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OXYGENIC PHOTOREDUCTION OF FERREDOXIN INDEPENDENTLY OF THE MEMBRANE-BOUND IRON-SULFUR CENTERS OF PHOTOSYSTEM I

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SUMMARY

An investigation of the photoreduction of soluble ferredoxin and membrane-bound Fe-S centers of chloroplasts yielded results that are incompatible with some basic postulates of the now prevalent concept of photosynthetic electron transport (the "Z scheme"). In the Z scheme, plastoquinone serves as an essential link in a linear electron transport chain from water via photosystem II to photosystem I and thence to the bound Fe-S centers, soluble ferredoxin and $NADP^+$. In this formulation the oxygenic photoreduction of ferredoxin and of the Fe-S centers should have the same sensitivity to the plastoquinone inhibitors, dibromothymoquinone (DBMIB) and dinitrophenol ether of iodonitrothymol (DNP-INT). We found that the photoreduction of ferredoxin and the Fe-S centers exhibited differential sensitivity to these inhibitors. Ferredoxin was fully photoreduced by water at inhibitor concentrations that abolished the photoreduction of the Fe-S centers. These findings suggest that the oxygenic photoreduction of ferredoxin does not involve the participation of the Fe-S centers or other components of photosystem I. Only when an artificial, direct donor to photosystem I is used is the reduction of ferredoxin invariably preceded by the reduction of the Fe-S centers.

A fundamental premise of the now prevalent concept of photosynthetic electron transport [known as the Z scheme (1,2)] is that of the two photosystems in chloroplasts, only photosystem I (PSI) can energize electrons originating from water to a potential adequate for the reduction of ferredoxin (Fd) and hence $NADP^+$ (3). According to the Z scheme, photosystem II (PSII) generates a strong oxidant; the collaboration of PSI is required to generate the strong reductant needed for the reduction of Fd and the more electronegative components of PSI (1,2).

$$\begin{array}{ccccccccccccc} H_2O & \xrightarrow{e^-} & PSII & \rightarrow\rightarrow & PQ & \rightarrow\rightarrow & PSI & \rightarrow\rightarrow & Fe\text{-}S & \rightarrow & Fd & \rightarrow & NADP^+ \\ \downarrow & & \uparrow & & & & \uparrow & & & & & & \\ O_2 & & h\nu & & & & h\nu & & & & & & \end{array}$$

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (dibromothymoquinone); DNP-INT, dinitrophenol ether of iodonitrothymol; DCIP, $DCIPH_2$, oxidized and reduced (by excess ascorbate) forms of 2,6-dichlorophenolindophenol; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (diuron); EPR, electron paramagnetic resonance.

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As summarized in this simplified diagram (where double arrows indicate other carriers) the essential features of the Z scheme also include (i) plastoquinone (PQ) as the obligatory link in electron transport between PSII and PSI, and (ii) the notion that the reduction of the strongly electronegative carriers of PSI represented by the membrane-bound Fe-S centers [E_m = -540 to -580 mV] (4,5) is a precondition for the oxygenic reduction of Fd [E_m = -420 mV] (6). ("Oxygenic reduction" will henceforth denote reduction by electrons that originate from water.)

We have recently reported (7,8) findings that are incompatible with these basic postulates of the Z scheme. When the obligatory PQ link between the two photosystems was inhibited by the use of the plastoquinone inhibitor DBMIB (9,10), chloroplasts unexpectedly exhibited an oxygenic reduction of Fd without a reduction of the Fe-S centers of PSI (7). The new evidence was uncovered because the sensitivity and specificity of electron paramagnetic resonance (EPR) spectroscopy for the iron-sulfur components of chloroplasts made it possible to measure simultaneously the photoreduction of Fd and that of the Fe-S centers of PSI (11).

These findings (7) suggested that contrary to the Z scheme, PSII can oxygenically photoreduce Fd without the collaboration of PSI. Because of the far reaching implications of such a conclusion, we deemed it desirable to test its validity by other experimental approaches. One of these, involving experiments with Tris-treated chloroplasts that lost the ability to photooxidize water but retained an otherwise functional PSII (and PSI), sustained the earlier interpretation (8). Only PSII appeared to be involved in the photoreduction of Fd by a substitute PSII donor; there was no evidence for a collaboration of PSI (8).

