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The site and mechanism of duroquinol oxidation by the cytochrome b_6-f complex in *Synechococcus* sp.

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Duroquinol (tetramethyl-*p*-hydroquinone) served as an excellent electron donor to Photosystem I and promoted methyl viologen photoreduction in 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea-poisoned cells of the thermophilic cyanobacterium *Synechococcus* sp. at a rate comparable to or, more likely, higher than that of the photoreduction with water as electron donor. The restored activity was inhibited by 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB). The inhibition is competitive and partially reversed by increasing duroquinol concentration. Spectrophotometric experiments with flashes demonstrated that duroquinol accelerates the reduction of P-700, cytochrome *c*-533 and cytochrome *f* in the DBMIB-poisoned cells similarly and in a way just opposite to the inhibitory action of DBMIB: the electron donor specifically increased the fraction of the three electron carriers that was reduced with the 2 ms half-time. The results indicate that duroquinol does not serve as a reductant of the plastoquinone pool, nor of P-700 and cytochrome *c*-533, but directly donates its electrons to the cytochrome b_6-f complex by binding to the plastoquinone-binding site and that DBMIB inhibits the quinol oxidation by binding competitively to the same site.

Introduction

A plastoquinone antagonist, DBMIB, blocks photosynthetic electron transport at the oxidation site of plastoquinone [1,2]. The binding of DBMIB to the cytochrome b_6-f complex is indicated by the sensitivity to the inhibitor of the plastoquinol-1-plastocyanin oxidoreductase activity mediated by the isolated complexes [3]. The DBMIB-inhibition was reversed by the addition of plastoquinone [2,4]. It has been suggested that the inhibitor blocks electron transport by competing with plastoquinone for a common reaction site of the cytochrome b_6-f complex [5,6].

Of known artificial electron donors to PS I, duroquinol is unique in that electron transport from duroquinol to PS I is highly sensitive to DBMIB, indicating a site of electron donation in the plastoquinone region [7,8]. The ability of duroquinol to donate electrons in this specific region of photosynthetic electron transport chain may be related to its lipophilic molecular structure as well as its midpoint potential (E_{m7} = 5 mV [9]) lower than that of plastoquinone (80 mV [10]). The detailed mode of the electron donation by duroquinol is, however, not known yet and a

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; PS, Photosystem; DQ, duroquinone.

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question remains as to whether duroquinol replaces plastoquinone as a direct electron donor to the cytochrome b_6-f complexes, or serves simply as a reductant of the plastoquinone pool.

In addition, there are reports suggesting that duroquinol feeds electrons directly to PS I. Trebst and Reimer [4] have observed a stimulation of electron flow in DBMIB-poisoned chloroplasts by duroquinol and suggested a bypass of the inhibition site by the electron donor. White et al. [8] who found that DBMIB suppresses the reduction of the Rieske iron-sulfur center and cytochrome f, but not that of P-700 and plastocyanin, by duroquinol proposed that duroquinol directly reduces plastocyanin.

In the present work, we have studied the site and mode of the electron donation from duroquinol to photosynthetic electron transport chains in the thermophilic cyanobacterium *Synechococcus* sp. in order to examine the mechanism of quinol oxidation by the cytochrome b_6-f complex. Duroquinol was found to serve as a good electron donor to PS I in the DCMU-poisoned cells. Inhibition of duroquinol-supported methyl viologen photoreduction by DBMIB was examined in details and effects of duroquinol on reduction kinetics of P-700, cytochrome c-553 and cytochrome f in the DBMIB-poisoned cells were investigated.

