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Role of NAD(P)H:Quinone Oxidoreductase Encoded by *drgA* Gene in Reduction of Exogenous Quinones in Cyanobacterium *Synechocystis* sp. PCC 6803 Cells

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Abstract—Insertion mutant Ins2 of the cyanobacterium *Synechocystis* sp. PCC 6803, lacking NAD(P)H:quinone oxidoreductase (NQR) encoded by *drgA* gene, was characterized by higher sensitivity to quinone-type inhibitors (menadione and plumbagin) than wild type (WT) cells. In photoautotrophically grown cyanobacterial cells more than 60% of NADPH:quinone-reductase activity, as well as all NADPH:dinoseb-reductase activity, was associated with the function of NQR. NQR activity was observed only in soluble fraction of cyanobacterial cells, but not in membrane fraction. The effects of menadione and menadiol on the reduction of Photosystem I reaction center ($P700^+$) after its photooxidation in the presence of DCMU were studied using the EPR spectroscopy. The addition of menadione increased the rate of $P700^+$ reduction in WT cells, whereas in Ins2 mutant the reduction of $P700^+$ was strongly inhibited. In the presence of menadiol the reduction of $P700^+$ was accelerated both in WT and Ins2 mutant cells. These data suggest that NQR protects the cyanobacterial cells from the toxic effect of exogenous quinones by their reduction to hydroquinones. These data may also indicate the probable functional homology of *Synechocystis* sp. PCC 6803 NQR with mammalian and plant NAD(P)H:quinone oxidoreductases (DT-diaphorases).

Key words: cyanobacterium *Synechocystis* sp. PCC 6803, NAD(P)H:quinone oxidoreductase, *drgA* gene, resistance to menadione, EPR-spectroscopy, $P700^+$ reduction

In prokaryotes and eukaryotes quinones are substrates of flavin enzymes, which reduce them to semiquinones (via a one electron mechanism) or hydroquinones (via a two electron mechanism). In the presence of oxygen, most quinones are oxidized with superoxide radical ($O_2^{\cdot-}$) formation and regeneration of the initial quinones [1]. Such redox conversions of quinones catalyzed by flavin enzymes lead to oxidative stress [2]. Hydroquinones are more stable molecules. They can form nontoxic conjugates with glucuronic acid and this is accompanied by loss of redox cycling [3].

Mechanisms responsible for inhibitory effects of quinones can differ; as a rule, they involve one electron reduction. In animal cells quinones are reduced to semiquinones by NADPH:cytochrome P450 reductase (EC 1.6.2.4) [4], xanthine oxidase (EC 1.1.3.22) [5], L-aspartate oxidase (EC 1.4.3.16) [6], and other enzymes. Lipophilic quinones with low redox potentials such as menadione and plumbagin easily penetrate into the thylakoid membrane of cyanobacteria and plant chloroplasts where they can accept electrons of photosynthetic electron transport at the level of ferredoxin:$NADP^+$ oxidoreductase (EC 1.18.1.2) [7], photosystems (PS) I and II, and also at the level of cytochromes b_6f [8, 9]. It was shown that the interaction of menadione with photoexcited chlorophyll molecules results in non-photochemical fluorescence quenching [10]. In cyanobacterial cells

Abbreviations: dinoseb) 2-*sec*-butyl-4,6-dinitrophenol; menadione) 2-methyl-1,4-naphthoquinone; metronidazole) 2-methyl-5-nitroimidazole-1-ethanol; plumbagin) 5-hydroxy-2-methyl-1,4-naphthoquinone; DBMIB) 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU) 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNOC) 4,6-dinitro-*o*-cresol; NDH-1) NAD(P)H-dehydrogenase (Complex I); NDH-2) NADH-dehydrogenase 2; NQR) NAD(P)H:quinone-oxidoreductase; Q_0) ubiquinone 0.

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quinones can be reduced by NAD(P)H-dehydrogenase (NDH-1), NADH-dehydrogenase 2 (NDH-2), and succinate dehydrogenase (SDH) [11, 12]. In thylakoid membranes menadiol interacts with the plastoquinone pool and it is involved in DBMIB-sensitive electron cycling which includes PS I [13].

