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Contrasts between oxygenic and anoxygenic photoreduction of ferredoxin: Incompatibilities with prevailing concepts of photosynthetic electron transport

(photosynthetic mechanisms/photosystems I and II)

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ABSTRACT **An investigation by paramagnetic resonance spectroscopy of the photoreduction of ferredoxin, oxygenically by water and anoxygenically by a direct electron donor to photosystem I, led to the unexpected findings that different reductive mechanisms may be involved. Ferredoxin photoreduced by water was not reoxidized in the light under aerobic conditions and, under anaerobic conditions, it was remarkably resistant to reoxidation in the dark. By contrast, ferredoxin photoreduced by a donor to photosystem I was readily reoxidized in the light by air and, under anaerobic conditions, by exposure to darkness. Furthermore, when electron transport linking photosystems I and II was inhibited by a plastoquinone antagonist, ferredoxin was photoreduced by water with no evidence for an accompanying photoreduction of the more electronegative bound iron-sulfur centers in chloroplasts. These findings are at variance with the now prevalent concepts of photosynthetic electron transport.**

A recent investigation (1) of the regulatory mechanisms of photosynthetic phosphorylation in chloroplasts disclosed that ferredoxin, a key protein in photosynthesis (2, 3), has an unusually strong affinity for electrons from water. Ferredoxin remained in a predominantly reduced steady state even when the photoinduced electron flow from water was severely restricted to a mere trickle, either by the use of far-red (715 nm) monochromatic illumination, which does not favor the functioning of the portion of the photosynthetic apparatus that photooxidizes water (photosystem II), or by the use of diuron [3-(3′,4′-dichlorophenyl)-1,1-dimethylurea)], a specific inhibitor of photooxidation by water (1).

A further investigation of the photoreduction of ferredoxin by isolated chloroplasts led to unexpected findings, reported in this paper, that the identity of the electron donor determined the stability of ferredoxin in the reduced state. Ferredoxin photoreduced by water was more resistant to reoxidation than was ferredoxin photoreduced by a donor that bypassed photosystem II and donated electrons directly to photosystem I. Moreover, with water as electron donor, the photoreduction of ferredoxin was found to be resistant to inhibition by 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), at concentrations that inhibited the photoreduction of the bound iron-sulfur centers (bound ferredoxins) associated with photosystem I (4–6). DBMIB, an antagonist of plastoquinone, is known to be an effective inhibitor of the linkage between photosystems I and II (7, 8).

These findings (*i*) suggest that photoreduction of ferredoxin by water may proceed by a mechanism different from that involved in the photoreduction of ferredoxin by a substitute electron donor to photosystem I and (*ii*) raise a question as to whether the photoreduction of the bound iron-sulfur centers is a prerequisite for the reduction of ferredoxin by water.

The possible existence of two different mechanisms of ferredoxin reduction in chloroplasts and the independence of at least one of them from the prior reduction of bound iron-sulfur centers is of considerable theoretical interest because both are excluded by the now-prevalent concepts of photosynthetic electron transport embodied in the so-called Z scheme (9). A major tenet of that scheme is that the reduction of ferredoxin, preceded by the reduction of the bound iron-sulfur centers (10), is driven by photosystem I in the same manner whether the electrons originate from water, via photosystem II, or are provided by a substitute donor directly to photosystem I (11).

METHODS

Chloroplasts were isolated from spinach leaves (*Spinacia oleracea*, var. Marathon or High Pack) grown in a greenhouse in a nutrient solution culture (12) and freshly harvested before each experiment. The preparation used consisted of osmotically disrupted ("broken") chloroplasts made by a procedure (13) that preserved the integrity of membrane structure needed for complete electron transport from water to $NADP^+$ and for ferredoxin-catalyzed cyclic photophosphorylation. Ferredoxin was isolated and purified (by R. K. Chain), and chlorophyll was measured as described (12, 14). Glucose oxidase (type VII) and bovine catalase were purchased from Sigma. DBMIB was a gift of A. Trebst.

In the oxygenic photoreduction of ferredoxin, the reductant was water and the reaction mixture contained no diuron. In the anoxygenic photoreduction of ferredoxin, the reductant was the ascorbate dichlorophenol-indophenol (DCIP) couple, supplied in combination with diuron to block the photooxidation of water (15). In some experiments, ascorbate/DCIP was added without diuron to test the effects of this couple on a system in which water could still function as a reductant.

