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DIFFERENT ROLES OF PLASTOQUINONE IN THE PHOTOREDUCTION OF FERREDOXIN AND OF MEMBRANE-BOUND IRON-SULFUR CENTERS OF CHLOROPLASTS

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SUMMARY: Photosynthetic electron transport in chloroplasts was inhibited by the plastoquinone antagonist, dibromothymoquinone (DBMIB) in two steps. Lower concentrations of DBMIB inhibited the photoreduction of the bound iron-sulfur centers of photosystem I without inhibiting the photoreduction of ferredoxin. Higher concentrations of DBMIB did inhibit the oxygenic photoreduction (i.e., by water) of ferredoxin and $NADP^+$, but their photoreduction was restored, wholly or partly, by each of four chemically diverse uncouplers, similar only in facilitating proton movement across membranes. By contrast, none of the uncouplers alleviated the DBMIB inhibition of the photoreduction of the bound Fe-S centers. These divergent responses to uncouplers are incompatible with the Z scheme but are consistent with the new concept of oxygenic and anoxygenic photosystems in plant photosynthesis (Proc. Natl. Acad. Sci. USA 78, 2942-2946, 1981).

Plastoquinone is the most abundant membrane-bound redox component of chloroplasts (1-3); its midpoint potential is about +80 mV at pH 7 (4). Because oxidoreductions of plastoquinone involve transfers of hydrogen atoms, plastoquinone is also the preeminent proton carrier in chloroplasts.

In the light-induced electron transport from water to ferredoxin, the soluble iron-sulfur protein that is the carrier of photosynthetic reducing power [E_m = -420 mV, ref. 5), plastoquinone is generally regarded as the essential link in electron transfer between two photosystems, PSII and PSI. In this so-called Z scheme (3,6) electrons liberated in the oxidation of water by PSII are transported via plastoquinone to PSI which alone has the capacity to energize them to a strong reducing potential, adequate for the reduction of ferredoxin. The Z scheme assigns no role to plastoquinone in the transport of protons released by the oxidation of water. These are thought to diffuse from the thylakoid membrane into the thylakoid lumen without the participation of plastoquinone (3,7).

We have recently presented an alternative concept of electron and proton transport in chloroplasts, embodied in oxygenic and anoxygenic photosystems (supplanting PSII and PSI, respectively) in which plastoquinone function is viewed differently (8-11). The new concept envisions several roles for plastoquinone,

Abbreviations: PSI and PSII, photosystems I and II; DBMIB, 2,5-dibromo-3 methyl-6-isopropyl-p-benzoquinone (dibromothymoquinone); FCCP, carbonyl cyanide trifluoromethoxyphenylhydrazone; EPR, electron paramagnetic resonance.

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including a role as an electron transfer link between two photosystems. However, unlike the Z scheme, this intersystem electron transfer is not considered as essential for the oxygenic reduction (i.e., reduction by electrons originating from water) of ferredoxin. The new concept envisions that the oxygenic photosystem not only photooxidizes water (like PSII in the Z scheme) but also reduces ferredoxin without the collaboration of PSI (the anoxygenic photosystem) whose role is limited to cyclic photophosphorylation. A new, important function assigned to plastoquinone in the oxygenic photosystem is to facilitate the translocation of protons from inside the thylakoid membrane into the thylakoid lumen. Protons are liberated simultaneously with electrons in the photooxidation of water, a reaction that takes place inside the thylakoid membrane (12), which is intrinsically impermeable to protons (13).

The new perspective on the role of plastoquinone was supported by experiments with plastoquinone inhibitors. Normally, electrons originating from water reduced both ferredoxin and redox components of PSI, but a partial inhibition of plastoquinone function that did not diminish the oxygenic reduction of ferredoxin, did inhibit the reduction of components of PSI (9), specifically, the membrane-bound iron-sulfur centers A and B whose strongly electronegative midpoint potentials fall in the range of -530 to -580 mV (14,15). A more complete inhibition of plastoquinone function blocked also the oxygenic reduction of ferredoxin, but this blockage was relieved by uncouplers (8), i.e., by agents that facilitate transmembrane proton transport (13,16).