There is the effective mechanism protecting mammalian cells against toxic effect of quinones. It involves cytosolic NAD(P)H:quinone oxidoreductase (DT-diaphorase; EC 1.6.99.2), which catalyzes two electron reduction of quinones and therefore prevents their one electron reduction catalyzed by other enzymes [14]. This enzyme is a homodimer with subunit molecular mass of 30.1 kD. Mammalian DT-diaphorase is sensitive to dicumarol [15]. Various xenobiotics, antioxidants, oxidants, heavy metals, and also UV and ionizing radiation induce expression of the *NQO1* gene encoding DT-diaphorase [16-20]. Mice carrying mutations in *NQO1* were characterized by increased sensitivity to menadione injections than wild type mice [21]. DT-diaphorase can catalyze reduction of a wide range of substrates. For example, this enzyme activates nitroaromatic compounds to toxic, mutagenic, and carcinogenic products during two electron reduction of the nitro group to an amino group [22]. This explains why mammalian cells characterized by high expression of DT-diaphorase (e.g., tumor cells) exhibit higher sensitivity to nitroaromatic inhibitors [23].

A homotetramer of NAD(P)H:quinone oxidoreductase (NQR) with subunit molecular mass of 21-27 kD has been found in higher plants [24, 25]. The gene encoding NQR in *Arabidopsis thaliana* was expressed in *Escherichia coli* [26]. The amino acid sequence of *A. thaliana* NQR does not share significant homology with mammalian DT-diaphorase. However, in spite of lack of significant homology with the mammalian enzyme, plant NQR catalyzes two-electron reduction of quinones; this suggest that the plant NQR might represent a functional homolog of mammalian DT-diaphorase [26]. Nevertheless, the function of NQR in plant cells remains unclear.

NQR is also recognized in the photoheterotrophic cyanobacterium *Synechocystis* sp. PCC 6803 (denominated further as *Synechocystis* 6803). The enzyme is a homodimer with subunit molecular mass of 23 kD. Purified NQR catalyzed oxidation of both NADPH and NADH. Since quinone derivatives were the most effective electron acceptors, the enzyme was referred to as NAD(P)H:quinone oxidoreductase [27]. Analysis of *Synechocystis* 6803 NQR primary structure revealed that this enzyme encoded by *drgA* [28] shares homology with bacterial NAD(P)H-nitroreductases [27, 29]. The inhibitory effects of some nitroaromatic compounds (such as dinoseb, DNOC, 2,4-dinitrophenol, metronidazole) on *Synechocystis* 6803 cells is associated with formation of toxic intermediates of their reduction by the DrgA protein. Mutants in *drgA* lacking NQR could not activate nitroaromatic compounds and they were highly resistant to nitroaromatic inhibitors [29].

In this study, we investigated the function of DrgA protein in *Synechocystis* 6803 cells by analyzing resistance of wild type and *drgA* mutant cells to quinone inhibitors menadione and plumbagin. We also investigated some biochemical characteristics of the *drgA* mutant and the effect of menadione on reduction of PS I reaction center under photoautotrophic conditions.

MATERIALS AND METHODS

Strains and cultivation conditions. A wild strain *Synechocystis* sp. PCC 6803 and Ins2 mutant carrying insertion of a kanamycin resistance gene in *BamHI* site of the *drgA* gene [28] were from the collection of the Department of Genetics, Lomonosov Moscow State University. Cells were cultivated at 30°C and constant illumination of 40 $\mu E \cdot m^{-2} \cdot sec^{-1}$ using liquid mineral medium BG-11 [30]. The mutant cells were grown in the presence of 20 μg/ml kanamycin (Sigma, USA). Cell growth was monitored by optical density at 750 nm using an Ultrospec II spectrophotometer (LKB, Sweden).

Preparation of cell-free extracts and membrane and soluble fractions. Cells at late logarithmic stage of growth were sedimented by centrifugation (4000*g*, 4°C). The sediment was washed twice with solution A [31] containing 25% (v/v) glycerol, 10 mM $MgCl_2$, 10 mM NaCl, and 20 mM sodium 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 in ice for 1 h was sonicated using a Soniprep-150 sonicator (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 at 140,000*g* for 1 h. The sediment was resuspended in solution A. Soluble fraction (supernatant) and membrane fraction were immediately placed into ice and used for determination of enzymatic activities.

