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Effects of dibromothymoquinone and bathophenanthroline on flash-induced cytochrome *f* oxidation in spinach chloroplasts

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The effects of several electron transport inhibitors on the magnitude and kinetics of cytochrome *f* oxidation induced by flash illumination were studied in the α- and γ-band regions. On the flash excitation only a fraction of cytochrome *f* present in the chloroplasts was oxidized with a half time of 0.1 to 0.3 msec and then reduced with a half time of 10 to 25 msec.

Dibromothymoquinone (DBMIB) at concentrations which severely suppressed the reduction of cytochrome *f* approximately doubled the magnitude of cytochrome *f* oxidation caused by a flash, mainly by inducing an additional slow oxidation of cytochrome *f* with a half time longer than 1 msec. Enhancement of the cytochrome *f* oxidation was also observed in the presence of bathophenanthroline. Such enhanced oxidation induced by the two inhibitors was largely diminished with the addition of reduced 2,6-dichlorophenolindophenol which accelerated cytochrome *f* reduction. In contrast, the inhibition of cytochrome *f* reduction by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was not associated with an increase in the magnitude of cytochrome *f* oxidation. However, addition of DBMIB to the DCMU-poisoned chloroplasts enhanced cytochrome *f* oxidation, suggesting that this is related to a block of the electron transport between plastoquinone and cytochrome *f*. The results are explained by assuming the occurrence of an electron carrier between plastoquinone and cytochrome *f*.

Key words: Bathophenanthroline — Cytochrome *f* — Dibromothymoquinone — Flash spectroscopy — Spinach chloroplasts.

Photosynthetic electron transport in chloroplast involves two photosystems. In the chain of electron carriers connecting the two photosystems, electrons are transferred from the reduced primary electron acceptor of photosystem II to the oxidized P-700 via plastoquinone, cytochrome *f* and plastocyanin. For details of the current state of knowledge on photosynthetic electron transport, the reader is referred the reviews in references *1*, *6*, *23*, *26*.

There are several reports suggesting the existence of an intermediary electron carrier between plastoquinone and cytochrome *f*. The ESR studies of Malkin and Aparicio demonstrated that chloroplasts contain a non-heme iron protein, and a relatively high redox potential (290 mV) favors its function between plastoquinone

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCIP, 2,6-dichlorophenolindophenol.

and cytochrome *f* (*18*). The protein was not detected in a mutant of *Lemna perpusilla* which has a block in the electron transport between plastoquinone and cytochrome *f* (*19*). Bering et al. have suggested that a lipophilic chelator, bathophenanthroline, which blocks electron transport mediated by both photosystems I and II, but not those mediated by either photosystem alone, attacked a non-heme iron protein located in the region of plastoquinone and cytochrome *f* (*2*).

In the present work, the kinetics of flash-induced oxidation-reduction reaction of cytochrome *f* in spinach chloroplasts was studied in the α- and γ-band regions. The effects on the cytochrome *f* response of inhibitors which block electron transport between photosystems I and II were examined. The results suggest the presence of an electron carrier between plastoquinone and cytochrome *f*.

Materials and methods

Spinach was obtained from a local market. The leaves were homogenized in a Waring blender in a chilled preparation medium containing 0.4 M sucrose, 20 mM *N*-Tris-(hydroxymethyl)methylglycine or phosphate (pH 7.8), 10 mM NaCl, 10 mM KCl and 5 mM $MgCl_2$. After filtration through eight layers of gauze, the homogenate was centrifuged at $3,500 \times g$ for 10 min. The precipitate was resuspended in the preparation medium, then centrifuged at $220 \times g$ for 5 min to remove cell fragments and large debris. Chloroplasts were collected from the supernatant by centrifugation at $3,500 \times g$ for 10 min, then suspended in a small amount of the preparation medium. All procedures were performed at 0–4°C.

