not accelerate it (Fig. 4). After dark starvation of mutant cells PSII activity sharply decreased (by 60-90%) and in most experiments cells died after their transfer from the dark into the condition of photoautotrophic growth.

Slowed rate of $P700^+$ reduction in Ins2 mutant cells could be attributed to reduced electron flow to the PQ pool and also to increased outflow of electrons to cytochrome and quinol oxidases [14, 15]. Addition of NaCN, an inhibitor of respiratory oxidases, to dark-starved wild type cells sharply increased the rate of $P700^+$ reduction even in the presence of DCMU (Fig. 5). However, in the dark-starved mutant cells CN^- addition insignificantly influenced the rate of $P700^+$ reduction (Fig. 5). Thus, slowed $P700^+$ reduction in the dark-starved Ins2 mutant cells is related to depletion of respiratory donors reducing the PQ pool rather than to increased electron outflow to cytochrome and quinol oxidases.

Wild type and mutant cells incubated in the dark for 24 h in the presence of 5 mM glucose were characterized by rapid reduction of $P700^+$. Since the rate of $P700^+$ reduction was insignificantly influenced by DCMU addition (Fig. 6), the contribution of PSII to PQ reduction after pulse illumination was negligible under these conditions. Thus, in wild type and Ins2 mutant cells adapted to heterotrophic conditions electrons from exogenous glucose enter the thylakoid membrane ETC at the same rate.

Reduction of $P700^+$ in thylakoid membranes isolated from wild type and Ins2 mutant. In *Synechocystis* 6803, the electron transport from respiratory substrates to the PQ pool involves NDH-1 and is sensitive to inhibition by rotenone [5]. To elucidate whether mutation in the *drgA* gene results in impairment of NDH-1-dependent electron transfer, we investigated effects of NADPH and rotenone on $P700^+$ reduction in thylakoid membranes isolated from wild type and Ins2 mutant cells. In contrast to intact cells, $P700^+$ reduction in the isolated thylakoid membranes of *Synechocystis* 6803 cells was characterized by biphasic kinetics. When light was switched off, rapid initial $P700^+$ reduction occurred; then this reduction slowed due to depletion of electrons derived from PSII. In thylakoids both of wild type and mutant cells reduction of $P700^+$ after illumination was totally inhibited by DCMU (Fig. 7, a and b), i.e., this reduction occurred due to non-cyclic electron transport from water. Addition of NADPH to DCMU-treated membranes caused roughly the same increase in $P700^+$ reduction rate in wild type and Ins2 mutant preparations (Fig. 7c). The reaction was sensitive to rotenone (Fig. 7d). The latter suggests involvement of NDH-1 complex. Thus, mutations in the *drgA* gene did not cause impairments in NADH-1-dependent electron transfer from NADPH to the PQ pool in thylakoid membranes of *Synechocystis* 6803.

MTT reductase activity in wild type and Ins2 mutant cells. Dehydrogenase activity of cells or isolated mito-