According to the Z scheme which places the redox components of PSI in a linear sequence preceding ferredoxin, uncouplers should affect equally the oxygenic reduction of ferredoxin and that of the Fe-S centers. By contrast, the new concept (8) predicts different effects of uncouplers. Functioning as transmembrane proton carriers, they should relieve the inhibition by plastoquinone inhibitors of the oxygenic photoreduction of ferredoxin, but since they cannot compensate for a blockage in intersystem electron transfer, uncouplers should not relieve the inhibition of the photoreduction of the Fe-S centers.

Reported here are experiments that substantiate these predictions and thereby lend further support to the concept of oxygenic and anoxygenic photosystems in plant photosynthesis.

METHODS

Chloroplasts were isolated from spinach leaves (Spinacia oleracea, var. Marathon) grown in a greenhouse in nutrient solution culture (17) and freshly harvested before each experiment. The preparation used consisted of osmotically disrupted ("broken") chloroplasts, depleted of soluble components (including Fd) but retaining the integrity of the thylakoid membrane structure needed for complete electron transport from water to $NADP^+$ and for ferredoxin-catalyzed cyclic photophosphorylation (18). Chlorophyll was estimated (17), ferredoxin was isolated and purified (19) and the photoreduction of $NADP^+$ was measured (20) as previously described. Glucose oxidase (type VII), bovine catalase, $NADP^+$, FCCP and gramicidin were purchased from Sigma Chemical Co. (St. Louis, MO). Nigericin was kindly supplied by Hoffman-La Roche Co, Nutley, NJ, and SF6847 by Sumitomo Chemical

Co., Ltd., Osaka, Japan. DBMIB (a gift of Prof. A. Trebst) was added as a methanol solution. Equal concentration of methanol was added to the control treatments.

The photoreduction of ferredoxin and of the membrane-bound iron-sulfur centers was measured by EPR spectroscopy. The chloroplasts (in their respective reaction mixtures) were placed in quartz EPR tubes (3 mm inside diameter) that had been gassed with nitrogen. The tubes were illuminated at a physiological temperature (293K) for 30 s and then, with illumination continued, immersed for 30 s in liquid nitrogen, contained in a silvered dewar with a window that admitted light. The frozen samples in the quartz tubes were further cooled in the EPR cavity with liquid helium to either 20K or 60K by an Oxford Instruments temperature controller (model DTB) and cryostat (model ESR 9) equipped with a quartz dewar cell (made by J. Scanlon, Solvang, CA). First derivative EPR spectra of the frozen samples were obtained with a Bruker Instrument Co. (Billerica, MA) X-band spectrometer (model ER200tt) [equipped with a 20 cm (8 inch) magnet] operated at a frequency of 9.45 GHz and were recorded after processing by a digital signal averager (model 1070, Nicolet Instruments Corp., Madison, WI).

Monochromatic illumination (650 nm) was provided by a light beam from a quartzline lamp (type DXN, 1000 W). The light beam was passed through heat-absorbing and interference filters (Baird-Atomic Co., Medford, MA).

RESULTS

In the EPR traces shown below, the extent of ferredoxin reduction is indicated by the amplitude of its characteristic signals at $\underline{g}$ = 1.89, 1.96 (main signal) and 2.05. The reduced Fe-S centers give signals at $\underline{g}$ = 1.86, 1.94 and 2.05 (center A) and at $\underline{g}$ = 1.89, 1.92 and 2.05 (center B). In fully reduced chloroplast preparations, the $\underline{g}$ = 1.86 signal of center A seems to undergo a $\underline{g}$-value shift to 1.89 although the other $\underline{g}$-values of center A remain unchanged (21-24). The EPR tubes were scanned at two temperatures, 20K and 60K. The scan at 20K gave signals of both reduced ferredoxin and the reduced Fe-S centers; at 60K the EPR signals of the Fe-S centers broaden and only the signals of reduced ferredoxin are measurable (25)

In previous experiments, high concentrations of chlorophyll (i.e., chloroplasts) were used to facilitate the measurements of the Fe-S signals (9), but low concentrations of chloroplasts (resulting in low plastoquinone: ferredoxin ratios) were used when only ferredoxin signals were to be measured (8). In the present experiments the same relatively high chloroplast concentration (0.3 mg chlorophyll/ml) was used in all cases.