The aim of this investigation was to test further the validity of the unorthodox conclusion that the Fe-S centers and, by extension, the other components of PSI, are not required for the oxygenic photoreduction of Fd. Our previous evidence for an oxygenic photoreduction of Fd without a reduction of the bound Fe-S centers (7) could be explained, from the perspective of the Z scheme, by an assumption that DBMIB has so diminished electron flow from water that Fd acted as an electron "sink" that drained electrons from all the preceding carriers in the photoredox chain, including the Fe-S

centers (1,2,12). Accordingly, in the present experiments with DBMIB, the illumination of chloroplasts was greatly extended (to 120s) to liberate enough electrons from water for the complete reduction of all carriers in the linear photoredox chain. Moreover, since Trebst et al. (13), taking cognizance of the shortcomings of DBMIB as a plastoquinone antagonist, have recently introduced new high-potency inhibitors of plastoquinone function, devoid of redox properties and other undesirable effects of DBMIB, we undertook to use one of them (DNP-INT) to reexamine the effects of inhibition of plastoquinone on the photoreduction of Fd and Fe-S centers.

We report here that, contrary to the conventional Z scheme formulation of linear electron transport in chloroplasts, we found a differential effect of plastoquinone inhibitors on the photoreduction of Fd and the bound Fe-S centers. Even under prolonged illumination, chloroplasts oxygenically photoreduced Fd at concentrations of plastoquinone inhibitors that blocked completely the photoreduction of the bound Fe-S centers.

METHODS

Chloroplasts were isolated from spinach leaves (Spinacia oleracea, var. Marathon) grown in a greenhouse in nutrient solution culture (14) 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 (15). Chlorophyll was estimated (14), ferredoxin was isolated and purified (16) (by R.K. Chain), and the photoreduction of $NADP^+$ was measured (17) as previously described. Glucose oxidase (type VII), bovine catalase, and $NADP^+$ were purchased from Sigma Chemical Co. (St. Louis, MO). DBMIB and DNP-INT (gifts of Prof. A. Trebst) were added as methanol solutions (DNP-INT was first dissolved in a drop of dimethyl formamide). Equal concentrations of methanol were 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. Unless otherwise indicated, the tubes were illuminated first 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 DTC) and cryostat (model ESR9) 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 Instruments Co. (Billerica, MA) X-band spectrometer (model ER200tt) [equipped with a 20-cm ("8-inch") magnet] operated at a frequency of 9.18 GHz and were recorded after processing by a digital signal averager (model 1070, Nicolet Instru. 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 presented below, the extent of ferredoxin reduction is indicated by the amplitude of characteristic EPR signals at g = 1.89, 1.96 (main signal), and 2.05. The reduced bound Fe-S centers give signals at g = 1.86, 1.94, and 2.05 (center A) and at g = 1.89, 1.92, and 2.05 (center B). In fully reduced chloroplast preparations, the g = 1.86 signal of center A seems to undergo a g-value shift to 1.89, although the other g-values of center A remain unchanged (18-21, 4,5). Because of the considerable overlap between the signals of the reduced ferredoxin and the reduced bound centers, 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 but at 60K the EPR signals of the Fe-S centers broadened and ceased to be detectable (11). Thus, the EPR scan at 60K served as a measure of reduced ferredoxin only; when no Fd was added, the scan at 20K measured only the reduction of bound Fe-S centers.

To obtain large signals of the membrane-bound Fe-S centers, high concentrations of chloroplasts (1 mg chlorophyll ml^{-1}) were used throughout. These resulted also in high concentrations of other membrane-bound components, including plastoquinone, and necessitated the use of relatively high concentrations of plastoquinone inhibitors (13). Effect of DBMIB on Oxygenic Photoreduction of Fe-S Centers and Fd. Prior investigations with varying concentrations of DBMIB disclosed that the photoreduction of the Fe-S centers by water was more sensitive to this inhibitor than the photoreduction of Fd (ref. 7 and other data not shown). Fig. 1 (top and middle traces) shows the oxygenic photoreduction of Fd (right) and of the Fe-S centers in the absence of Fd (left), after 30s illumination. DBMIB drastically inhibited the photoreduction of the Fe-S centers but did not diminish the photoreduction of Fd (compare top and middle traces in Fig. 1). However, no inhibition of the photoreduction of the Fe-S centers (or Fd) was detected after 120s illumination (Fig. 1, bottom traces).