Absorbance changes were determined with a laboratory-constructed single beam spectrophotometer (*22*). The measuring beam from a Nikon monochrometor P-250 traversed the sample in a four-sided transparent cuvette (1×1 cm) at right angle to the actinic flash. The flash with a half time of 20 μsec came from a xenon flash lamp and passed through a Toshiba VR-65 cut-off filter. The intensity of the flash was saturating. The photomultiplier (Hamamatsu TV, R-636) was protected against the actinic light with a Corning band-pass filter 4-96. Unless otherwise stated, flashes were repeated every 2 sec. Usually, 128 signals were averaged with a Nicolet signal averager 1074 with plug-ins SD 72A and SW 78/74 to improve the signal/noise ratio.

For the assay, the chloroplasts were suspended in the preparation medium at concentrations of 20 to 25 μg chlorophyll/ml. The reaction medium usually contained 4 mM ascorbate to facilitate reduction of cytochrome *f* during flash intervals. Other additions were indicated in the figure legends. DBMIB was a gift from Dr. A. Trebst.

Results

Fig. 1 shows the time courses of absorbance changes invoked by the illumination of chloroplasts with a short saturating flash. Valinomycin or gramicidin D was added to eliminate the electrochromic band shift of photosynthetic pigments responding to the membrane potential formed across the thylakoid membrane (*9*, *26*). Flash excitation induced a rapid absorbance decrease followed by a slower