The photoreduction of ferredoxin and of the membrane-bound iron-sulfur centers was measured by electron paramagnetic resonance (EPR) spectroscopy. The chloroplasts (in their respective reaction mixtures) were placed in quartz EPR tubes (3 mm, inside diameter) which were either illuminated directly or, when oxygen was to be excluded, after having been gassed with nitrogen. The EPR tubes were always illuminated first at a physiological temperature (293 K) for 30 sec before they were frozen by a 30-sec immersion in liquid nitrogen contained in a silvered dewar flask with a window to be used for illumination, when desired. Freezing was either under continuing illumination or in darkness. In some experiments, a dark interval at 293 K was sandwiched between the illumination and the dark freezing step. Monochromatic illumination (715 or 664 nm) was provided by a light beam from a Quartz-

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Abbreviations: DCIP, $DCIPH_2$, oxidized and reduced forms of dichlorophenol-indophenol; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone); EPR, electron paramagnetic resonance.

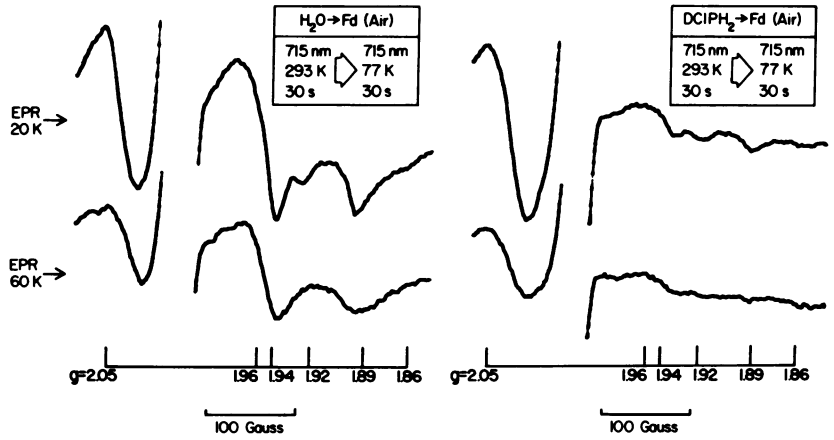


FIG. 1. Donor-dependent stability of ferredoxin photoreduced under aerobic conditions. (*Left*) Water was the electron donor; (*Right*) $DCIPH_2$ (kept in the reduced form by an excess of ascorbate) was the electron donor. The EPR tubes were illuminated for 30 sec at room temperature (293 K) and illumination was continued for another 30 sec while the tubes were immersed in liquid nitrogen (77 K). The extent of ferredoxin reduction is indicated by the amplitude of the EPR signals with $g = 1.89$, 1.96, and 2.05. EPR spectra were recorded at 20 and 60 K. Spectrometer field setting, 3450 ± 200 G; microwave power, 10 mW; modulation amplitude, 10 G. With water as donor, the reaction mixture contained spinach chloroplasts (equivalent to 1.0 mg of chlorophyll per ml), 50 mM *N*-Tris(hydroxymethyl)methylglycine buffer (pH 8.2), 10 mM $MgCl_2$, 2.5 mM ADP, 2.5 mM K_2HPO_4, 0.01 mM spinach ferredoxin, and 3% methanol. With $DCIPH_2$ as donor, the reaction mixture contained, in addition, 10 mM sodium ascorbate, 0.1 mM DCIP, and 0.01 mM diuron. Illumination was monochromatic (715 nm; 1×10^5 ergs·cm^{-2}·sec^{-1}. The gas phase was air.

line lamp (type DXN, 1000 W). The light beam was passed through heat-absorbing and interference filters (Baird).

First-derivative EPR spectra of the frozen samples were obtained with a Bruker (Billerica, MA) EPR spectrometer (model ER200tt) [equipped with a 20-cm (8-inch) magnet] and recorded after processing by a digital signal averager (Nicolet, Madison, WI; model 1070). The frozen samples in the quartz EPR tubes were further cooled with liquid helium to either 20 or 60 K by an Oxford temperature controller (model DTC) and cryostat (model ESR9) equipped with a quartz dewar cell (made by J. Scanlon, Solvang, CA).

