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Electron paramagnetic resonance studies of photosynthetic electron transport: Photoreduction of ferredoxin and membrane-bound iron–sulfur centers*

(photosynthesis/reducing power/electron carriers)

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ABSTRACT **Electron paramagnetic resonance spectrometry was used to investigate, at physiological temperatures, light-induced electron transport from membrane-bound iron–sulfur components (bound ferredoxin) to soluble ferredoxin and $NADP^+$ in membrane fragments (from the blue-green alga, *Nostoc muscorum*) that had high rates of electron transport from water to $NADP^+$ and from an artificial electron donor, reduced dichlorophenolindophenol ($DCIPH_2$) to $NADP^+$. Illumination at 20° resulted in the photoreduction of membrane-bound iron–sulfur centers A and B. Photoreduction by water gave electron paramagnetic resonance signals of both centers A and B; photoreduction by $DCIPH_2$ was found to generate a strong electron paramagnetic resonance signal of only center B.**

When water was the reductant, the addition and photoreduction of soluble ferredoxin generated additional signals characteristic of soluble ferredoxin without causing a decrease in the amplitude of the signals due to centers A and B. The further addition of $NADP^+$ (and its photoreduction) greatly diminished signals due to the bound iron–sulfur centers and to soluble ferredoxin. An outflow of electrons from center B to soluble ferredoxin and $NADP^+$ was particularly pronounced when $DCIPH_2$ was the reductant. These observations provide the first evidence for a light-induced electron transport between membrane-bound iron–sulfur centers and ferredoxin-$NADP^+$. The relationship of these observations to current concepts of photosynthetic electron transport is discussed.

The now well-documented broad importance of iron–sulfur proteins to photosynthesis (reviewed in ref. 1) became apparent in two stages. First, when photoreduced chloroplast ferredoxin was found to be both the electron donor (with a midpoint potential of −420 mV) for $NADP^+$ reduction (2–4) and the native catalyst for cyclic photophosphorylation in chloroplasts (5, 6), and second, when electron paramagnetic resonance (EPR) spectroscopy at cryogenic temperatures led to the detection in chloroplasts of a photoreducible membrane-bound iron–sulfur center (bound ferredoxin) distinct from soluble ferredoxin (7). Other EPR experiments with isolated chloroplasts and photosystem I preparations yielded evidence for two bound iron–sulfur centers; center A (synonymous with bound ferredoxin) characterized in the reduced state by resonances at g = 1.86, 1.94, and 2.05 and a midpoint potential of about −530 mV, and center B, characterized in the reduced state by resonances at g = 1.89, 1.92, and 2.05 and a midpoint potential of about −580 mV (7–11).

EPR spectroscopy at cryogenic temperatures was also used to identify in chloroplasts and algal preparations another photoreducible component (component X) with resonances at g = 1.78, 1.88, and 2.08 (12–16). The photoreduction of component X was detectable only after prior reduction of centers A and B with dithionite. The chemical nature of component X is unknown; its EPR spectrum differs from that of known iron–sulfur proteins. McIntosh and Bolton (15) and Evans *et al.* (16) regarded component X as the primary acceptor of electrons released by the concurrent photooxidation of P700, the reaction center chlorophyll of photosystem I; photoreduced component X is thought to reduce centers A and B. Bearden and Malkin (17–22) assigned the role of primary electron acceptor in photosystem I to center A which, unlike the more electronegative center B, undergoes a photoreduction at cryogenic temperatures concurrently with the photooxidation of P700. There is as yet no evidence bearing on the role of center B in photosystem I although it appears that center B may be interacting with center A under certain experimental conditions.

Aside from the identity of the primary electron acceptor of photosystem I, there is a general assumption, unsupported so far by any experimental evidence, of electron transfer between the bound iron–sulfur centers of photosystem I and soluble ferredoxin. The reduction of centers A and B was investigated under drastic experimental conditions—i.e., under illumination at cryogenic temperatures (≤77 K) and in the presence of a strong nonphysiological reductant (dithionite)—that are not compatible with the reduction of ferredoxin and $NADP^+$ either by the physiological reductant water or by substitute electron donors such as the reduced dye, 2,6-dichlorophenolindophenol ($DCIPH_2$). Photoreduction of centers A and B by water or $DCIPH_2$ has not hitherto been demonstrated.

This paper reports EPR evidence for the photoreduction of centers A and B and soluble ferredoxin at physiological temperatures with water or $DCIPH_2$ as the electron donor by membrane fragments (fraction C) from the blue-green alga *Nostoc muscorum* noted for their high rates of electron transport from water to $NADP^+$ and from $DCIPH_2$ to $NADP^+$ (23). EPR evidence is presented for electron transfer between center B and soluble ferredoxin and for the effect of $NADP^+$ on the steady-state photoreduction levels of ferredoxin and the membrane-bound iron–sulfur centers. The relationship of these observations to current concepts of photosynthetic electron transport is discussed.

METHODS

Cells of the blue-green alga *N. muscorum* (strain 7119) grown in an N_2/CO_2 atmosphere with N_2 as the sole source of nitrogen were used as the source of membrane fragments (fraction C) that were prepared as described (23) except that 10 mM sodium ascorbate was included in the suspending solution in addition

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Abbreviations: EPR, electron paramagnetic resonance; $DCIPH_2$, 2,6-dichlorophenolindophenol.

