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EFFECTS OF DIBROMOTHYMOQUINONE ON OXIDATION-REDUCTION REACTIONS AND THE MIDPOINT POTENTIAL OF THE RIESKE IRON-SULFUR CENTER IN PHOTOSYNTHETIC ELECTRON TRANSPORT OF *SYNECHOCOCCUS* SP.

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The effects of 2,5-dibromo-3-methyl-*p*-benzoquinone (DBMIB) on the reduction kinetics of flash-oxidized P-700 and cytochrome *c*-553 were studied in the thermophilic cyanobacterium *Synechococcus* sp. (1) The reduction kinetics of P-700 showed two exponential phases with half-times of 0.2 and 2 ms at the recording time used (Nanba, M. and Katoh, S. (1983) Biochim. Biophys. Acta 725, 272–279). DBMIB strongly slowed down the 2 ms reduction phase but not the 0.2 ms phase. (2) The content of an electron donor which transfers its electrons to P-700 with the half time of 0.2 ms was estimated to be comparable to that of cytochrome *f*. (3) The magnitudes of the 0.2 ms reduction phase and cytochrome *c*-553 oxidation decreased as the flash interval was shortened below 2 s in the poisoned cells. Assuming a rapid equilibrium of electrons in the electron donor pool of Photosystem I, the midpoint potential of the 0.2 ms donor was estimated as 280 mV by comparing its percent reduction with that of cytochrome *c*-553 at three different flash intervals. (4) A similar value was obtained for the midpoint potential of the 0.2 ms donor in the cells in which the plastoquinone pool had been oxidized by dark starvation. It is concluded that the 0.2 ms reduction phase of P-700 is due to the electron donation from the Rieske iron-sulfur center and that DBMIB inhibits strongly but incompletely the reduction of the iron-sulfur center with electrons from the plastoquinone pool, whereas the inhibitor has no effect on the midpoint potential and Photosystem-I-dependent oxidation of the iron-sulfur center.

Introduction

The occurrence of the Rieske iron-sulfur center in photosynthetic electron transport of green plants has first been reported by Malkin and Aparicio [1], who observed an EPR signal with a *g*-value of 1.89 and a midpoint potential of 290 mV in spinach chloroplasts. The Rieske center is present in the cytochrome b_6-*f* complexes isolated from spinach [2] or cyanobacterium [3] and a 20 kDa polypeptide of the complex was identified as the subunit which carries the iron-sulfur center [4]. The Rieske center is an essential component for the plastoquinol-plastocyanin (cytochrome *c*-553) reductase activity of the complex [5]. Little is, however, known about the reaction kinetics of the Rieske iron-sulfur center in situ, which are important for the understanding of mechanism of electron transport from plastoquinol to Photosys-

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone); DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll, UHDBT, 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole; Cyt, cytochrome.

tem I. Redox changes of the iron-sulfur center are difficult to monitor spectrophotometrically due to its weak and broad absorption bands [6]. The EPR method measures the $g = 1.89$ signal at cryogenic temperature and thus is not feasible for detailed kinetical analysis of redox changes of the iron-sulfur center.

In the previous work, we resolved reaction kinetics of P-700, cytochrome *c*-553 and cytochrome *f* from flash-induced absorption changes in the intact cells of the thermophilic cyanobacterium *Synechococcus* sp. by means of the computer subtraction [7]. The reduction kinetics of P-700 shows a small lag and then three successive exponential phases with half-times of 40 μs, 0.2 ms and 2 ms. The 0.2 ms reduction phase is ascribed to electron transfer from the Rieske iron-sulfur center to P-700 because it occurs after cytochrome *f* is oxidized ($t_{1/2} = 40$ μs) and is followed by electron donation from plastoquinone ($t_{1/2} = 2$ ms). It was concluded that the Rieske iron-sulfur center is oxidized by cytochrome *f* and in turn is reduced by plastoquinone with half-times of 0.2 and 2 ms, respectively.

DBMIB is a plastoquinone analog which inhibits linear electron transport in the plastoquinone region [8,9]. The inhibitor specifically blocks the oxidation but not the reduction of the plastoquinone pool at low concentrations, although it additionally suppresses electron flow from Photosystem II to the plastoquinone pool at higher concentrations [9,10]. The binding of DBMIB with the cytochrome b_6-*f* complex, possibly at the plastoquinone binding site, was indicated by the sensitivity to the inhibitor of the plastoquinol-1-plastocyanin oxidoreductase activity of the isolated complex [2]. A direct interaction between DBMIB and the Rieske iron-sulfur center in spinach chloroplasts was suggested by the observation that the $g = 1.89$ signal disappears with a concomitant appearance of a new EPR signal with a *g*-value of 1.94 in the presence of DBMIB [11–13]. The Rieske iron-sulfur protein isolated from the complex does not, however, show any alteration of its EPR signal on the addition of DBMIB [5]. The site and mode of the DBMIB inhibition has yet to be studied.