Determination of enzymatic activities. NAD(P)H:Q_0-reductase and NAD(P)H:dinoseb-reductase activities were registered at 340 nm (by decrease in NAD(P)H absorbance, $\varepsilon = 6.2\ mM^{-1} \cdot cm^{-1}$) and 470 nm (reduced dinoseb absorbance, $\varepsilon = 6.9\ mM^{-1} \cdot cm^{-1}$), respectively. The reaction mixture contained 20 mM Tris-HCl, pH 7.5, 100 μM NADPH or NADH, and electron acceptors (100 μM Q_0 or 50 μM dinoseb). All measurements were carried out at 30°C using a Hitachi-557 spectrophotometer (Hitachi, Japan).

EPR-spectroscopy. Cells photoautotrophically grown for 3 days were sedimented by centrifugation and suspended in BG-11 medium containing 10 mM Hepes,

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 (chlorophyll concentration, 50 μg/ml). EPR-signal I was generated by impulse illumination (0.1 sec) of the cell suspension with white light (2000 $\mu E \cdot m^{-2} \cdot sec^{-1}$). There were the following EPR conditions: power, 20 mW; amplitude modulation, 0.3 mT; frequency modulation, 100 kHz; time constant, 10 μsec. DCMU (20 μM) and menadione (20 μM) were added in the cell suspension just before registration of EPR spectra.

RESULTS

Biochemical characteristics of the Ins2 mutant. Purified *Synechocystis* 6803 NQR was characterized by high NAD(P)H:quinone-reductase and low NAD(P)H-nitroreductase activities [27]. For evaluation of the contribution of NQR to reduction of quinone and nitroaromatic compounds in *Synechocystis* 6803 cells, we assayed NAD(P)H:Q_0-reductase and NAD(P)H:dinoseb-reductase activities of wild type (WT) cells and of Ins2 mutant lacking NQR. Both activities were higher when NADPH was used as electron donor (Table 1). Results of several independent experiments demonstrated that in the Ins2 mutant NADPH:Q_0-reductase activity was significantly lower than in WT cells. Results of a typical experiment show (Table 1) that more than 60% of NADPH:Q_0-reductase activity (and in some experiments up to 80%) of WT cells is associated with the function of NQR encoded by the *drgA* gene. In WT cells NAD(P)H:dinoseb-reductase activity was lower than NAD(P)H:Q_0-reductase activity. In the Ins2 mutant NAD(P)H:dinoseb-reductase activity was not detected (Table 1). Thus, all NAD(P)H:dinoseb-reductase activity of *Synechocystis* 6803 is associated with the function of NQR. This is consistent with previous data on the key role of the protein product of the *drgA* gene in reduction of nitroaromatic compounds [29].

The hydrophobicity profile of NQR suggests that it is not an integral membrane protein. Nevertheless, the possibility of NQR binding to thylakoid or cytoplasmic membrane could not be ruled out. So, to localize NQR in *Synechocystis* 6803 cells we studied the distribution of NADPH:Q_0-reductase and NADPH:dinoseb-reductase activities between soluble and membrane fractions of WT and Ins2 mutant cells. Table 1 shows that all NADPH:dinoseb-reductase activity and a major proportion of NADPH:Q_0-reductase activities are associated with soluble fraction of *Synechocystis* 6803 WT cells. Since DrgA protein was not found among *Synechocystis* 6803 periplasm [32], we conclude that it is located in the cytoplasm and does not form tight bonds with the cyanobacterial membrane.

Resistance of wild type and Ins2 mutant cells to menadione and plumbagin. We previously demonstrated that Ins2 mutant cells carrying an insertion in the *drgA* gene are characterized by higher resistance to nitroaromatic inhibitors [29]. Since DrgA protein exhibits high quinone reductase activity, the mutant cells could have altered sensitivity to some quinone type inhibitors such as menadione and plumbagin.