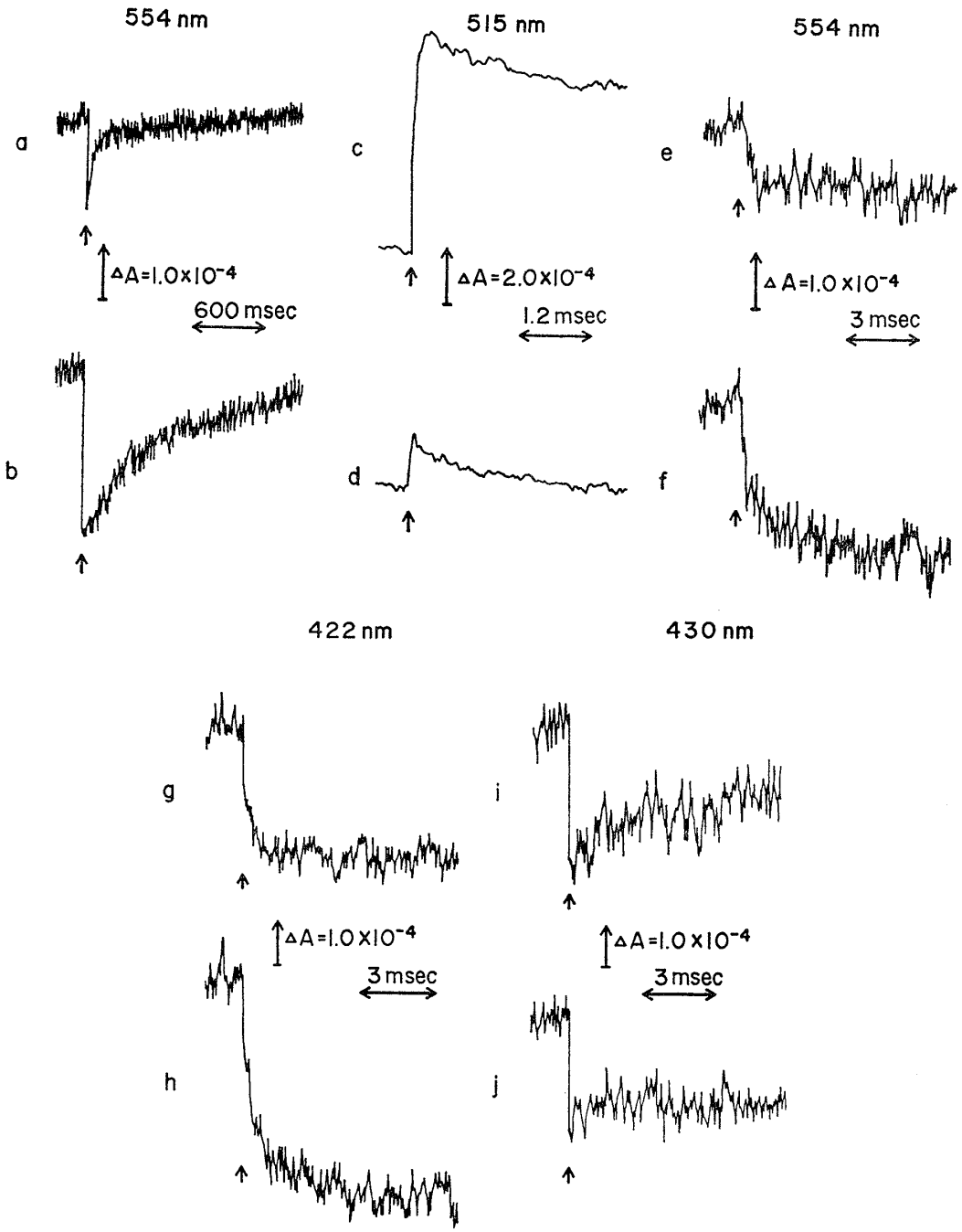

Fig. 1. *Time courses of flash-induced absorbance changes in spinach chloroplasts.* The reaction mixture contained 5 μM valinomycin, except for traces c and d. Traces a, e, g and i, no addition; traces b, f, h and j, 10 μM DBMIB; trace c, 50 mM KCl; trace d, 50 mM KCl plus 5 μM gramicidin D. Arrows indicate when the flash was fired.

return to the original level at 554 nm (trace a). The difference spectrum for the absorbance decrease shows a negative peak at 554 nm which can be ascribed to oxidation of cytochrome *f* (Fig. 2). The half times for the decay of the signals fell in the range of 10 to 25 msec, with an average value of 20 msec, in agreement with

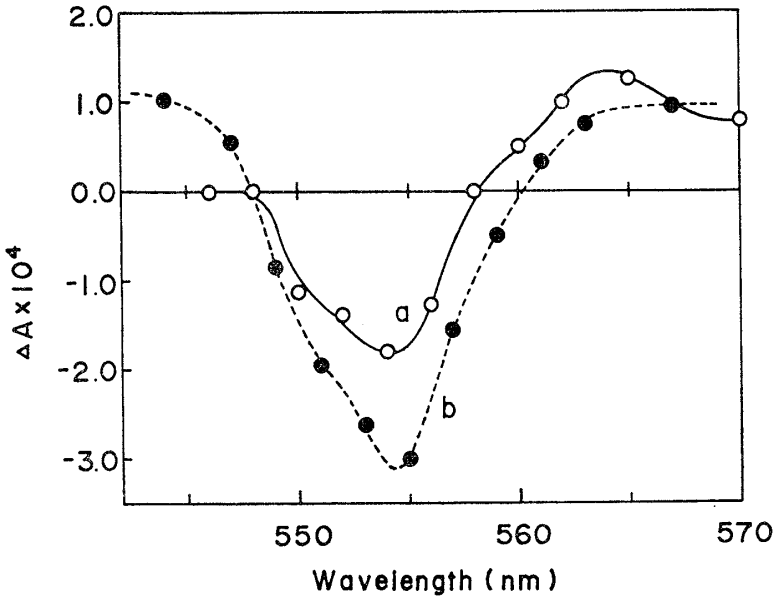

Fig. 2. *Difference spectra of flash-induced absorbance changes in the α-band region.* The reaction mixture contained 5 μM valinomycin. Magnitudes of flash-induced absorbance changes were plotted against wavelengths. Curves a and b were determined in the absence and presence of 10 μM DBMIB.

the half times for cytochrome *f* reduction reported by Biggins (*3*), Nishimura (*20*) and Haehnel (*8*, *9*).