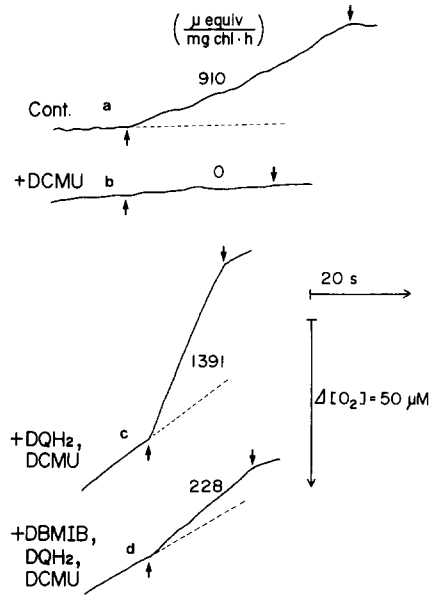

Fig. 1. Methyl viologen photoreduction in *Synechococcus* cells. Oxygen uptake was determined with a Clark-type oxygen electrode at 55°C. Cells were suspended in the fresh growth medium supplemented with 25 mM Hepes-NaOH (pH 7.5), 2 mM methyl viologen and 1 mM KCN, and illuminated with white light of $3.3 \cdot 10^2$ W·m^{-2}. Numbers indicate the rates of electron flow (μequiv/mg Chl per h) estimated by assuming an e/O_2 ratio of 2 (see text). Upward and downward arrows indicate times when light was turned on and off, respectively. (a) No addition; (b) 10 μM DCMU was added; (c) 10 μM DCMU and 0.5 mM duroquinol (DQH_2) were added; (d) 10 μM DCMU, 0.5 mM duroquinol and 2 μM DBMIB were added.

Methods

The thermophilic cyanobacterium *Synechococcus* sp. was grown for 24 h at 55°C [11]. Flash-induced absorption changes in cells were measured as described previously [12–14]. Methyl viologen photoreduction was assayed with a Clark-type oxygen electrode as described in Ref. 12. The reaction medium contained 25 mM Hepes-NaOH (pH 7.5), 2 mM methyl viologen, 1 mM KCN and cells equivalent to about 10 μg chlorophyll/ml.

Duroquinol was prepared according to the procedure described in Ref. 7. Chlorophyll was determined as described by Mackinney [15].

Results

Effects of duroquinol on methyl viologen photoreduction

Methyl viologen serves as a good electron acceptor of PS I in the *Synechococcus* cells [12]. Photoreduction of methyl viologen with water as electron donor results in a net oxygen uptake as reduced methyl viologen is rapidly reoxidized with oxygen (Fig. 1, trace a). The activity was completely inhibited by 10 μM DCMU (trace b). Trace c shows that the addition of duroquinol to the DCMU-inhibited cells restored oxygen uptake at a rate considerable higher than that of the original activity in unpoisoned cells.

With water as electron donor a rate of 910, while with duroquinol a rate of 1390 μequiv./mg Chl per h was observed, taking into account that with water four, with duroquinol two electrons per consumed O_2 molecule are carried through the photosynthetic electron transport system. This is the case if catalase is inhibited by KCN, and superoxide dismutase is acting [7,16,17]. If super-

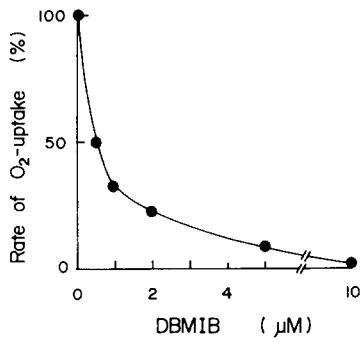

Fig. 2. Effects of DBMIB on the rates of methyl viologen photoreduction supported by duroquinol. Experimental conditions were the same as in Fig. 1. Light-dependent oxygen uptake was measured in the presence of 10 μM DCMU, 0.5 mM duroquinol and indicated concentrations of DBMIB. The 100% rate of methyl viologen photoreduction determined in the absence of DBMIB was 725 μmol O_2/mg Chl per h.

oxide dismutase does not act, superoxide radical anions, formed from O_2 and reduced methyl viologen, could directly oxidize duroquinol [7,16]. The e/O_2-ratio would maximally drop to 1, and the calculated rate would be half (695 instead of 1390 in Fig. 1c). Even then, the rate with duroquinol would be high. It is concluded, therefore, that duroquinol can penetrate into *Synechococcus* cells and serve as an excellent electron donor to PS I.