RESULTS

In the EPR traces presented below, the extent of ferredoxin reduction is indicated by the amplitude of its characteristic EPR signals at $g = 1.89$, 1.96 (main signal), and 2.05. The reduced membrane-bound iron-sulfur centers (bound ferredoxins) give, in the reduced state, signals at $g = 1.86$, 1.94, and 2.05 (center A) and $g = 1.89$, 1.92, and 2.05 (center B) (4–6, 16, 17). Because of the considerable overlap between the signals of reduced ferredoxin and reduced centers A and B, the EPR tubes were scanned at two temperatures, 20 and 60 K. The scan at 20 K gave signals both of reduced ferredoxin and reduced bound iron-sulfur centers but at 60 K the EPR signals of the bound iron-sulfur centers broadened and ceased to be detectable (18). Thus, the EPR scan at 60 K was used to measure only reduced ferredoxin.

Effect of Electron Donors on the Stability of Reduced Ferredoxin in the Presence of Oxygen. There is abundant evidence that, in isolated chloroplasts, electron donors to photosystem I, such as the ascorbate/DCIP couple, will photoreduce ferredoxin (and $NADP^+$) when the physiological electron flow from water via photosystem II is blocked by the use of diuron (see review, ref. 11). Inherent in, and basic to, the Z scheme is the premise that photoreduction of ferredoxin by ascorbate/DCIP mirrors the photoreduction of ferredoxin by photosystem I with electrons that originate from water.

This was found not to be the case (Fig. 1). With water as the electron donor, ferredoxin accumulated predominantly in the reduced state despite the presence of oxygen, which is known

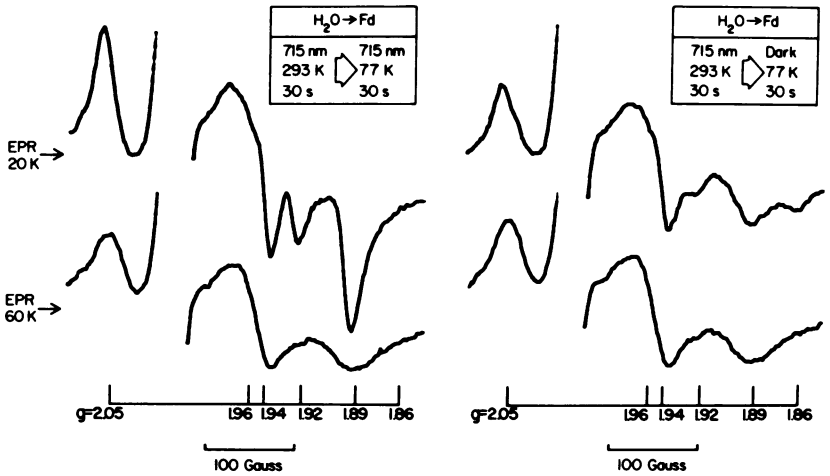


FIG. 2. Stability in the dark of ferredoxin photoreduced under anaerobic conditions by water. Immediately following illumination for 30 sec at room temperature, the EPR tubes were immersed in liquid nitrogen either under continuing illumination (*Left*) or in the dark (*Right*) for another 30 sec. The reaction mixture was the same as for the water donor system in Fig. 1 except that it was gassed with nitrogen and contained, in addition, an oxygen trap consisting of 10 mM glucose and 7500 units of catalase and 13 units of glucose oxidase per ml.