As described previously (*5*, *9*, *17*), illumination with a short saturating flash failed to oxidize all cytochrome *f* present in the chloroplasts. In the present work, 25 to 40% of cytochrome *f* underwent oxidation. The magnitude of the cytochrome *f* signal was found to increase significantly on addition of DBMIB, an inhibitor of electron transport between plastoquinone and cytochrome *f* (*24*). As expected from its inhibition site, DBMIB markedly retarded the decay of the signal (trace b). Note that the signal size approximately doubled in the presence of the inhibitor. The difference spectrum depicted in Fig. 2 clearly shows that DBMIB increased the magnitude of cytochrome *f* oxidation.

In order to examine the effects of DBMIB on the oxidation kinetics of cytochrome *f*, absorbance changes were determined with faster recording times. However, the flash illumination of chloroplasts induced several fast absorption changes which interfered with the determination of the oxidation kinetics in the α-band region. The electrochromic shift of photosynthetic pigments could be eliminated by gramicidin D (*9*). Elimination of the shift, however, disclosed another fast absorbance increase at 515 nm which decayed with a half time of less than 1 msec (trace d in Fig. 1). Its fast rise rate as well as its flat difference spectrum between 515 and 540 nm (not shown) are characteristics of the P-700 oxidation (see ref. *5*). In addition, there might be a minor contribution of plastocyanin oxidation to the absorbance changes in this wavelength region (*5*, *9*). The dual wavelength method, in which absorbance change at 540 nm was subtracted from that at the α-band maximum, would minimize the contribution of the P-700 and plastocyanin responses since their spectra are almost flat between 540 and 560 nm (*11*, *13*). However, this method would sense the photoresponse of C-550, a band shift with a positive

peak at 542 nm and a negative peak at 550 nm (*12*, *25*). Because of the superposition of other absorbance changes and the limited resolution time of our apparatus, we could only estimate the upper limit for the half time of cytochrome *f* oxidation as being 300 μsec in the α-band region.

Experiments were therefore extended to the γ-band region where C-550 has no absorption band (*12*, *16*, *25*) and the magnitude of the plastocyanin signal is less significant than that in the α-band region (*13*). Trace g in Fig. 1 shows that the flash induced a biphasic absorbance decrease at 422 nm. The rate of the fast decrease could not be resolved in our apparatus. This, together with its difference spectrum showing a maximum at 430 nm (Fig. 3 curve a), suggests that the fast component represents photooxidation of P-700. However, the decay of this component, when determined at 430 nm, was not as fast as the decay of the P-700 response was expected to be, suggesting superposition of other absorbance changes, such as photoreduction of cytochrome *b*-563, on the P-700 signal (trace i in Fig. 1). On the other hand, a negative peak at 422 nm in the difference spectrum for the slower decrease clearly indicates that this kinetical component is due to cytochrome *f* oxidation (Fig. 3 curve c). The half times of the slower components ranged between 100 and 300 μsec. The values are compatible with the upper limit estimated in the α-band region, and also with the half times for cytochrome *f* oxidation reported by Hildreth (*10*), Dolan and Hind (*7*), and Bouges-Bocquet (*5*).

DBMIB increased the magnitude of the slow component of the absorbance decrease in the γ-band region (curve d in Fig. 3). On the other hand, the fast absorbance decrease centered at 430 nm was diminished to some extent on addition of DBMIB (curve b in Fig. 3). Obviously, the inhibitor is effective only for enhancing the cytochrome *f* oxidation and not that of P-700 oxidation.