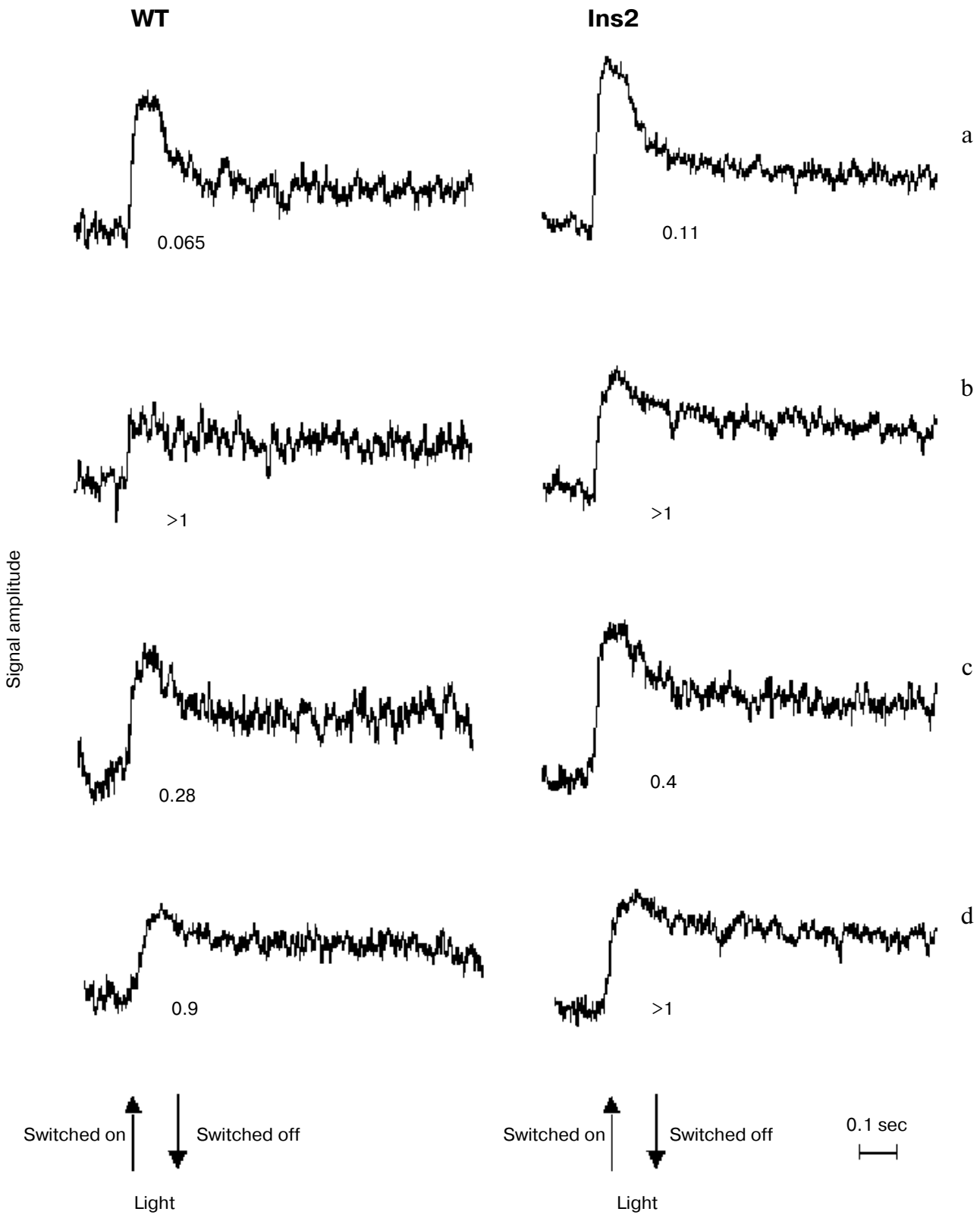

Fig. 7. Oxidation–reduction of P700 in isolated membranes of wild type (WT) and Ins2 mutant cells: a) without DCMU; b) 20 μM DCMU; c) 20 μM DCMU, 800 μM NADPH; d) 20 μM DCMU, 800 μM NADPH, 40 μM rotenone. Conditions are the same as for Fig. 2.