The disappearance of DBMIB inhibition of the photoreduction of Fe-S centers after 120s illumination seemed consistent with the supposition that the inhibition seen after the shorter (30s) illumination was attributable to a "drainage" of electrons from the Fe-S centers to Fd as the terminal acceptor. However, the results could also be

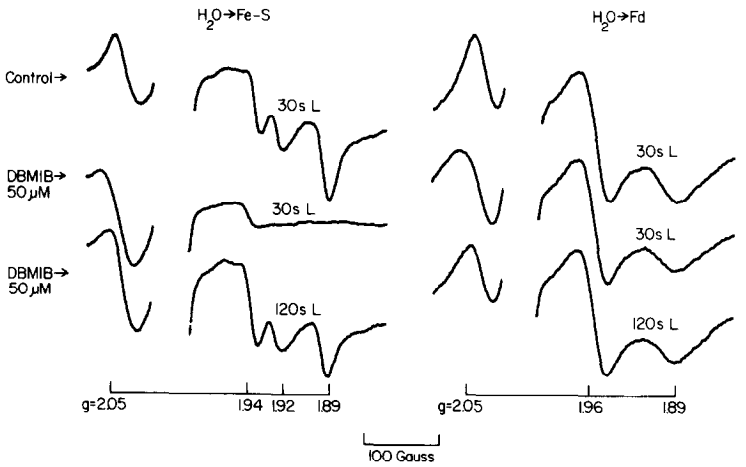

Fig. 1. Effect of DBMIB on photoreduction by water of bound iron-sulfur centers (Fe-S) or soluble ferredoxin (Fd), measured by electron paramagnetic resonance (EPR) spectroscopy. The reaction mixture, equilibrated with N_2, contained osmotically disrupted chloroplasts (equivalent to 1 mg chlorophyll per ml), 50 mM Tricine buffer (pH 7.7), 5.0 mM $MgCl_2$, 50 mM KCl, 2.5 mM ADP, 2.5 mM K_2HPO_4, 10 mM glucose, glucose oxidase, catalase and 12% methanol. Spinach ferredoxin (0.010 mM) and DBMIB were added as indicated. The EPR tubes were illuminated for 30s or 120s at 293K and immediately frozen in liquid N_2 under continuing illumination (650 nm, 5 X 10^5 ergs cm^{-2} s^{-1}). EPR spectra were recorded at 20K for $H_2O \rightarrow$ Fe-S and at 60K for $H_2O \rightarrow$ Fd. Spectrometer field setting, 3450 ± 200G; microwave power 10 mW, modulation amplitude 10G; gain, 1 X 10^5. EPR signals of dark controls were subtracted by computer. (Free radical region around g = 2.0 not shown.)

explained in another way. DBMIB being a quinone can itself be photoreduced via PSII by water (13,22-25); DBMIB can also reduce cytochrome f, plastocyanin and the P700 component of PSI (26). It thus appeared possible that prolonged illumination obscured the differential inhibitory effect of DBMIB by activating an artificial electron transport system in which DBMIB was first photoreduced by water and in turn photoreduced components of PSI, including the Fe-S centers. Such an artificial electron transport system would likely to have been sluggish and its operation would have been favored by prolonged illumination.

Evidence that DBMIB did indeed act as a carrier of electrons from water is given in Fig. 2. The top traces show the combined signals of photoreduced Fe-S centers and Fd after 120s illumination in the presence of DBMIB. The lower traces show that the addition of diuron, which blocked the photooxidation of water also prevented the photoreduction of the Fe-S centers and Fd.

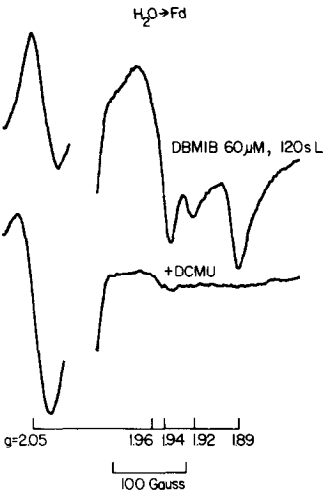

Fig. 2. Inhibition by diuron (DCMU) of oxygenic photoreduction of Fd and bound Fe-S centers in the presence of DBMIB. Illumination 120s at 293K. EPR spectra recorded at 20K included signals of Fd and the Fe-S centers. 10^{-5} M DCMU added where indicated. Other conditions as in Fig. 1.

In sum, the redox properties of DBMIB (13,22) appeared to generate secondary effects which complicated the assessment of the role of plastoquinone in the native oxygenic electron transport of chloroplasts. We undertook therefore to seek further evidence on this point, using DNP-INT, a potent plastoquinone inhibitor devoid of redox properties (13,22).