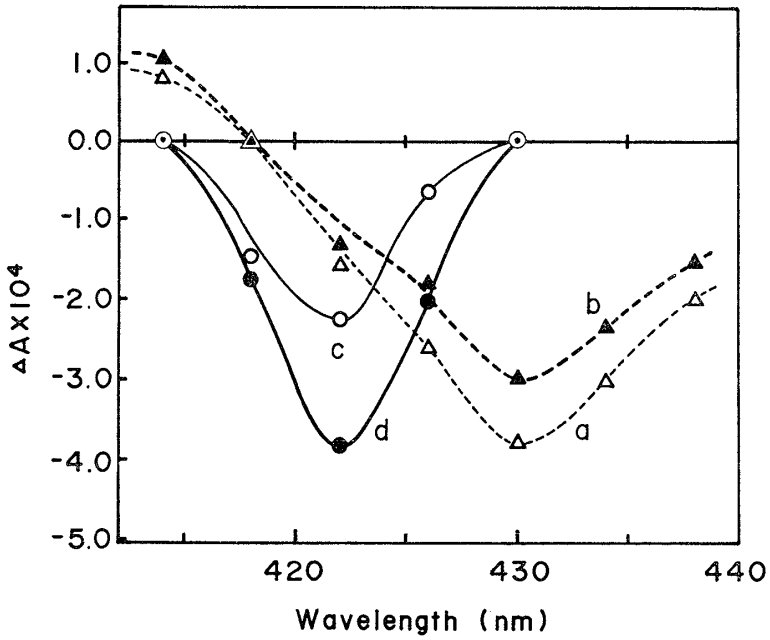

Fig. 3. *Difference spectra of flash-induced absorbance changes in the γ-band region.* Absorbance changes were determined in the presence of 5 μM valinomycin. Curves a and b, fast components; curves c and d, slow components. Curves b and d were determined in the presence of 10 μM DBMIB.

Note that the kinetics of cytochrome *f* oxidation in the presence of DBMIB was biphasic. Traces f and h in Fig. 1 show a slow oxidation of cytochrome *f* with a half time of 1 to 2 msec subsequent to the initial fast oxidation, both in the α- and the γ-band regions. Thus, DBMIB appears to increase the magnitude of cytochrome *f* oxidation mainly by inducing an additional slow oxidation of the cytochrome.

Fig. 4 summarizes the effects of different DBMIB concentrations on the cytochrome *f* response determined at 554 nm. A decrease in the rate of cytochrome *f* reduction was apparent below 0.1 μM, whereas about 0.5 μM of DBMIB was required to increase the signal size appreciably. This suggests that cytochrome *f* oxidation was enhanced only when the inhibition of cytochrome *f* reduction had proceeded to some extent. The maximum enhancement was attained at 10 μM DBMIB, at which the cytochrome reduction was almost completely suppressed.

The inhibition site of DBMIB can be bypassed as the DCIP-ascorbate couple was added as an electron donor (*4*). Fig. 5 shows that the rate of cytochrome *f* reduction was markedly accelerated by the addition of 50 μM DCIP to the reaction mixture which already contained 4 mM ascorbate. The half time for the cytochrome *f* reduction in the presence of DCIP was less than 2 msec and only slightly affected by DBMIB. Note that DBMIB-induced increases in the magnitude of cytochrome *f* oxidation were largely diminished in the presence of the donor couple. These observations suggest that the enhancement of cytochrome *f* oxidation is associated with the inhibition of cytochrome *f* reduction.

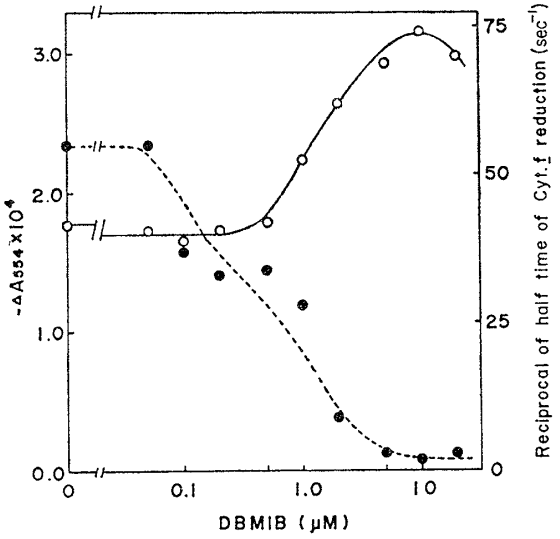

Fig. 4.