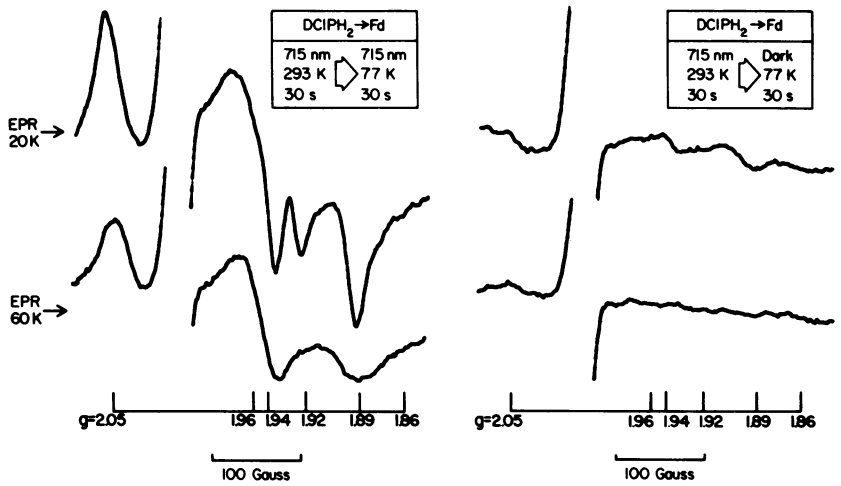


FIG. 3. Instability in the dark of ferredoxin photoreduced under anaerobic conditions by a donor to photosystem I. Anaerobic conditions, illumination, and freezing in the dark were as in Fig. 2. The reaction mixture was the same as for the $DCIPH_2$ system in Fig. 1.

to oxidize reduced ferredoxin (19). By contrast, with the ascorbate/DCIP couple as donor, there was little accumulation of reduced ferredoxin in the presence of oxygen.

It was possible that, apart from reoxidation by oxygen, the failure of ferredoxin to accumulate in the reduced state when ascorbate/DCIP was the donor may have resulted from the oxidation of reduced ferredoxin by the dehydroascorbate that was formed as ascorbate reduced DCIP. This possibility was tested by the addition of the ascorbate/DCIP couple to chloroplasts that were photoreducing ferredoxin with water (i.e., when no diuron was added). The addition of the ascorbate/DCIP couple did not bring about the reoxidation of ferredoxin. As long as water was the electron donor, a substantial portion of ferredoxin remained in the reduced state (data not included).

The strong affinity of ferredoxin for electrons from water was confirmed. In agreement with previous findings (1), far-red (715 nm) illumination, which mainly activates photosystem I and can sustain only a low rate of electron flow from water, maintained ferredoxin in a predominantly reduced state (Fig. 1).

Effect of Electron Donors on the Stability of Reduced Ferredoxin in the Absence of Oxygen. In these and subsequent experiments, the effect of donor on the stability of reduced ferredoxin was tested under anaerobic conditions—i.e., under nitrogen and in the presence of a glucose oxidase trap for oxygen that would be liberated by the photooxidation of water.

When the chloroplasts were handled in the same way as under aerobic conditions—namely, when illumination was continued during the 30-sec freezing period in liquid nitrogen—the exclusion of oxygen did indeed increase the stability of the reduced state of all iron-sulfur centers, the membrane-bound centers A and B as well as soluble ferredoxin, regardless of whether water or ascorbate/DCIP was the electron donor (Figs. 2 *Left* and 3 *Left*). However, a donor effect on the stability of photoreduced ferredoxin was detected when chloroplasts were frozen in the dark after prior illumination at room temperature.

As shown in Fig. 2, with water as donor, ferredoxin remained in the reduced state, regardless of whether the chloroplasts were frozen in the dark or under continuing illumination. By contrast, with ascorbate/DCIP as donor, ferredoxin remained in the reduced state only when the chloroplasts were frozen under continuing illumination; when the chloroplasts were frozen in the dark (Fig. 3 *Right*), the photoreduced ferredoxin became reoxidized.

The possibility that the formation of dehydroascorbate by the ascorbate/DCIP couple may have been responsible for the anaerobic reoxidation of reduced ferredoxin during the dark freezing step was tested by the addition of ascorbate/DCIP (but no diuron) to chloroplasts that were photoreducing ferredoxin with water. When the electron flow from water was not blocked by diuron, the presence of ascorbate/DCIP did not cause a