Table 2 shows that the Ins2 mutant was characterized by higher sensitivity to menadione and plumbagin than WT. During cultivation on solid medium, 2 μM mena-

Table 1. NAD(P)H:quinone-reductase and NAD(P)H:dinoseb-reductase activities in cell-free preparations and in membrane and soluble fractions of WT and Ins2 mutant *Synechocystis* 6803 cells

	Q_0-reductase activity, nmol/min per mg protein		Dinoseb-reductase activity, nmol/min per mg protein	
	NADPH	NADH	NADPH	NADH
Cell-free preparations:				
WT	198	35.7	23	1.1
Ins2	78	27.5	0	0
Membrane fraction:				
WT	28	n.d.*	0	n.d.
Ins2	14	n.d.	0	n.d.
Soluble fraction:				
WT	474	n.d.	56	n.d.
Ins2	210	n.d.	0	n.d.

* n.d., not determined.

Table 2. Resistance of WT and Ins2 mutant cells to menadione and plumbagin on solid medium under photoautotrophic conditions

Strain	Inhibitory concentration, μM*	
	menadione	plumbagin
WT	22 ± 0.4	2.7 ± 0.5
Ins2	2.2 ± 0.2	1.1 ± 0.3

* Minimal inhibitory concentration suppressing cell growth on the solid medium.

dione inhibited growth of the mutant cells whereas inhibition of WT cell growth required much higher concentration (>20 μM). In the case of plumbagin the differences between concentrations required for inhibition of growth of WT and the mutant cells were less pronounced: inhibitory concentration of plumbagin was 2-3-fold higher for WT than for the Ins2 mutant. Similar differences were also found during cultivation of WT and Ins2 mutant cells in liquid medium under photoautotrophic conditions (data not shown). Thus, in the absence of functionally active NQR *Synechocystis* 6803 cells are characterized by higher sensitivity to menadione and plumbagin.

Effect of menadione on reduction of reaction center of PS I in WT and Ins2 mutant cells. Menadione can easily penetrate into the thylakoid membrane where it can be involved into electron cycling that includes PS I [13]. Using EPR spectroscopy, we investigated the effect of menadione and menadiol on reduction of PS I reaction center in response to an impulse of white light.

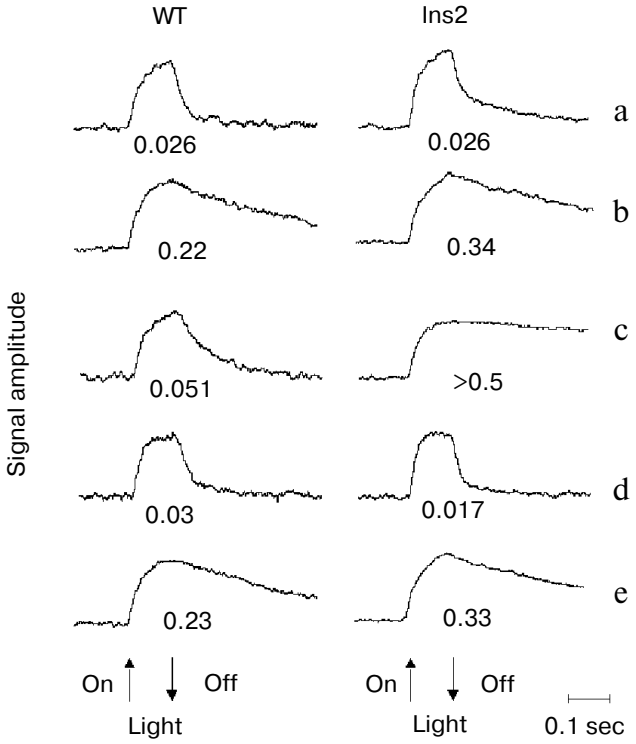
In the absence of DCMU, $P700^+$ (oxidized under white light) was rapidly reduced by electrons from PS II, and also from respiratory substrates localized in the cytoplasm [33]. The rates of $P700^+$ reduction in WT and Ins2 mutant cells were roughly the same (figure, (a)). Addition of DCMU, blocking electron flow from PS II, delayed $P700^+$ reduction in both WT and Ins2 mutant cells (figure, (b)). Nevertheless, DCMU did not cause total inhibition of $P700^+$ reduction; this may be explained by electron cycling which involves PS I and electron transport from respiratory substrates. Similar results were obtained during illumination of cells with far red light (λ > 700 nm), which is preferentially absorbed by PS I (data not shown).