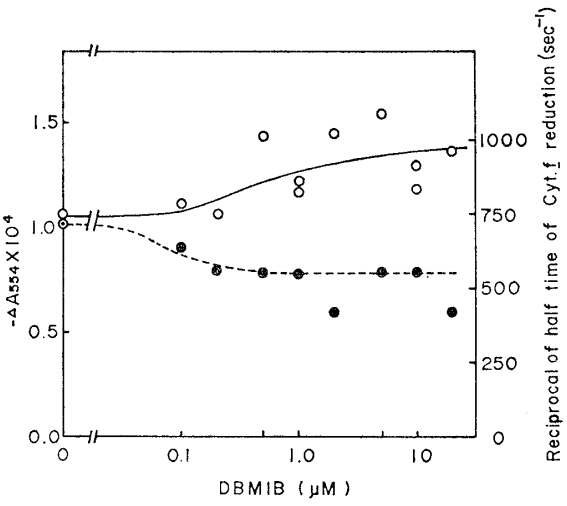

Fig. 5.

Fig. 4. *Effects of DBMIB on the magnitude of cytochrome* f *oxidation and the rates of cytochrome* f *reduction.* Absorbance changes were determined at 554 nm in the presence of 5 μM valinomycin. Open circles, magnitudes of cytochrome *f* oxidation; closed circles, reciprocals of half times of cytochrome *f* reduction.

Fig. 5. *Effects of the DCIP-ascorbate couple on the magnitude of cytochrome* f *oxidation and the rate of cytochrome* f *reduction determined in the presence of different concentrations of DBMIB.* Experiments were carried out as described in Fig. 4, except that 50 μM DCIP was added. Open and closed circles indicate magnitudes of cytochrome *f* oxidation and reciprocals of half times of cytochrome *f* reduction, respectively.

This assumption was supported by the results of experiments in which a lipophilic chelator, bathophenanthroline, was employed. Bering et al. have suggested that this chelator, like DBMIB, blocks electron transport in the region of plastoquinone and cytochrome *f* (*2*). In accord with their assumption, bathophenanthroline suppressed the reduction of cytochrome *f* (Fig. 6). The chelator also induced a significant increment in the magnitude of cytochrome *f* oxidation in the concentration range where the inhibition of cytochrome *f* reduction became apparent. The DCIP-ascorbate couple was again effective in diminishing the chelator-induced enhancement of cytochrome *f* oxidation. Note that the magnitudes of cytochrome *f* oxidation tended to decrease with increasing concentrations of bathophenanthroline in the presence of the DCIP-ascorbate couple. This suggests that bathophenanthroline also inhibits the cytochrome *f* oxidation. The small enhancement obtained with this substance would be partly ascribed to this secondary effect.

However, an inhibition of cytochrome *f* reduction was not always associated with an increase in the magnitude of cytochrome *f* oxidation. As shown in Fig. 7, no appreciable increase in the signal size was observed at any concentration of DCMU added, whereas the cytochrome *f* reduction was strongly inhibited at concentrations above 0.1 μM. In this experiment, flashes were fired every 8 sec, since longer intervals were found to be required to attain the maximum extent of cytochrome *f* oxidation in the presence of DCMU than in the presence of DBMIB. Presumably, the inhibition of electron transport by DBMIB is incomplete or DBMIB

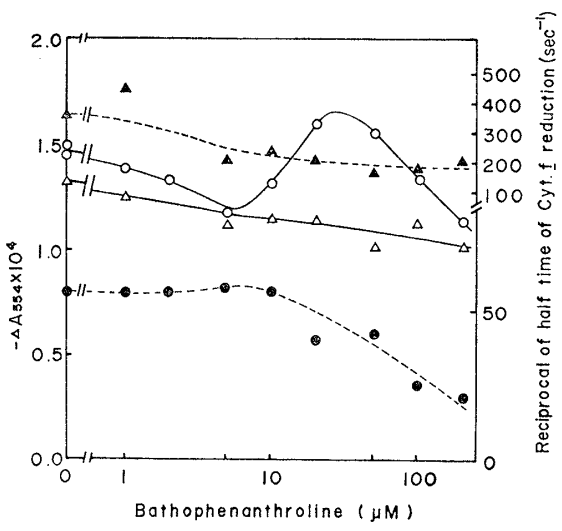

Fig. 6.