Fig. 1 shows that, as evidenced by the disappearance of the $\underline{g}$ = 1.92 signal and the diminution of the 1.89, 2.05, and the overlapping 1.94,1.96 signals, DBMIB, a widely used inhibitor of plastoquinone function (26,27), has totally abolished the reduction of the Fe-S centers without abolishing the oxygenic reduction of ferredoxin (compare top left and top right traces, Fig. 1). The addition of the uncouplers, gramicidin, nigericin, FCCP and SF 6847 [a ditertiary phenol derivative (28)] did not restore the reduction of the Fe-S centers, but it appeared to increase the extent of ferredoxin reduction (Fig. 1).

The possibility existed that in the experiments represented by Fig. 1, the reduction of the Fe-S centers was only partly inhibited but no Fe-S signals were detected because ferredoxin, having a more positive redox potential, acted as a "sink" that drained electrons from the Fe-S centers. The effect of DBMIB on the

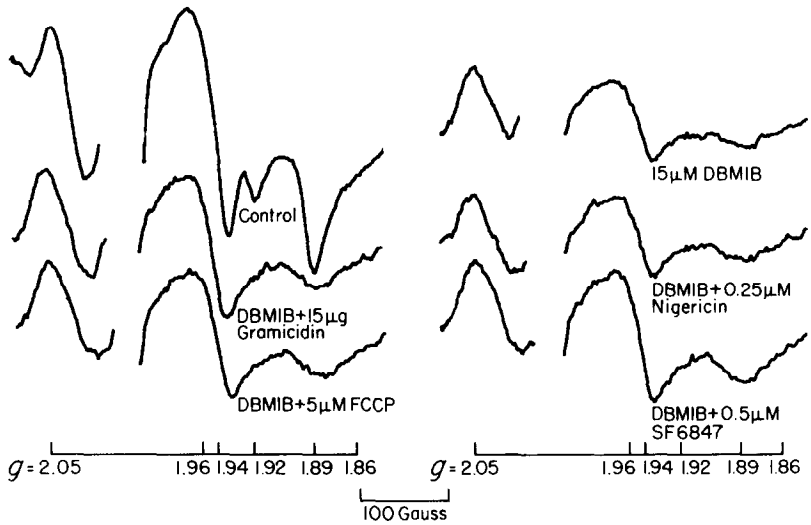

Fig. 1. Uncouplers do not alleviate DBMIB inhibition of the photoreduction by water of bound iron-sulfur centers in the presence of soluble ferredoxin (Fd). The photoreduction of ferredoxin and the Fe-S centers was measured by electron paramagnetic resonance (EPR) spectroscopy (see Methods). The reaction mixture, placed in EPR tubes and equilibrated with N_2, contained osmotically disrupted chloroplasts (equivalent to 300 μg of chlorophyll per ml), 50 mM N-tris (hydroxymethyl) methylglycine (Tricine) buffer (pH 7.7), 10 μM spinach ferredoxin, 5 mM $MgCl_2$, 50 mM KCl, 2.5 mM ADP, 2.5 mM K_2HPO_4, 10 mM glucose, glucose oxidase, catalase and 10% methanol. DBMIB and uncouplers were added as indicated.

The EPR tubes were illuminated for 30 sec at 293K and immediately frozen in liquid N_2 under continuing illumination (650 nm, 5 x 10^5 ergs cm^{-2} sec^{-1}). EPR spectra were recorded at 20K. Spectrometer field setting, 3450 + 200 G; microwave power, 10 mW; modulation amplitude, 10G; gain, 1 x 10^5. (The free radical signals in the g = 2.0 region are omitted.)

reduction of the Fe-S centers was therefore also investigated in the absence of ferredoxin. As shown in Fig. 2, DBMIB again totally abolished the reduction of the Fe-S centers, and the inhibition by DBMIB was not relieved by any of the four uncouplers used.

The concentration of DBMIB used to abolish the reduction of the Fe-S centers was 10 uM (Fig. 2). Increasing the DBMIB concentration to 20 uM has totally abolished the oxygenic reduction of ferredoxin as well (compare top left and top right traces in Fig. 3). Here, however, ferredoxin reduction was restored by each of the four uncouplers used (Fig. 3).

The respective concentrations of DBMIB needed for the total inhibition of the oxygenic reduction of the Fe-S centers and ferredoxin varied with different preparations of chloroplasts but the pattern of inhibition was consistent in that higher concentration DBMIB were always required for the inhibition of ferredoxin reduction than for the inhibition of the reduction of the Fe-S centers.