Addition of menadione caused an increase in the rate of $P700^+$ reduction in WT cells and a sharp decrease in this parameter in the Ins2 mutant (figure, (c)). Menadiol (menadione reduced by sodium dithionite) added at the same concentration accelerated $P700^+$ reduction in both WT and Ins2 mutant cells (figure, (d)). Direct addition of sodium dithionite to DCMU-treated cells did not increase the rate of $P700^+$ reduction (figure, (e)).

DISCUSSION

The data suggest that resistance of photoautotrophically grown *Synechocystis* 6803 cells to menadione and plumbagin depends on the presence of functionally active *drgA* protein product, which can effectively reduce exogenous quinones. Mutant cells lacking NQR were characterized by low level of NADPH:quinone-reductase activity and increased sensitivity to the quinone inhibitors—menadione and plumbagin. Comparison of the effect of menadione and menadiol on $P700^+$ reduction in photoautotrophically grown WT and Ins2 mutant cells suggests that NQR encoded by *drgA* is responsible for two electron reduction of quinones to hydroquinones.

The toxic effect of menadione and plumbagin is usually associated with oxidative stress, which appears as the result of superoxide formation during cyclic reactions of one electron oxidation/reduction of quinones [2]. It is possible that two-electron reduction of quinones to hydroquinones catalyzed by NQR prevents their one-electron reduction inducing oxidative stress. Taking into consideration that the major proportion of NADPH:



Kinetics of P700 oxidation–reduction, registered by EPR spectroscopy in photoautotrophically grown WT and Ins2 mutant *Synechocystis* 6803 cells in the absence (a) and in the presence of 20 μM DCMU (b-e): a) control; b) 20 μM DCMU; c) 20 μM menadione; d) 20 μM menadiol; e) 1 mM sodium dithionite. Numbers show time of $P700^+$ half-reduction, sec

quinone-reductase activity of photoautotrophically grown WT cells is associated with NQR function, it is reasonable to suggest that DrgA protein forms a cytoplasmic barrier providing rapid detoxification of exogenous quinones. However, certain evidence exists that menadiol as well as semiquinone may reduce soluble oxygen with formation of superoxide radical and H_2O_2 [34]. So it is possible that in the absence of NQR the effect of menadione in cyanobacterial cells is mainly determined by appearance of alternative pathways of electron transport, resulting in inhibition of various metabolic processes in the cell. For example, in *Escherichia coli* cells plumbagin inhibits respiratory activity by scavenging electrons from NADH-dehydrogenase [35]. In is possible that in *Synechocystis* 6803 cells lacking NQR, menadione is actively reduced by electrons from the thylakoid membrane electron transport chain and this inhibits photosynthetic and respiratory electron transport.

Besides higher sensitivity to menadione and plumbagin, the *drgA* mutant was characterized by higher resistance to nitroaromatic inhibitors such as dinoseb, DNOC, and metronidazole. This may be attributed to inability of Ins2 mutant cells to activate nitroaromatic compounds; their activation is accompanied by appearance of highly toxic intermediates [29]. In this study we have demonstrated that all NADPH:dinoseb-reductase activity of *Synechocystis* 6803 cells is associated with NQR function. Thus, soluble NQR, encoded by the *drgA* gene, is involved in protection of *Synechocystis* 6803 cells against the inhibitory effect of menadione; it also activates nitroaromatic compounds. Both these properties of DrgA protein are also typical for mammalian DT-diaphorase [7]. It is possible that NQR, encoded by *drgA* gene, is a functional homolog of DT-diaphorase in *Synechocystis* 6803 cells, which prevents toxic effect of quinone type inhibitors by reducing them to hydroquinones.

One of the functions of mammalian DT-diaphorases may consist of maintenance of the reduced state of membrane quinones (ubiquinone and tocopheryl-quinone) [36]. Our data on the increased rate of photo-induced reduction of $P700^+$ in the presence of menadiol also suggest that NQR in *Synechocystis* 6803 may be involved in plastoquinone reduction in the thylakoid (and also in the cytoplasmic) membrane of the cyanobacterial cell.

In contrast to DT-diaphorase, NQR activity of *Synechocystis* 6803 cells was insensitive to dicumarol and flavones. The amino acid sequence of *drgA* protein product lacks adenine- and phosphate-binding motifs typical for DT-diaphorases [27]. So, it is possible that NQR represents a part of a larger protein complex which functions in cytoplasm of cyanobacteria.

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