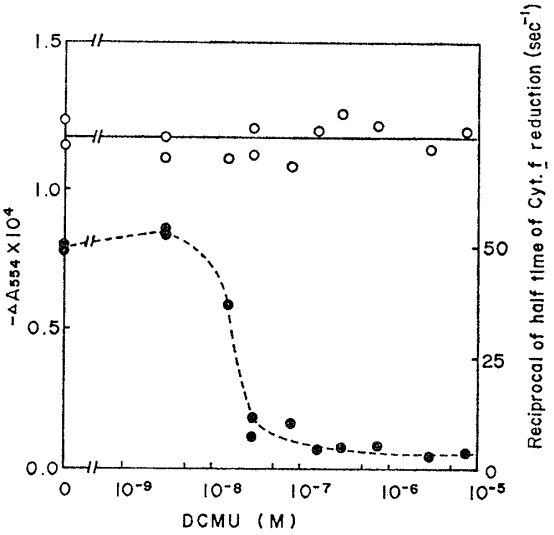

Fig. 7.

Fig. 6. *Effects of different concentrations of bathophenanthroline on the magnitude of cytochrome* f *oxidation and the reciprocal of the half time of cytochrome* f *reduction.* Experiments were carried out as described in Fig. 4, except that bathophenanthroline was used in place of DBMIB. Open and closed symbols indicate magnitudes of cytochrome *f* oxidation and reciprocals of half times of cytochrome *f* reduction, respectively. 50 μM DCIP was added in the experiments indicated by triangles.

Fig. 7. *Effects of different concentrations of DCMU on the magnitude of cytochrome* f *oxidation and the rate of cytochrome* f *reduction.* Experiments were carried out as described in Fig. 4, except that DCMU was used in place of DBMIB. Open and closed circles indicate magnitudes of cytochrome *f* oxidation and reciprocals of half times of cytochrome *f* reduction, respectively. Flashes were fired every 8 sec.

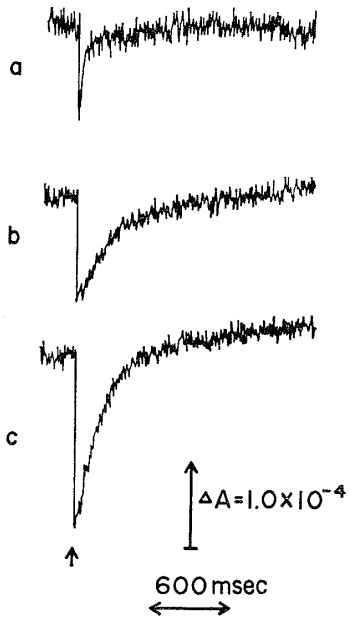

Fig. 8. *Effects of DBMIB on the magnitude of cytochrome* f *oxidation in the presence of DCMU.* Absorbance changes were determined at 554 nm in the presence of 5 μM valinomycin. Trace a, no addition; b, 7.5 μM DCMU; c, 7.5 μM DCMU plus 10 μM DBMIB.

also mediates electron transfer from ascorbate or photosystem II to cytochrome *f* (*15*). However, the absence of any increase in the magnitude of cytochrome *f* oxidation in the presence of DCMU is not due to an incomplete re-reduction of cytochrome *f* during the dark intervals, since prolongation of the dark intervals failed to increase the extent of cytochrome *f* oxidation by flash.