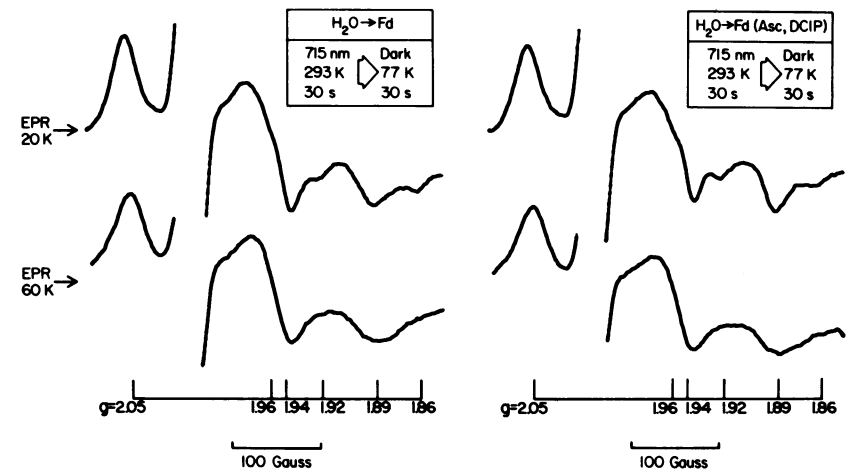


FIG. 4. Lack of effect of added ascorbate/DCIP on the stability of ferredoxin photoreduced by water and frozen in the dark. Experimental conditions were as for Fig. 2 *Right*, except that 10 mM sodium ascorbate and 0.1 mM DCIP were added where indicated.

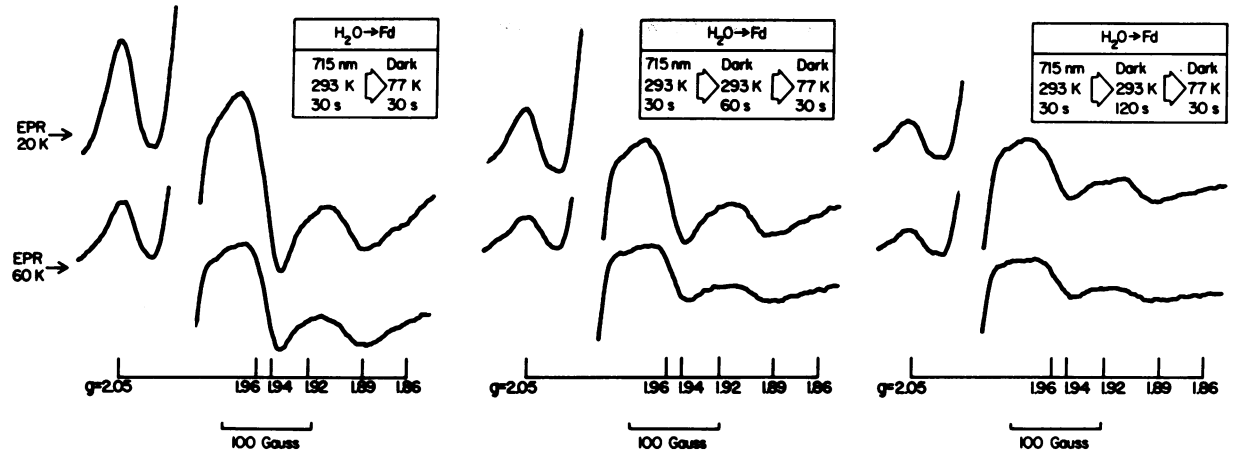


FIG. 5. Stability of ferredoxin photoreduced by water to dark exposure at room temperature. Experimental conditions were as in Fig. 2 *Right* except that, where indicated, a dark interval at room temperature (293 K) was sandwiched between illumination and the dark freezing step.

reoxidation of ferredoxin in the dark (Fig. 4). It appeared unlikely, therefore, that the contrast between the stability in the dark of ferredoxin photoreduced by water and the instability in the dark of ferredoxin photoreduced by ascorbate/DCIP was a consequence of the reoxidation of reduced ferredoxin by dehydroascorbate.

Stability in the Dark and at Room Temperature of Ferredoxin Photoreduced by Water. The stability of ferredoxin photoreduced by water was further investigated by interposing a dark interval at room temperature prior to the freezing step, during which the ambient temperature dropped from room temperature (293 K) to 77 K. As shown in Fig. 5 *Middle*, after a 60-sec dark interval at room temperature and subsequent freezing in the dark, about half of the ferredoxin previously photoreduced by water remained in the reduced state. An appreciable portion of ferredoxin photoreduced by water remained in the reduced state even after a 120-sec dark interval at room temperature (Fig. 5 *Right*).