Fig. 1, trace d, shows that methyl viologen photoreduction restored by duroquinol was strongly inhibited by DBMIB. This indicates that duroquinol donates electrons in the plastoquinone region in the cyanobacterial cells. As described in Introduction, however, there are indications that duroquinol also gives electrons directly to P-700 or plastocyanin [4,8]. In order to determine the site(s) of the electron donation from duroquinol, we have studied the effect of various concentrations of DBMIB on the duroquinol-restored methyl viologen photoreduction. Fig. 2 shows that methyl viologen photoreduction supported by 0.5 mM duroquinol was almost completely inhibited by 10 μM DBMIB. The results indicate that duroquinol cannot directly donate an electron to P-700 or cytochrome *c*-553 (which replaces plastocyanin in the cyanobacterium [13,18]) at a significant rate. Fig. 2 also shows that methyl viologen photoreduction supported by duroquinol is inhibited by 50% at 0.5 μM DBMIB. This is significantly lower than the DBMIB concentration (1 μM or more), which is needed to half-inactivate methyl viologen photoreduction with water as electron donor (Ref.

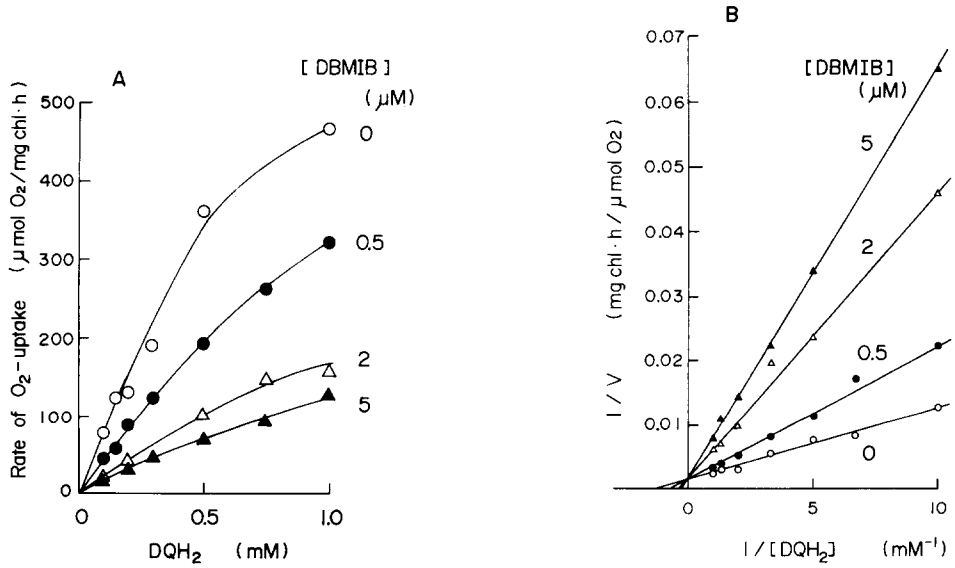

Fig. 3. (A) Effects of duroquinol (DQH_2) on the rates of methyl viologen photoreduction in the presence of various concentrations of DBMIB. (B) Double-reciprocal plots of the data in (A). The activity was measured in the presence of 10 μM DCMU and indicated concentrations of duroquinol and DBMIB. Other experimental conditions were the same as in Fig. 1, except that O_2 uptake was determined at 40°C.

12 and data not presented). A higher sensitivity to DBMIB of the duroquinol-dependent activity as compared with the activity with water as electron donor has been previously reported with chloroplasts [7,8].

Fig. 3A shows effects of a wide range of duroquinol concentrations on rates of methyl viologen photoreduction determined in the presence of three different concentrations of DBMIB. It is seen that the rate of oxygen uptake increased with increasing concentrations of duroquinol at all DBMIB concentrations examined. Higher concentrations of duroquinol could not be tested because of its poor solubility in water. The double-reciprocal plots of the data clearly show that the DBMIB-inhibition is competitive; the maximum rates of electron transport obtained at the infinite concentration of duroquinol (the intercepts on the ordinate) are not at all affected by DBMIB (Fig. 3B). The data strong suggest that DBMIB and duroquinol compete for a common binding site, so that DBMIB suppresses duroquinol-supported electron flow, while duroquinol reverses the DBMIB inhibition.

Experiments shown in Fig. 3 were carried out at 40°C. Similar experiments were performed at 55°C and kinetic parameters determined at the two temperatures are shown in Table I. An apparent K_m for duroquinol estimated in the absence of DBMIB was much larger than the K_i for DBMIB. This explains why the reversal of the DBMIB inhibition by duroquinol is only partial. Rates of methyl viologen photoreduction with water as electron donor range between 1000 and 1500 μequiv/mg Chl per h at 55°C (Ref. 12 and data not presented). Table I shows that the maximum rate of electron transport from duroquinol to methyl viologen at 55°C estimated by assuming an e/O_2 ratio of 2 is approximately twice as large as rates of electron transport from water.