In the present work, flash-induced absorption changes of P-700, cytochrome *c*-553 and cytochrome *f* in *Synechococcus* cells were studied (1) to examine whether the electron carrier which donates its electrons to P-700 with the 0.2 ms half-time is indeed the Rieske iron-sulfur center and, if so, (2) to determine the inhibition site of DBMIB in respect of the Rieske iron-sulfur center. The effects of the cell starvation on the 0.2 ms reduction phase of P-700 were also investigated.

Materials and Methods

The thermophilic cyanobacterium *Synechococcus* sp. was grown for 24 h at 55°C as described previously [14,15]. For spectrophotometric measurement, the cells were suspended in the fresh culture medium containing 25 mM Hepes-NaOH (pH 7.5) and 20 mM fructose to give a final chlorophyll *a* concentration of about 10 μg/ml. The suspension was kept under illumination with white light of 1000 lux at 22–24°C prior to measurement [7,16]. The addition of fructose and the prior illumination were necessary to keep the plastoquinone pool in the fully reduced state during the course of experiments [15]. To eliminate the fast electrochromic absorption change, 5 μM gramicidin D and 50 mM KCl were added just before measurement [7,16]. Also added was 2 mM Na-ascorbate to keep DBMIB in the reduced form. Otherwise, DBMIB, which is highly autooxidizable at 55°C, caused a slow oxidation of the plastoquinone pool [17]. Because O_2 was rapidly consumed in the presence of DBMIB and ascorbate, absorption changes were measured under the anaerobic condition. The starvation of cells was carried out by aerating the suspension in the dark, and fructose and ascorbate were not added.

Absorption changes were measured with a single beam spectrophotometer as described previously. [7,15,18]. A xenon lamp was a source of the actinic flash with a half-duration of 5 μs but with a rather long tailing of about 80 μs [7]. The flash was passed through Toshiba VR-65 and VR-66 filters. The photomultiplier was protected against the actinic light with two Corning 4-96 filters. Photoresponses of P-700, cytochrome *c*-553 and cytochrome *f* in the Soret band region were resolved by the computer subtraction as described previously [7]. In order to obtain signals amenable to the subtraction method, the flash illumination was

repeated and 200–400 signals were averaged. All measurements were carried out at 55°C. DBMIB was a gift from Dr. A. Trebst in University of Bochum, and Dr. M. Nishimura, Kyushu University. Chlorophyll was determined as described by Mackinney [19].

Results

The effects of DBMIB on photosynthetic electron transport in *Synechococcus* cells have been studied previously [15]. At 10 μM, DBMIB nearly completely inhibits electron transport from H_2O to methyl viologen via Photosystem I and II and strongly suppresses the reduction of flash-oxidized *c*-type cytochrome(s) measured at 553 nm. The photoresponse of P-700, a soluble cytochrome *c*-553 and a membrane-bound cytochrome *f* were recently resolved from flash-induced absorption changes in the Soret band region by the computer subtraction [7]. Fig. 1 shows kinetics of flash-induced changes of P-700 and cytochrome *c*-553 thus resolved. On the flash excitation, P-700 and cytochrome *c*-553 were oxidized very rapidly and then were rereduced to the original level about 10 ms after the flash was fired (traces a and c). The reduction kinetics of P-700 were biphasic, consisting of two first-order phases with half times of about 0.2 and 2 ms [7]. (The initial small lag and the 40 μs reduction phase were not resolved at the recording time used.) It is seen that 10 μM DBMIB strongly inhibited the slow but not the fast reduction phase of P-700 (trace c). The semilogarithmic plot of the reduction kinetics indicated that the rate of the fast reduction phase was not affected by the addition of the inhibitor (data not shown). The results indicate that an electron donor which reduces P-700 with the 0.2 ms half time (the 0.2 ms donor) is present between the plastoquinone pool and P-700 and thus electron transfer from the 0.2 ms donor to Photosystem I is insensitive to DBMIB.