Interestingly, further addition of DBMIB to the DCMU-poisoned chloroplasts invoked an enhancement of cytochrome *f* oxidation as large as that caused by DBMIB alone (Fig. 8). The rate of cytochrome *f* re-reduction was only moderately accelerated by DBMIB. This clearly indicates that the enhancement of cytochrome *f* oxidation caused by the flash is specifically related to the inhibition of electron transport by DBMIB. DCMU and DBMIB block electron transport before and after the plastoquinone pool, respectively (*4*, *24*). Thus, the enhancement of cytochrome *f* oxidation must be related to the inhibition of electron transport at a site between plastoquinone and cytochrome *f*.

Discussion

The results obtained in the present work indicate that the reaction kinetics of cytochrome *f* invoked by a flash is complex. A short saturating flash could not oxidize all the cytochrome *f* present in the chloroplasts. However, addition of DBMIB markedly increased the magnitude of the oxidation mainly by inducing an additional slow oxidation. The appearance of this slow kinetic component was accompanied by an inhibition of cytochrome *f* reduction and could be effectively prevented by the addition of the DCIP-ascorbate couple which accelerated the reduction rate. These observations strongly suggest that the slow oxidation component is usually masked by an opposing reductive reaction and becomes apparent only when cytochrome *f* reduction is suppressed by an inhibitor.

However, the overall reduction rate of cytochrome *f* (half time, 20 msec) is

not fast enough to cancel the slow oxidation component with a half time of 1 to 2 msec. Moreover, an inhibition of the cytochrome *f* reduction by DCMU is not associated with the enhancement of the cytochrome *f* oxidation. Clearly, an inhibition of the overall reduction of cytochrome *f* is not the cause of the appearance of the slow oxidation component. In this respect, of special interest is the finding that the magnitude of cytochrome *f* oxidation in the DCMU-poisoned chloroplasts increased on further addition of DBMIB. This strongly indicates that the enhancement of cytochrome *f* oxidation is related to a block in the electron transport between plastoquinone and cytochrome *f*.

In order to explain the effects of DBMIB, we assume that an electron carrier X is located between the plastoquinone pool and cytochrome *f*. The electron transfer from plastoquinone to X is the rate-limiting step of the electron transport chain, whereas that from X to cytochrome *f* is fast enough to cancel the slow oxidation of cytochrome *f*. The slow oxidation component appears when reduction of cytochrome *f* by reduced X is prevented by DBMIB.

A small but reproducible enhancement of cytochrome *f* oxidation also occurred in the presence of bathophenanthroline. The observation that the increase in magnitude of cytochrome *f* oxidation was closely associated with the inhibition of its reduction indicates a direct action of the lipophilic chelator on X. Bering et al. (*2*) have suggested that this chelator blocked electron transport in the region of plastoquinone and cytochrome *f* by attacking a non-heme iron protein. The occurrence of a non-heme iron protein in chloroplasts has been reported by Malkin and Aparicio (*18*). Thus, the high-potential non-heme iron protein would be a likely candidate for X.

The biphasic oxidation kinetics of cytochrome *f* observed in the presence of DBMIB suggests a heterogeneity of photosystem I with respect to cytochrome *f*. The kinetically different components of cytochrome *f* oxidation might be due to either the difference in the reaction center of photosystem I or the physically different localization of cytochrome *f* in chloroplasts (*21*). An alternative mechanism is that the slow component of cytochrome *f* oxidation reflects damage to the electron transport caused during chloroplast preparation. The association of plastocyanin with the chloroplast membrane has been shown to be sensitive to mechanical treatments of chloroplast (*14*). However, we have shown previously that oxidation of cytochrome *f* in the presence of DBMIB was largely abolished by the $HgCl_2$ or sonic treatment of chloroplasts (*17*). This indicates the participation of plastocyanin in both the fast and slow components of cytochrome *f* oxidation. Further experiments are needed to clarify the significance and mechanism of the slow oxidation process of cytochrome *f*.

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