TABLE I

KINETIC PARAMETERS OF DBMIB INHIBITION ON DUROQUINOL-SUPPORTED METHYL VIOLOGEN PHOTOREDUCTION

	Assay temperature: 40°C	55°C
V_{max} (μequiv/mg Chl per h)	1280	2870
K_m for duroquinol	0.7 mM	0.6 mM
K_i for DBMIB	0.8 μM	0.2 μM

Effects of duroquinol on reduction kinetics of PS I electron donors

Reaction kinetics of P-700, cytochrome *c*-553 and cytochrome *f* have been previously resolved from flash-induced absorption changes in *Synechococcus* cells by means of the computer subtraction [13]. The site of electron donation from duroquinol to PS I can be more directly determined by measuring effects of DBMIB and duroquinol on flash-induced absorption changes of PS I electron donors in *Synechococcus* cells. A previous work has shown that *N,N'*-tetramethyl-*p*-phenylenediamine accelerates only P-700 reduction in DBMIB-poisoned cells, leaving the reduction kinetics of cytochrome *c*-553 and cytochrome *f* inhibited [19]. Fig. 4 shows that there is no

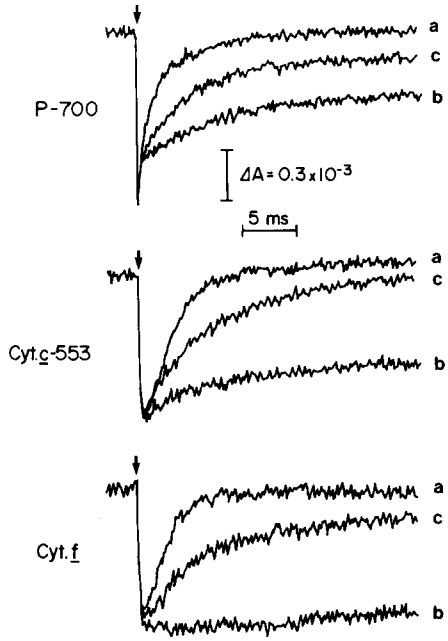

Fig. 4. Effects of duroquinol on the reduction kinetics of P-700, cytochrome *c*-553 and cytochrome *f* in DBMIB-inhibited cells. The reaction medium contained 25 mM Hepes-NaOH (pH 7.5), 5 μM gramicidin D, 50 mM KCl and 2 mM sodium ascorbate. Flashes were fired at 0.5 Hz and 200 signals were averaged. Arrows mark flash illumination. Measurements were carried out at 55°C and photoresponses of P-700, cytochrome *c*-553 and cytochrome *f* were resolved as described in Ref. 13. (a) No addition; (b) 10 μM DBMIB and 0.5 mM duroquinol.

specific interaction between P-700 (or P-700 and cytochrome *c*-553) and duroquinol: Reduction of flash-oxidized P-700, cytochrome *c*-553 and cytochrome *f*, which had been strongly suppressed by 10 μM DBMIB (traces b), was similarly accelerated by the addition of duroquinol (traces c). The results support the conclusion that duroquinol cannot bypass the cytochrome b_6-*f* complexes.

The effects of DBMIB and duroquinol on the reduction kinetics of cytochrome *f* were examined in more detail (Fig. 5). We have shown previously that flash-oxidized cytochrome *f* undergoes monophasic reduction with a half-time of about 2 ms and DBMIB does not affect the half-time but diminishes the amount of cytochrome *f* that is reduced with the 2 ms kinetics because the reduction of the Rieske center is inhibited in the DBMIB-bound cytochrome b_6-*f* complexes, but not in unpoisoned complexes [13,20]. Fig. 5 shows that duroquinol accelerated cytochrome *f* reduction mainly by restoring the 2 ms reduction component in DBMIB-poisoned cells. Duroquinol also increased the magnitude of the 2 ms reduction phase of cytochrome *c*-553 and P-700 in the inhibited cells (not shown). The effect of duroquinol that is just opposite to that of DBMIB is consistent with the conclusion that duroquinol competes with DBMIB for a common binding site.