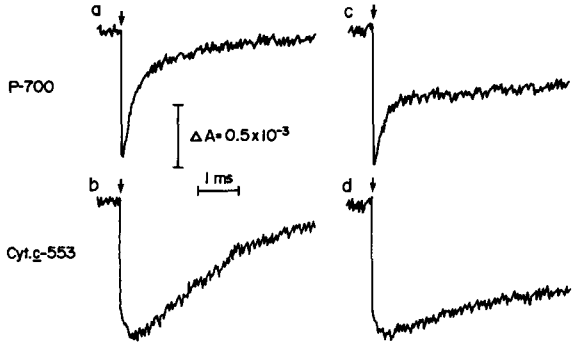

Fig. 1. Effects of DBMIB on the reduction kinetics of P-700 and cytochrome *c*-553. Cells were suspended in fresh growth medium containing 25 mM Hepes-NaOH (pH 7.5)/5 μM gramicidin D/50 mM KCl/20 mM fructose/2 mM Na ascorbate. Chlorophyll concentration was 6.8 μg/ml. Flash repetition rate was 0.5 Hz and 400 signals were averaged. Arrows mark flash illumination. a and b, no addition; c and d, 10 μM DBMIB was added.

The reduction kinetics of cytochrome *c*-553 and cytochrome *f* are both monophasic, and their half-reduction-times of 2 ms agree well with that of the slow reduction phase of P-700, leading to a conclusion that the reduction of the three electron carriers involves a common rate-limiting reaction, i.e., plastoquinone oxidation [7]. In fact, DBMIB inhibited reduction of cytochrome *c*-553 (trace d) and cytochrome *f* (not shown) as strongly as the slow reduction phase of P-700.

Fig. 1 shows, however, that the magnitudes of P-700 and cytochrome *c*-553 oxidation were little affected on addition of DBMIB. Because the experiments were carried out at the flash repetition rate of 0.5 Hz, this indicates that P-700 and cytochrome *c*-553 are fully rereduced during the flash interval of 2 s even in the presence of 10 μM DBMIB.

Aoki and Katoh [17] showed that DBMIB inhibits electron transport from plastoquinone to Photosystem I strongly but incompletely in *Synechococcus* cells; illumination with Photosystem I light caused a slow oxidation of the plastoquinone pool (measured by the area over the fluorescence induction curve) in the presence of DBMIB at concentrations up to 10 μM. We have, therefore, examined the mode of the DBMIB inhibition by varying the flash intervals and the results are illustrated in Fig. 2. The inhibitory effect of DBMIB was clearly demonstrated at the flash intervals below 2 s; the magnitudes of the cytochrome *c*-553 oxidation decreased with increasing flash repetition rate. Because the extent of the cytochrome oxidation reflects the amount of the cytochrome which has been reduced during the flash interval, the results show that DBMIB

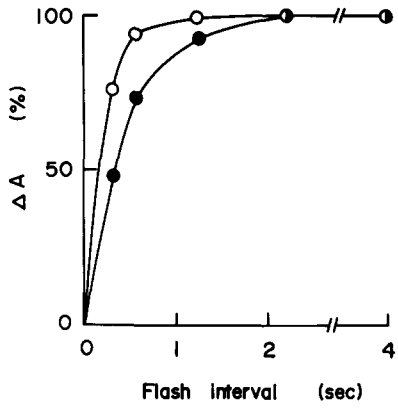

Fig. 2. Magnitudes of cytochrome *c*-553 oxidation and the 0.2 ms reduction phase of P-700 determined in the presence of 10 μM DBMIB at different flash intervals. Experimental conditions were the same as in Fig. 1, except that the flash repetition rate was varied as indicated and 200 signals were averaged. Open circle, magnitude of cytochrome *c*-553 oxidation. Closed circle, magnitudes of the 0.2 ms reduction phase estimated by the exponential analysis [7]. Signal sizes measured in the absence of the inhibitor were taken as 100%.

strongly suppresses the reductive reaction of cytochrome *c*-553. Note that the magnitude of the 0.2 ms reduction phase was also diminished as the flash interval was shortened. The shortening of the flash interval had no effect on the signal size in unpoisoned cells. We conclude, therefore, that DBMIB inhibits reduction of the 0.2 ms donor and cytochrome *c*-553 strongly but incompletely.