Effect of DNP-INT on Oxygenic Photoreduction of Fe-S Centers and Fd. Preliminary investigations with varying concentrations of DNP-INT showed that the photoreduction of the Fe-S centers was more sensitive to this inhibitor than the photoreduction of Fd (data not shown). Of special significance were the findings that the oxygenic photoreduction of Fd was not inhibited at DNP-INT concentrations (relatively high due to the high chlorophyll concentration, cf. ref. 13) that completely inhibited the photoreduction of the Fe-S centers (Fig. 3). Moreover, regardless whether illumination was short (30s) or long (120s), DNP-INT blocked completely the photoreduction of the Fe-S centers without diminishing the photoreduction of Fd (Fig. 3).

Sensitivity to diuron inhibition confirmed that water was indeed the electron donor for photoreduction of Fd at concentrations of DNP-INT that blocked completely the photoreduction of the Fe-S centers (Fig. 4).

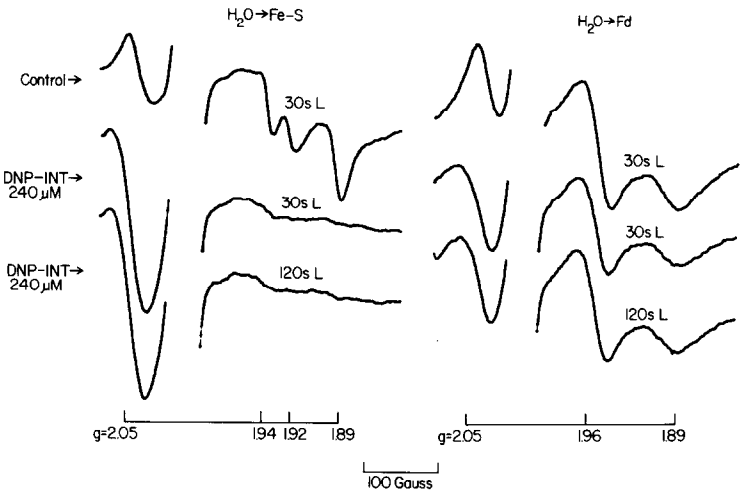

Fig. 3. Effect of DNP-INT on photoreduction by water of bound iron-sulfur centers or soluble ferredoxin. Experimental conditions as in Fig. 1 except that DNP-INT replaced DBMIB (no EPR signals were observed in the dark).

These results clearly demonstrated that the oxygenic photoreduction of Fd is not obligatorily tied to a prior, sequential reduction of PSI components represented by the Fe-S centers. Normally, electrons originating from water reduced both Fd and the Fe-S centers but when plastoquinone function was inhibited, Fd was photoreduced by water without the involvement of the Fe-S centers of PSI. Thus, in the oxygenic photoreduction of Fd, the photoreduction of the Fe-S centers was not a precondition but a parallel event that was more sensitive to inhibitors of plastoquinone function.

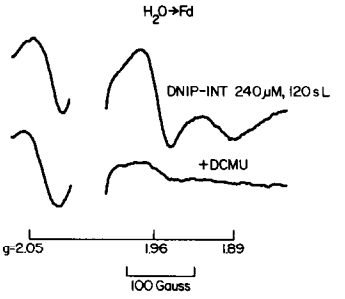

Fig. 4. Inhibition by diuron (10^{-5} M DCMU) of Fd photoreduction in the presence of DNP-INT. Experimental conditions as in Fig. 3, bottom right trace.

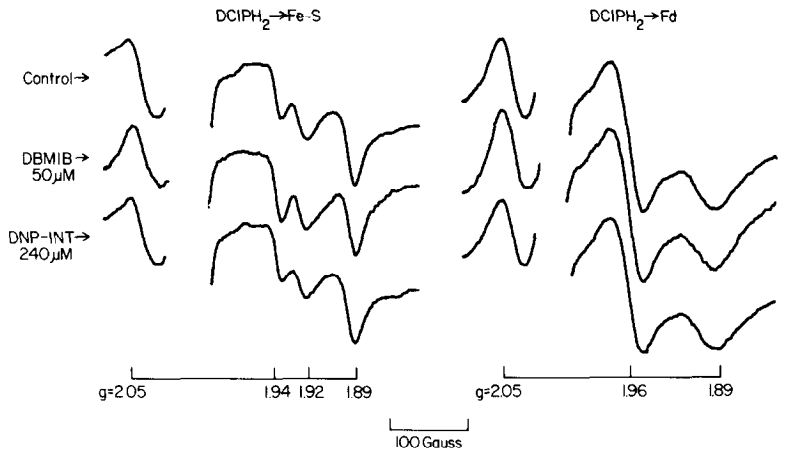

Fig. 5. No effect of plastoquinone inhibitors on photoreduction of Fd and Fe-S centers by a photosystem I electron donor ($DCIPH_2$). Experimental conditions as in upper and middle traces of Fig. 1 except that 1 X 10^{-5} M DCMU, 10 mM sodium ascorbate and 0.1 mM DCIP were present throughout and DBMIB and DNP-INT added as indicated.