Effect of DBMIB on Photoreduction of Ferredoxin and Bound Iron-Sulfur Centers by Water. The striking stability of the reduced state of ferredoxin when the donor was water and the contrasting instability of reduced ferredoxin under the same conditions when the donor was ascorbate/DCIP rendered it unlikely that the electron transport pathway from water to ferredoxin included the same segment through photosystem I and bound iron-sulfur centers that is involved in photoreduction of ferredoxin by ascorbate/DCIP. It thus became desirable to investigate the role of photosystem I and bound iron-sulfur centers in the photoreduction of ferredoxin by water in the presence of DBMIB, an inhibitor that blocks the connecting electron transport between photosystems II and I (8, 11).

In the absence of DBMIB, electrons from water photoreduced both soluble ferredoxin and the bound iron-sulfur centers (Fig. 6 *Left*). Unexpected results, however, were obtained in the presence of 10 μM DBMIB (Fig. 6 *Center*); as evidenced by signals at $g = 2.05$, 1.96, and 1.89, ferredoxin was photoreduced by water, but the absence of the $g = 1.92$ signal and the diminished amplitude of the signals at $g = 2.05$, 1.94, and 1.89 indicate little photoreduction of the bound iron-sulfur centers. When the concentration of DBMIB was increased to 20 μM, the photoreduction of ferredoxin by water was severely inhibited; there was no trace of reduction of the bound iron-sulfur centers (Fig. 6 *Right*).

Similar results were obtained with chloroplasts prepared from leaves harvested at different seasons. Despite seasonal variations in the requisite concentrations of DBMIB, there were no exceptions to the finding that, at DBMIB concentrations that inhibited the photoreduction of the bound iron-sulfur centers by water, soluble ferredoxin was still being photoreduced by water.

DISCUSSION

The currently prevalent concept of the generation of photosynthetic reducing power (represented by NADPH) envisions a light-induced, noncyclic (linear), electron flow from water

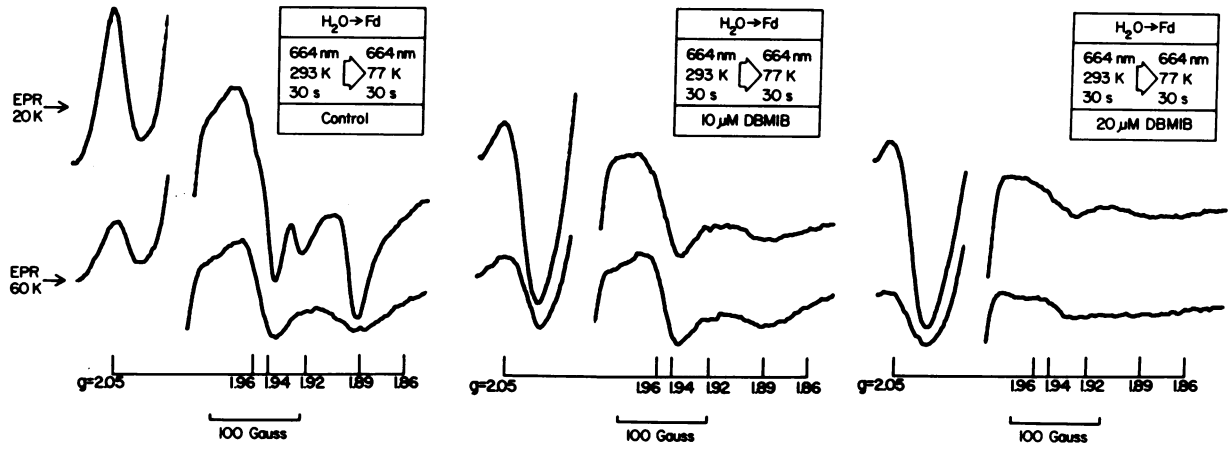
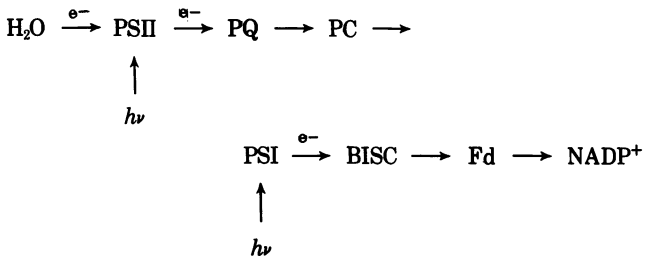