Previous experiments (8) in which low concentrations of chloroplasts were used (50 ug chlorophyll/ml) yielded evidence that the alleviation by uncouplers of plastoquinone inhibition is demonstrable not only by measurements of steady state

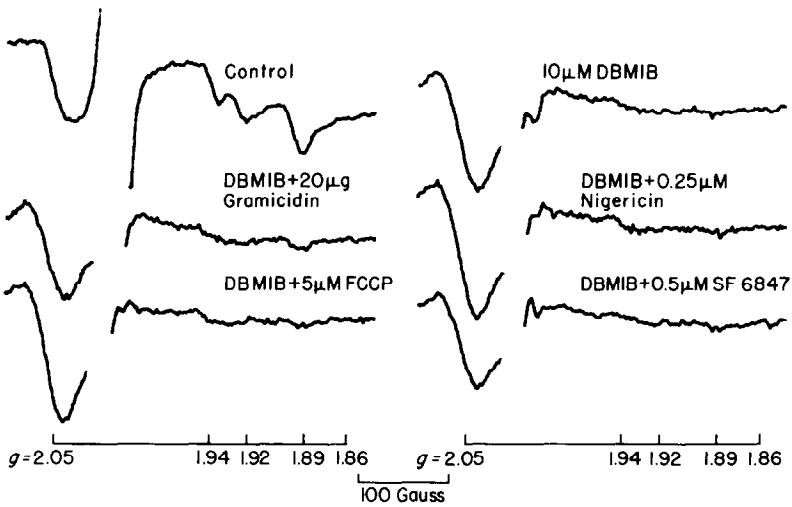

Fig. 2. Uncouplers do not alleviate DBMIB inhibition of the photoreduction by water of bound iron-sulfur centers even in the absence of soluble ferredoxin. Experimental conditions as in Fig. 1, except that ferredoxin was omitted.

ferredoxin reduction but also by the more demanding $NADP^+$ reduction that reflects ferredoxin turnover. Fig. 4 shows that at the relatively high concentrations of chlorophyll (0.3 mg/ml) used in the present experiments, uncouplers also alleviated DBMIB inhibition of oxygenic $NADP^+$ reduction.

DISCUSSION

The results of this study run counter to the fundamental postulate of the Z scheme that the oxygenic reduction (e.i., by electrons originating from water) of ferredoxin can be accomplished only through an intersystem electron transfer from

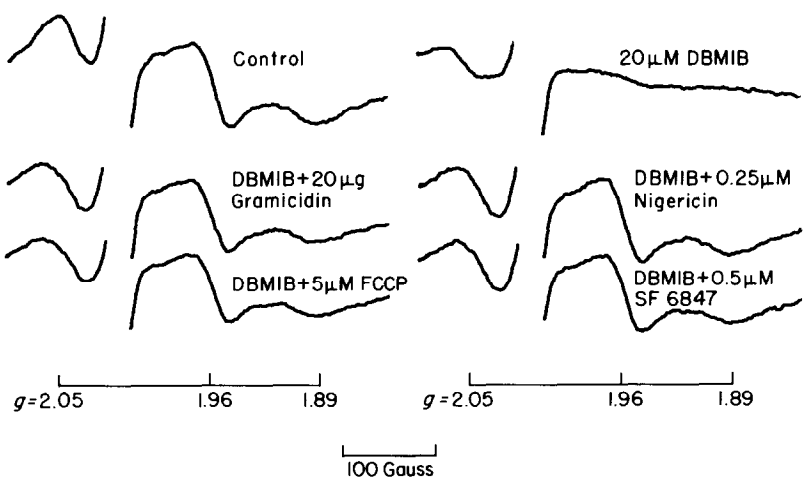

Fig. 3. Uncouplers alleviate DBMIB inhibition of the photoreduction by water of ferredoxin. Experimental conditions as in Fig. 1, except that EPR spectra were recorded at 60K.