Fig. 2 also shows that the accumulation of the reduced cytochrome *c*-553 during the flash interval is significantly faster than that of the reduced 0.2 ms donor. This can be ascribed to difference in the midpoint potential of the two electron donors because the electron donation from the 0.2 ms donor to P-700 ($t_{1/2} = 0.2$ ms) is two or three orders of magnitude faster than electron flow from the plastoquinone pool to the Photosystem I electron donors in the presence of 10 μM DBMIB and thus one can assume an equilibrium of electrons among P-700, cytochrome *c*-553, cytochrome *f* and the 0.2 ms donor according to their midpoint potentials. The data suggest therefore that the midpoint potential of the 0.2 ms donor is somewhat lower than that of cytochrome *c*-553, which was determined as 315 mV at 50°C [20].

In order to estimate the midpoint potential of the 0.2 ms donor by comparing the redox state of the 0.2 ms donor with that of cytochrome *c*-553 at each flash interval, it is necessary to know the total contents of the 0.2 ms donor and cytochrome *c*-553 in cells. The first column of Table I shows the amounts of P-700, cytochrome *c*-553 and cytochrome *f* oxidized by the flash. In this experiment, the 0.2 ms reduction phase amounted to 47% of the total P-700 oxidized, or to 16.9 nM P-700.

It is to be mentioned here that the reduction kinetics of P-700 show a lag lasting several tens microseconds due to the flash tailing, during which most of cytochrome *c*-553 and about a half of cytochrome *f* are oxidized. The above value would be an underestimation, if a fraction of the 0.2 ms donor undergoes oxidation during the lag of P-700 reduction as well. The amount of the 0.2 ms donor was, therefore, compared with that of cytochrome *f*, which is present in the stoichiometric amount of the Rieske iron-sulfur center in the cytochrome b_6-*f* complex [2,3]. The cytochrome *f* content was determined previously by measuring the oxidized minus reduced difference spectrum of the isolated thylakoid membranes, but the values obtained varied somewhat with cultures [21]. In the present work, therefore, the relative amount of cytochrome *f* present in the same sample was estimated by measuring absorption changes induced by illumination with a strong continuous light. DCMU and DBMIB were added to ensure complete

TABLE I

AMOUNTS OF P-700, CYTOCHROME *C*-553 AND CYTOCHROME *F* OXIDIZED BY ILLUMINATION WITH FLASH AND CONTINUOUS ILLUMINATION

Experimental conditions for the flash experiment were the same as in Fig. 1. Experiments with continuous light were carried out with the same spectrophotometer by replacing the xenon lamp by a 100 W halogen-tungsten lamp ($1.8 \cdot 10^2$ J·m^{-2}·s^{-1}) and 20 μM DCMU and 10 μM DBMIB were added. Chlorophyll concentration was 6.8 μg·ml^{-1}. Correction was made for the flattening effect [7].

	Flash (nM)	Continuous light (nM)	Flash / Continuous light
P-700	36.0	51.1	0.70
Cyt. *c*-553	29.1	28.1	1.03
Cyt. *f*	14.6	19.1	0.76

oxidation of all the electron donors of Photosystem I. The photoresponses of P-700, cytochrome *c*-553 and cytochrome *f* were then resolved by subtracting overlapping absorption changes. The contents of the three electron carriers thus determined agree well with the previous estimation, confirming that P-700 is present in excess of cytochrome *c*-553, while the cytochrome *f* content is slightly less than that of cytochrome *c*-553 [21]. The cytochrome *f* content of 19.1 nM is larger than that of the 0.2 ms donor but, as stated above, this is explained by a partial oxidation of the 0.2 ms donor during the lag of P-700 reduction. Thus, it is reasonable to assume that the content of the 0.2 ms donor is similar to that of cytochrome *f*. Table I also shows that all cytochrome *c*-553 present in cells is oxidized by the flash.

Table II shows the percent reduction of cytochrome *c*-553 and the 0.2 ms donor estimated from the data presented in Fig. 2. The midpoint potentials of the 0.2 ms donor estimated at three flash intervals agree with each other and the mean value of 277 mV is comparable to that of the Rieske iron-sulfur center in *Rhodopseudomonas sphaeroides* (285 mV, $n = 1$) [22] or in spinach chloroplasts (290–310 mV, $n = 1$) [1,11]. The content as well as the midpoint potential of the 0.2 ms donor thus determined provide strong supports for our conclusion that the 0.2 ms reduction phase of P-700 is due to electron transfer from the Rieske iron-sulfur center.