FIG. 6. Differential effect of DBMIB on the photoreduction of the bound iron-sulfur centers and ferredoxin by water. Experimental conditions were as in Fig. 2 *Left* except that illumination was by 664-nm light (2×10^5 ergs·cm^{-2}·sec^{-1}) and, where indicated, DBMIB was added. The EPR signals from DBMIB itself were subtracted by the data processor.

to ferredoxin that involves the collaboration of two photosystems joined by a chain of electron carriers (9, 11). As outlined in the abbreviated scheme below, after being energized by photosystem II (PSII) to an intermediate reducing power, electrons from water are transported via plastoquinone (PQ) and plastocyanin (PC) to photosystem I (PSI), which energizes the electrons to their ultimate reducing power and makes possible the reduction of the strongly electronegative early intermediates and bound iron-sulfur centers (BISC) (10, 20, 21); the latter in turn reduce ferredoxin (Fd) and ferredoxin finally reduces $NADP^+$ in a reaction catalyzed by ferredoxin-$NADP^+$ reductase (22).



Basic to this scheme is the notion that the electron transport path from the reaction center of photosystem I to the bound iron-sulfur centers and ferredoxin is the same, regardless of whether electrons to photosystem I are provided by a direct donor like ascorbate/DCIP or originate from water via photosystem II (11). In either case, ferredoxin would be reduced by the same mechanism and should have the same properties.

Our results are incompatible with this notion. When photoreduced by ascorbate/DCIP under aerobic conditions, ferredoxin was readily reoxidized by oxygen and, under anaerobic conditions, by exposure to darkness. By contrast, ferredoxin photoreduced by water remained reduced in the presence of oxygen and was remarkably resistant to reoxidation in the dark under anaerobic conditions. These donor-dependent differences argue against an identical mechanism of ferredoxin reduction in both cases.

A related basic premise of the scheme above is that the reduction of ferredoxin, whose midpoint potential is −420 mV (2, 23), depends on reduction of the bound iron-sulfur centers, whose midpoint potentials are in the range −530 to −580 mV (17, 24). Our earlier results with cyanobacterial membrane fragments (18) and our present results with chloroplasts are consistent with this view only when a direct donor to photosystem I is used. Thus, with ascorbate/DCIP as donor, photoreduction of ferredoxin was regularly accompanied by photoreduction of the bound iron-sulfur centers (Fig. 3 *Left*).

With water as donor, the results were different. In the absence of DBMIB, the photoreduction of ferredoxin was indeed accompanied by a demonstrable reduction of bound iron-sulfur centers (Fig. 6 *Left*). However, when electron transport through plastoquinone, which links photosystems I and II, was inhibited by DBMIB, ferredoxin was photoreduced by water without an accompanying photoreduction of the bound iron-sulfur centers (Fig. 6 *Center*). Thus, the photoreduction of the bound iron-sulfur centers by water seems to be an event occurring at the same time but whose blocking by DBMIB does not prevent the photoreduction of ferredoxin by water.

It appears that only in an artificial system in which a direct donor to photosystem I has replaced water may a prior reduction of the bound iron-sulfur centers be a prerequisite for the subsequent reduction of ferredoxin. The present findings, and those to be published separately, suggest that, under physiological conditions when water is the electron donor, the photoreduction of ferredoxin utilizes a mechanism different from that involved in the photoreduction of ferredoxin by an artificial donor to photosystem I.

To recapitulate, the different, donor-dependent stabilities of reduced ferredoxin and the evidence that, in the presence of DBMIB, ferredoxin can be photoreduced by water without a concomitant reduction of bound iron-sulfur centers suggest that photoreduction of ferredoxin by water involves a mechanism different from that in photoreduction of ferredoxin by ascorbate/DCIP. The anoxygenic photoreduction of ferredoxin (and $NADP^+$) by ascorbate/DCIP or other photosystem I donors may reflect an experimental perturbation of the cyclic electron transport driven by photosystem I. Evidence for the simultaneous functioning of cyclic and noncyclic electron transport in chloroplasts has been presented elsewhere (13, 25).

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