No Effect of Plastoquinone Inhibitors on Photoreduction of Fd and Fe-S Centers by a PSI Donor. The differential sensitivity of the oxygenic photoreduction of Fd and Fe-S centers toward plastoquinone inhibitors argued against the involvement of the Fe-S centers (or other components of PSI) as electron carriers in the photoreduction of Fd by water. However, this interpretation did not rule out -- in fact it predicted -- a carrier role for the Fe-S centers in the photoreduction of Fd under special experimental conditions when photooxidation of water is blocked by diuron and a direct donor to PSI (e.g., $DCIPH_2$) bypassing plastoquinone is used (7). In such an artificial anoxygenic photoreduction of Fd via PSI, no inhibition by DBMIB or DNP-INT of the reduction of the Fe-S centers was expected and none was found (Fig. 5).

DISCUSSION

The results of this study are incompatible with some basic postulates of the now popular Z scheme of photosynthetic electron transport. In the Z scheme, plastoquinone serves as an essential link in the linear electron transport from water via PSII to PSI and thence to the Fe-S centers and Fd. Our evidence is in conflict with this formulation. The photoreduction of the Fe-S centers and Fd by water exhibited differential sensitivity to plastoquinone inhibitors, a finding inconsistent with the sequential

positions of Fe-S centers and Fd in the linear photoredox chain envisaged by the Z scheme (see diagram in Introduction). Fd was still photoreduced by water at inhibitor concentrations that abolished the photoreduction of the Fe-S centers.

The new findings are compatible with, and supportive of, an updated concept of photosynthetic electron and proton transport in chloroplasts that views differently the nature of the two photosystems and the role of plastoquinone (27). Here an oxygenic photosystem (supplanting PSII) photoreduces Fd without the collaboration of PSI. PSI (renamed the anoxygenic photosystem) is viewed as a parallel, synchronous photosystem responsible for cyclic electron flow and photophosphorylation and dependent only on a trickle of electrons from water (via the oxygenic photosystem) for regulation (15). (The designations oxygenic and anoxygenic were introduced to avoid confusion with the widespread notions that PSII photooxidizes water but cannot reduce Fd whereas PSI reduces Fd but cannot photooxidize water.)

According to the new concept, to be elaborated elsewhere, the main function of plastoquinone is not to serve as a link between two photosystems but to facilitate vectorial proton translocation in <u>each</u> of the two photosystems (27). Plastoquinone may also bridge electron transport between the oxygenic and anoxygenic photosystems but this is a secondary function which, as shown here, can be abolished without impairing the photoreduction of Fd by the oxygenic photosystem.

Until recently it was widely held that a single oxygenic photosystem (PSII) could not generate a reductant strong enough to reduce Fd. Recently, however, PSII was found capable of reducing pheophytin (E_m = ca. -610 mV) without the collaboration of PSI (28-30). Because of its lipophilic properties and strongly electronegative potential, pheophytin might serve as an intermediate in the photoreduction of Fd by the oxygenic photosystem (27).

Two other points warrant mentioning. First, our results are not based on a small fraction of the total possible reduction of Fd or the bound Fe-S centers. Similar EPR signal intensities were obtained when excess dithionite (plus illumination) was used for the reduction of chloroplasts and/or Fd (data not shown). Second, the relative proportions of Fe-S centers and Fd in our experiments were not too dissimilar from those in

intact chloroplasts. In intact spinach chloroplasts the Fd:chlorophyll ratio is about 1:400 (6) and the ratio of Fe-S centers (A+B) to chlorophyll is about 1:200 [based on ratios of P700:chlorophyll = 1:400 (31); center A:chlorophyll 1:400 (31) and center A:center B = 1:1 (32)]. In our experiments, the molar ratio Fd:chlorophyll was 1:100 and that of Fe-S centers (A+B): chlorophyll is estimated to be 1:200.

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