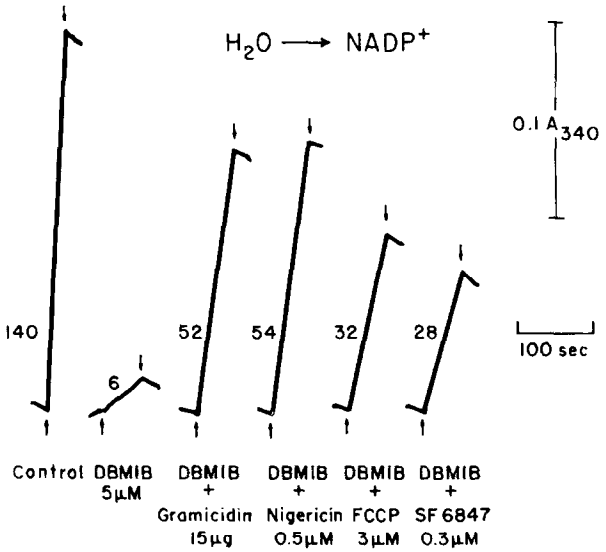

Fig. 4 Uncouplers alleviate DBMIB inhibition of $NADP^+$ photoreduction. The reaction mixtures were as in Fig. 1 except that 2 mM $NADP^+$ was present throughout and N_2, glucose, glucose oxidase, and catalase were omitted. The reaction mixtures were illuminated at room temperature in cuvettes (2.0 mm light path) open to air. Monochromatic illumination: 650 nm; 5×10^4 ergs cm^{-2} sec^{-1}. Arrow up, light on; arrow down, light off. Numbers give rates of $NADP^+$ reduction (umol per mg of chlorophyll per hr.).

PSII to PSI, mediated by plastoquinone. A partial inhibition of plastoquinone function by DBMIB blocked the intersystem electron transfer from PSII to PSI and prevented the oxygenic reduction of redox components of PSI, specifically, the bound Fe-S centers A and B, without, however, impairing the oxygenic reduction of ferredoxin (Fig. 1). Only at higher concentrations of DBMIB that inhibited plastoquinone function more completely was the oxygenic reduction of ferredoxin also inhibited (Fig. 3).

The main finding in this study was the divergent effect of uncouplers on the oxygenic reduction of ferredoxin and the Fe-S centers. At the higher DBMIB concentrations that abolished the reduction of ferredoxin (Fig. 3), the reduction was restored by the addition of gramicidin, nigericin, FCCP or SF 6847, four chemically diverse uncouplers, similar only in their ability to facilitate proton transport across membranes. By contrast, the inhibition of the reduction of the Fe-S centers by lower concentrations of DBMIB was totally unrelieved by the same uncouplers (Fig. 2).

These divergent responses to uncouplers are incompatible with the linear sequence of electron transport in the Z scheme in which the reduction of the Fe-S centers of PSI is a precondition for the subsequent reduction of ferredoxin. The Z scheme predicts that agents that restore (in an inhibited system) electron flow from water to ferredoxin should also restore electron flow to the Fe-S centers. This prediction was not borne out. Uncouplers restored the oxygenic reduction of

ferredoxin (Fig. 3), and to a large extent that of $NADP^+$ (Fig. 4), but did not restore the reduction of the Fe-S centers (Fig. 2).

The present findings while inconsistent with the Z scheme are consistent with the multiple roles of plastoquinone inherent in the concept of oxygenic and anoxygenic photosystems (8). In the oxygenic photosystem the main function assigned to plastoquinone is translocation of protons liberated inside the membrane by photooxidation of water (see Fig. 5 in ref. 8). DBMIB at higher concentrations, inhibited proton translocation and thereby blocked ferredoxin and $NADP^+$ reduction. Their reduction was restored by uncouplers, i.e., agents that facilitate proton transport. The second function of plastoquinone, that of intersystem electron transfer (see Fig. 7 in ref. 8) was not alleviated by uncouplers because these agents could not restore intersystem electron transport.

Consistent with the new perspective on photosynthetic electron and proton transport but not with the Z scheme are recent findings in other laboratories that (i) PSII and PSI are spatially separated in chloroplast membranes (29,30), (ii) the ratio of PSII to PSI varies widely in thylakoid membranes (31), and (iii) PSII can generate a strong reductant, adequate even for the reduction of pheophytin (E_m ca. -610 mV) (32) let alone the reduction of ferredoxin (E_m = -420 mV). These findings are inconsistent with basic tenets of the Z scheme but are in harmony with the concept of two synchronous photosystems that are (except for regulatory connections) essentially autonomous, one (the oxygenic) responsible for noncyclic photophosphorylation that generates reduced ferredoxin and ATP, and the other (the anoxygenic) for cyclic photophosphorylation that generates only ATP (8).

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