Comparison of the results shown in Fig. 5 with those shown in Fig. 2 reveals, however, an unexpected feature of the effect of duroquinol. The addition of 500 μM duroquinol to the cells which had been inhibited by 10 μM DBMIB restored more than half of the 2 ms reduction component of cytochrome *f* (Fig. 5). The same concentration of duroquinol was, however, much less effective in reactivating methyl viologen photoreduction (Fig. 2). Restoration of the cytochrome *f* reduction was always significantly larger than that of methyl viologen photoreduction with different combinations of the duroquinol and DBMIB concentrations (not shown). The effect of duroquinol may be related to the different experimental conditions used for measurement of methyl viologen photoreduction and cytochrome *f* reduction (see Discussion).

Discussion

The present work shows that duroquinol can be used as an electron donor to PS I in *Synechococcus* cells. Duroquinol restores methyl viologen photoreduction in DCMU-poisoned cells. Rates of electron transport thus restored is comparable to or, more likely, higher than that of electron transport from water to methyl viologen. Thus duroquinol penetrates into the cyanobacterial cells and serves as an excellent electron donor to PS I.

The results obtained here show that duroquinol donates its electrons specifically to the cytochrome b_6-*f* complexes. Methyl viologen photore-

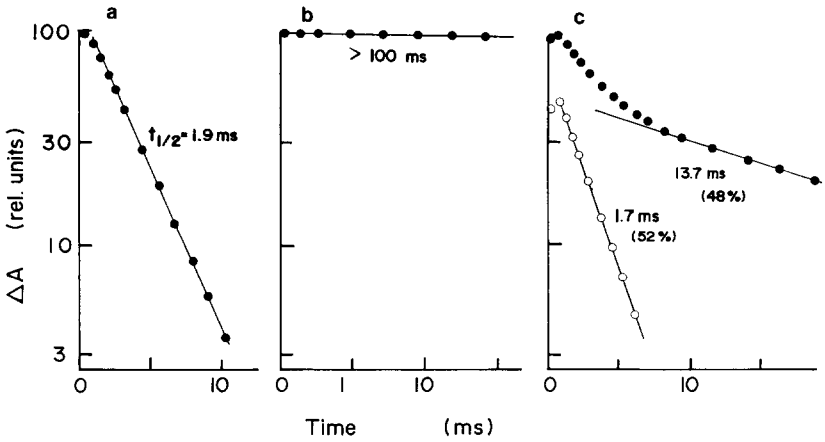

Fig. 5. Effects of DBMIB and duroquinol on the reduction kinetics of cytochrome *f*. Experimental conditions were the same as in Fig. 4, except that 10 μM DCMU was added. (a) No addition; (b) 10 μM DBMIB; (c) 10 μM DBMIB and 0.5 mM duroquinol.

duction supported by duroquinol was almost completely inhibited by 10 μM DBMIB. Spectrophotometric experiments clearly demonstrated that duroquinol does not interact directly with P-700 or cytochrome *c*-553. Evidently, duroquinol cannot bypass the cytochrome b_6-*f* complexes.

Detailed kinetic analyses of the DBMIB inhibition of steady-state electron transport from duroquinol to methyl viologen revealed a competitive relationship between duroquinol and the inhibitory quinone. The results obtained from spectrophotometric experiments that duroquinol reverses the 2 ms reduction of cytochrome *f* in a manner just opposite to the inhibitory action of DBMIB are consistent with the competition between the reductant and the inhibitor. We conclude that duroquinol does not serve as a reductant of the pool plastoquinone but feeds its electrons directly to the cytochrome b_6-*f* complex by binding to a quinone-binding site of the complex and DBMIB inhibits the quinol oxidation by competing for the same site.

Supporting evidence comes form the observations that duroquinol serves as electron donor for isolated cytochrome b_6-*f* complexes without pool quinone and that quinols do not efficiently reduce quinones in a hydrophobic environment [21]. Our conclusion is also consistent with the previous findings that the DBMIB inhibition is reversed on addition of plastoquinone [2] and the DBMIB is a good reductant of the isolated cytochrome b_6-*f* complexes in single turnovers but is inhibitory in multiple turnovers [22].