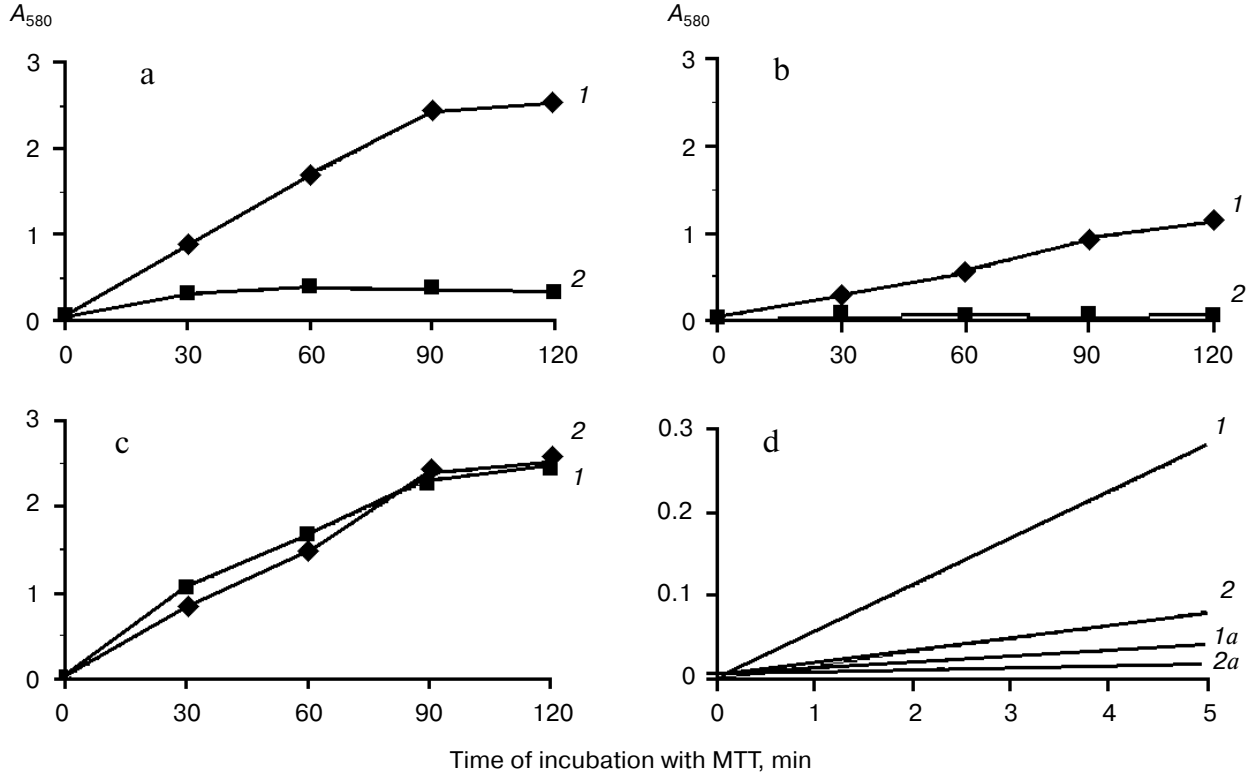

Fig. 8. MTT reduction by wild type (*1*) and Ins2 mutant (*2*) cells grown photoautotrophically (a) and preincubated in the dark for 24 h without glucose (b) and in the presence of 5 mM glucose (c); d) MTT reduction in sonicated wild type (*1*, *1a*) and Ins2 mutant (*2*, *2a*) cells with NADPH (*1*, *2*) and NADH (*1a*, *2a*) as electron donors.

chondria can be evaluated by tetrazolium salt (e.g., MTT) reduction to formazan [16]. The rate of MTT reduction in photoautotrophically grown wild type cells was 3-4 times higher than in Ins2 mutant cells (Fig. 8a). Depletion of respiratory substrates after 24 h starvation in the dark caused a sharp decrease in MTT reduction by wild type and Ins2 mutant cells (Fig. 8b). After incubation in the dark for 24 h in the presence of 5 mM glucose, the rate of MTT reduction in the mutant cells increased to the level observed in the wild type cells (Fig. 8c).

Thus, a significant proportion of total dehydrogenase activity in photoautotrophically grown *Synechocystis* 6803 cells is related to NQR function. However, it was also possible that low MTT-reductase activity in Ins2 mutant cells could be attributed to lower content of respiratory substrates. So we investigated the rate of MTT reduction in sonicated wild type and mutant cells. NAD(P)H:MTT-reductase activity of Ins2 mutant was significantly lower than that of wild type and NADPH was a more effective electron donor than NADH. In Ins2 mutant, NADPH:MTT reductase activity did not exceed 30% of the level observed in wild type (Fig. 8d). These results suggest that under photoautotrophic conditions, *drgA* mutant cells are characterized by reduced NADPH oxidizing ability.

DISCUSSION

Data of the present study demonstrate that the mutation in the *drgA* gene encoding soluble NQR did not impair activity of PSII and PSI in photoautotrophically grown *Synechocystis* 6803 cells and did not influence NADPH-dependent rotenone-sensitive $P700^+$ reduction, which involves NDH-1 in thylakoid membranes [5]. Enzymes involved in glucose oxidation also remained unimpaired in the *drgA* mutant cells because wild type and the mutant cells adapted to heterotrophic conditions (dark, glucose) were characterized by the same rate of $P700^+$ reduction after pulse illumination. However, in the presence of DCMU blocking electron flow from PSII the rate of $P700^+$ reduction by electrons derived from exogenous glucose was lower in Ins2 mutant cells than in wild type cells. This suggests reduced ability for glucose oxidation in cells with mutated *drgA* gene.

In *Synechocystis* 6803 cells, glucose oxidation occurs via the pentose phosphate pathway [17]. The rate of pentose phosphate pathway depends on $NADP^+$ concentration. According to data of NADPH-dehydrogenase activity assay, mutation in the *drgA* gene decreased the ability of photoautotrophically grown *Synechocystis*

6803 cells for NADPH oxidation. This suggests that NQR is involved in regulation of NADPH oxidation in the cytoplasm of cyanobacteria, which supplies glucose oxidation in the pentose phosphate pathway with $NADP^+$.

Thus, slowed reduction of the reaction center of PSI in the mutant cells lacking soluble NQR may be attributed to a decrease in cytoplasmic $NADP^+$. Besides reduced rate of glucose oxidation in the pentose phosphate pathway, $NADP^+$ deficit may result in reduced succinate concentration and decreased SDH activity, which is the main electron supplier to the PQ pool in the dark [7].

NQR is not the only enzyme capable of NADPH oxidation. Under photoautotrophic conditions, NADPH is oxidized in the Calvin cycle and so differences in the rate of PQ pool reduction in wild type and Ins2 mutant cells were observed only in the presence of DCMU (under conditions of inhibition of non-cyclic electron transport). In the dark, NDH-1 and NDH-2 are the main regulators of $NADP^+$/NADPH and NAD^+/NADH ratios, respectively, in *Synechocystis* 6803 cells [7]. In fact, under respiratory conditions (dark, glucose) there were no differences in the rate of $P700^+$ reduction in wild type and Ins2 mutant cells. The data suggest that in cyanobacteria NADPH-dehydrogenase activity of NQR is, probably, required under stress conditions to switch metabolism from photoautotrophic to heterotrophic pathways.

This work was supported by the Russian Foundation for Basic Research (grant No. 03-04-49217).

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