EPR experiments suggested that DBMIB affects the Rieske center in situ in a way to decrease its midpoint potential [11]. We attempted, therefore, to measure the midpoint potential of the 0.2 ms donor in the absence of DBMIB. The electron flow from the plastoquinone pool to the electron donors of Photosystem I is slowed down when the pool is oxidized. The plastoquinone pool of photosynthetic electron transport is shared by respiratory chains in the cyanobacterium and hence can be oxidized with O_2 when endogenous reductants are exhausted by the dark aeration of the cells [15,17,23]. Retardation of cytochrome 553 reduction in the starved cells has been observed previously [15].

Fig. 3 shows effects of the starvation on flash-induced absorption changes at 434 nm and 415 nm. In this experiment, the photoresponses of cytochrome *c*-553 and P-700 were not resolved because the redox state of the plastoquinone pool changed gradually during the course of the computer manipulations. Absorption changes at 434 nm and 415 nm can be, however, ascribed mostly to redox changes of P-700 and cytochrome *c*-553, respectively, because overlapping absorption changes are small [7]. The starvation of cells for 1 h resulted in a considerable retardation of the relaxation of flash-induced absorption changes at the two wavelengths. In addition, the magnitude of the photoresponse at 415 nm was diminished significantly in the starved cells, indicating that cyto-

TABLE II

THE MIDPOINT POTENTIAL OF THE 0.2 ms DONOR DETERMINED IN THE PRESENCE OF DBMIB

The data presented in Fig. 2 were used to estimate percent reduction of cytochrome *c*-553 and the Rieske iron-sulfur center. The midpoint potential of the 0.2 ms donor was calculated by the use of the Nernst equation, assuming the midpoint potential of cytochrome *c*-553 as 315 mV (55°C).

Flash interval (s)	Percent reduction		Midpoint potential (mV)
	Cyt. *c*-553	Rieske Fe-S	
0.3	75.7	51.7	285
0.5	92.9	74.4	273
1.2	98.6	94.3	274
			277 (mean value)

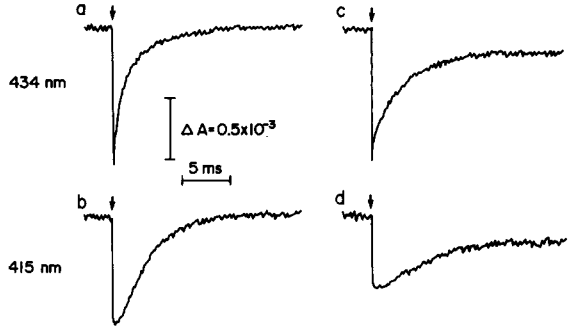

Fig. 3. Effects of dark starvation of cells on flash-induced absorbance changes at 434 nm (P-700) and 415 nm (cytochrome *c*-553). Cells were incubated at 55°C in the light (white light, 1000 lux) for 1 h (a and b), and then in the dark with vigorous aeration for 1 h (c and d) in the absence of fructose. Flashes were fired at 1 Hz and 250 signals were averaged. Other conditions were the same as in Fig. 1.

TABLE III

THE MIDPOINT POTENTIAL OF THE 0.2 ms DONOR DETERMINED IN DARK-STARVED CELLS

Experimental conditions were the same as in Fig. 3.

Dark time (h)	Percent reduction		Midpoint potential (mV)
	Cyt. *c*-553	0.2 ms comp.	
0	100	100	–
0.5	95	83	277
1	68	49	292
1.5	45	20	282
			284 (mean value)

chrome *c*-553 cannot be fully rereduced during the flash interval of 1 s. Although the total signal size of P-700 oxidation was not affected, the magnitude of the 0.2 ms reduction phase was reduced in the starved cells. In this case again, the rate of the 0.2 ms reduction phase remained constant irrespective of the degrees of the cell starvation.

Table III summarizes the results obtained from the cells which had been starved for different periods of time. The midpoint potential of the 0.2 ms donor was fairly constant in the cells which had been starved to different extents and agree well with that obtained from the DBMIB-poisoned cells. We conclude therefore that the midpoint potential of the Rieske center is not significantly affected by DBMIB.

Discussion

The present investigation characterizes an electron carrier which donates electrons to P-700 with a half-time of 0.2 ms in *Synechococcus* cells. It was demonstrated that the electron carrier has a high midpoint potential similar to that of the Rieske iron-sulfur center in chloroplasts [1], chromatophore [22] and mitochondria [24]. The content of the electron carrier in cells is comparable to that of cytochrome *f*, and thus to that of the iron-sulfur center. The results strongly support our view that the electron carrier is the Rieske iron-sulfur center functioning in linear electron transport between cytochrome *f* and the plastoquinone pool.