The quinone-binding site may be located near the Rieske iron-sulfur center because the center is considered to be the initial electron acceptor of the cytochrome b_6-*f* complex [21,23]. Photoaffinity labelling of the isolated complexes with a radioactive azido derivative of plastoquinone has shown that the label has a high affinity toward, among others, a 20 kDa protein which carries the Rieske center [24]. Koike et al. [25] have found that DBMIB and bathophenanthroline markedly increases the magnitude of cytochrome *f* oxidation invoked by short saturating flashes in spinach chloroplasts and proposed that the inhibitors block electron transfer from an electron carrier, presumably the Rieske center, to cytochrome *f*. EPR studies have shown that DBMIB interacts with the Rieske center, causing a change in the EPR signal and the redox potential of the iron-sulfur center [26] and the change is reversed by the addition of plastoquinone [27].

There is, however, a report that DBMIB has no effect on the EPR spectrum of the Rieske center [28]. Our previous kinetical studies on the effect of DBMIB on reduction kinetics of P-700 and cytochromes in *Synechococcus* cells demonstrated that the DBMIB blocks reduction of the Rieske center with electron from plastoquinone but affects neither its midpoint potential nor its oxidation by cytochrome *f* [14]. This is consistent with the competitive mode of the DBMIB inhibition found here. The DBMIB-enhanced oxidation of cytochrome *f* in chloroplasts was therefore attributed to inhibition of electron transport from a plastoquinone molecule bound to the cytochrome b_6-*f* complex, rather than from the Rieske center, to cytochrome *f* [14]. No enhancement of cytochrome *f* oxidation is observed in *Synechococcus* cells where, among others, oxidation rate of cytochrome *f* is much faster than its reduction rate.

Cytochrome *b*-c_1 complexes of photosynthetic bacteria have two ubiquinone-binding sites. Ubiquinone bound to one site (Q_z) is oxidized by the Rieske iron-sulfur center to a semiquinone form, which in turn gives its electron to cytochrome *b*, while another site is involved in the cytochrome *b* oxidation [22]. It may be assumed that the cytochrome b_6-*f* complexes contains similar two quinone-binding sites and both duroquinone and DBMIB bind to the quinone oxidation site. The role of cytochrome b_6 in quinol oxidation in *Synechococcus* cells is not clear because the cyanobacterium shows no detectable photoresponse of the cytochrome [13].

Duroquinol was found to be rather ineffective in reactivating methyl viologen photoreduction at a concentration which restores about half the 2 ms reduction component of cytochrome *f* in DBMIB-poisoned cells (see Figs. 2 and 5). This may be ascribed to the different experimental conditions used for measurement of the two reactions: Methyl viologen photoreduction was assayed under continuous light but cytochrome *f* reduction was determined with repetitive flashes fired at 0.5 Hz. There would be three explanations. (1) The first one assumes that the effect of

DBMIB varies with redox states of the inhibitory quinone, the quinol being a reductant and the semiquinone or quinone being an inhibitor. The inhibitory form of DBMIB binds stably to the binding site but is slowly replaced with the quinol. Then, while the cytochrome b_6-f complexes are mostly blocked under continuous light, the 2 ms reduction of cytochrome f takes place in a significant fraction of the complexes in which the inhibitor is replaced by the quinol during the dark time of 2 s between flashes. (2) The inhibitory effect of DBMIB is considered to depend upon the redox state of the PS I donors such as cytochrome f or the Rieske center in the second explanation. If DBMIB is more strongly inhibitory with the oxidized donors than with the reduced donors, the cytochrome f reduction is less sensitive to DBMIB than the steady-state electron transport because cytochrome f and the Rieske center are rereduced during the flash intervals in the flash experiments but the PS I donors are largely kept in the oxidized state under continuous illumination. (3) The third explanation assumes the occurrence of a bound electron carrier between the Rieske center and the DBMIB inhibition site, which gives its electron to the Rieske center with a 2 ms half-time and in turn is reduced slowly during the flash intervals. Interestingly, highly purified cytochrome b_6-f complexes contain a tightly bound plastoquinone molecule [29] and the complexes which are largely depleted of the bound plastoquinone are inactive in duroquinol-plastocyanin oxidoreductase activity but the activity is reconstituted by the addition of plastoquinone [30]. We expect that the duroquinol effect found here would supply an important clue for elucidation of the mechanism of the DBMIB inhibition and the quinol oxidation. Experiments along this line are in progress.

Acknowledgement

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