Manifestation of the electron donation from the Rieske center in the reduction kinetics of P-700 relies on the relative contents of the electron donor pool of Photosystem I, rates of electron transfer between the donors in *Synechococcus* cells and the duration of the flash used. We confirmed the previous observation [21] that *Synechococcus* contains P-700 nearly twice in excess of cytochrome *c*-553 or cytochrome *f*. We also showed previously that P-700 is oxidized on average 1.5-times during the single excitation with the flash having a long tailing [7]. Although most of cytochrome *c*-553 and about a half of cytochrome *f* are oxidized during the flash excitation, a substantial amount of P-700 still remains in the oxidized form after the flash and thus accepts electrons successively from cytochrome *f*, the Rieske center and the plastoquinone pool. In *Anabaena* heterocysts, the electron transfer from the Rieske center seems to appear in the reduction kinetics of cytochrome *c*(*f*) [25], presumably reflecting difference in the stoichiometry and turnover rates of the Photosystem I electron donors.

The present study also provide important information on the site and mode of the inhibition of linear electron transport by DBMIB. DBMIB strongly inhibits the reduction of cytochrome *c*-553 and the Rieske iron-sulfur center. The inhibition was, however, incomplete; for instance, the half-time of the cytochrome *c*-553 reduction was increased from 2 to more than 100 ms in the presence of 10 μM DBMIB so that, under the repetitive flash excitation, the extent of the cytochrome reduction is a function of the flash interval.

The EPR experiment showed that the Rieske iron-sulfur center undergoes oxidation even in the presence of DBMIB when spinach chloroplasts are illuminated for 30 s at 20°C and then the EPR signal is measured at 15 K [13]. The results, however, cannot exclude a possibility that DBMIB inhibits oxidation of the Rieske center incompletely so that the oxidized center accumulates slowly during the long illumination period used. In contrast, our results unequivocally demonstrate that electron transfer from the Rieske center to P-700 is totally insensitive to the inhibitor; neither the magnitude nor the rate of the 0.2 ms reduction phase of P-700 was affected by DBMIB. The results are consistent with the inhibition mechanism which assumes that DBMIB competes with plastoquinone for the binding site of the cytochrome b_6-*f* complex [9,12,26].

The observation that DBMIB has no significant effect on the midpoint potential of the Rieske center is at variance with the EPR experiment, which suggests that the inhibitor decreases the midpoint potential of the Rieske center in spinach chloroplasts to 180 mV [11]. On addition of DBMIB, the $g = 1.89$ signal of the reduced iron-sulfur center is replaced by a new EPR signal with a g-value of 1.94. During the reductive titration, the $g = 1.94$ signal appears with a midpoint potential of 180 mV and then disappears with a midpoint potential of 20 mV [11]. This, together with the $n = 1$ behaviour, suggests that the transition is related to changes in the redox state of DBMIB involving a semiquinone form. More has to be learned about the $g = 1.94$ signal because there are reports that DBMIB has no effect on the EPR spectrum of the iron-sulfur center in lettuce chloroplasts [27] or in the isolated Rieske protein [5].

Koike et al. [28] observed that the extent of flash-induced cytochrome *f* oxidation amounts to only 25–40% of the total cytochrome *f* present in spinach chloroplasts but is increased approx. 2-fold in the presence of DBMIB. Similar enhanced photooxidations of cytochrome *f* in chloroplasts [29] and of cytochrome c_1 in chromatophores [30] by 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) has been reported. The effects of the poisons were interpreted in terms of inhibition of electron transport from the Rieske center to cytochrome *f* or an increase in the midpoint potential of the iron-sulfur center [28–30]. While the inhibition mechanism of UHDBT remains to be studied in future, our results clearly demonstrate that DBMIB affects neither the oxidation rate nor the midpoint potential of the Rieske center. Consistently, the fraction of cytochrome *f* that undergoes oxidation on the flash excitation is not increased by DBMIB in *Synechococcus cells*. Then a question arises as to why DBMIB enhances the cytochrome *f* photooxidation in chloroplasts. Our present working hypothesis is that there would be an another electron carrier between the plastoquinone pool and the Rieske center which transfers its electrons to cytochrome *f* to affect the extent of its oxidation in chloroplasts but not in *Synechococcus* cells where oxidation reactions of cytochrome *f* are much faster. A likely candidate would be a plastoquinone molecule bound to the cytochrome b_6/f complexes, because it is considered to be replaced by DBMIB. Experiments to examine this hypothesis is in progress.

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