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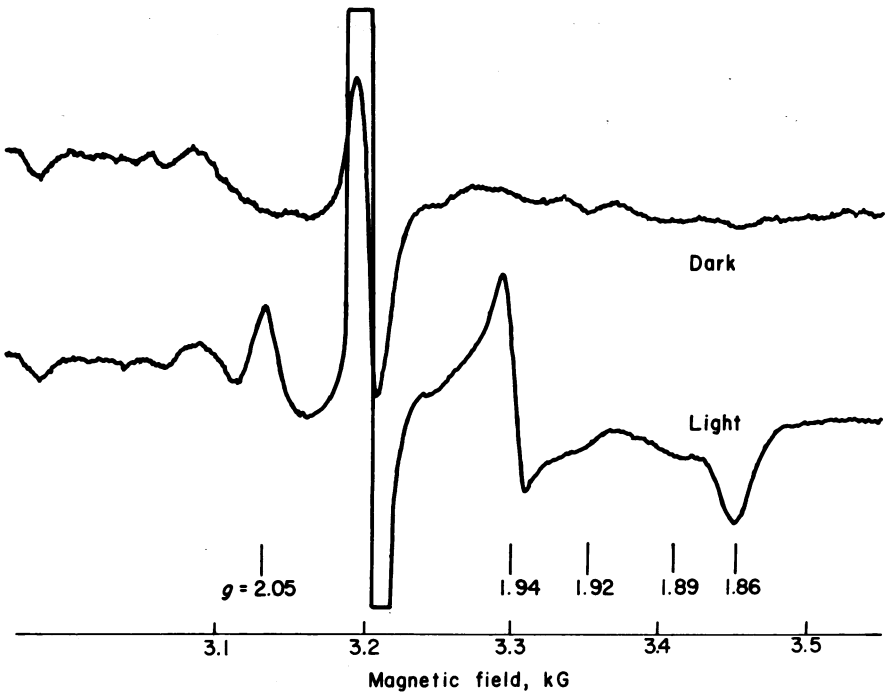

FIG. 1. Photoreduction of bound iron–sulfur center A ($g_x = 1.86$, $g_y = 1.94$, $g_z = 2.05$) of *Nostoc* membrane fragments at 77 K. The reaction mixture contained (per 1.0 ml): *Nostoc* fraction C membrane fragments equivalent to 0.5 mg of chlorophyll *a*; Tricine [*N*-Tris(hydroxymethyl)methylglycine] buffer (pH 7.7), 50 μmol; sodium ascorbate, 10 μmol; and $MgCl_2$, 10 μmol. Monochromatic 650-nm illumination (at 77K), 5×10^5 ergs·cm^{-2}·sec^{-1}. EPR spectra were recorded at 20 K with the following instrument settings: microwave frequency, 9.25 GHz; power, 10 mW; magnetic field modulation frequency, 100 kHz; amplitude, 10 G; scanning rate, 5 G/sec; time-constant, 0.2 sec; amplifier gain, 2×10^5.

to sucrose, buffer, and $MgCl_2$. Chlorophyll *a* was measured (24), and ferredoxin and ferredoxin–$NADP^+$ reductase were isolated and purified from spinach leaves by procedures reported from this laboratory (25, 26).

The algal membrane fragments (in their respective reaction mixtures) were placed in quartz EPR tubes (3 mm inside diameter) and illuminated either at a physiological (293 K) or cryogenic temperature (77 K). Physiological temperature illumination included 20 sec at 293 K followed without interruption by illumination for another 30 sec through a window of a silvered Dewar flask containing liquid nitrogen in which the EPR tube was immersed to be frozen. For cryogenic temperature illumination, the samples were first frozen in the dark in liquid nitrogen and then were illuminated at 77 K for 60 sec. Monochromatic illumination (650 or 715 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.).

After illumination, first-derivative EPR spectra of the samples were obtained with a Bruker Instruments Co. EPR spectrometer (model ER 200 tt) equipped with a 20-cm ("8 inch") double-yoke magnet and an X-band resonator in the TE_{102} mode. The samples in the quartz tubes were cooled with liquid helium to the desired temperature by an Oxford Instruments Inc. cryostat (model ESR9) and temperature controller (model DTC).

RESULTS

EPR Spectrum of *Nostoc* Membrane Fragments Illuminated at 77 K. Illumination at cryogenic temperatures of different chloroplast and algal preparations (7, 18, 27) has consistently produced an EPR spectrum characteristic of reduced iron–sulfur center A (bound ferredoxin) as was first observed in spinach chloroplasts (7). We undertook, therefore, to determine whether this feature was also characteristic of our *Nostoc* membrane fragments. This proved to be the case. Only center A, with g values of 2.05, 1.94, and 1.86, was photoreduced under illumination at 77 K (Fig. 1). The same EPR spectrum was obtained with either red (650 nm) or far-red (715 nm) monochromatic illumination. The addition of soluble ferredoxin (with or without $NADP^+$) prior to freezing and illumination had no effect.

The photoreduction of center A was accompanied by a corresponding (off scale) increase of a free-radical signal in the g = 2.00 region that is due to photooxidized P700 (18).

These observations established the similarity of our *Nostoc* membrane fragments to chloroplasts from different species (18, 27) and cell-free preparations from green and blue-green algae (27) insofar as illumination at that temperature is concerned. It appears that, in the absence of a strong reductant such as dithionite, illumination at cryogenic temperatures produces the same effect on all membrane preparations associated with oxygenic photosynthesis: photoreduction of bound iron–sulfur center A and a concurrent photooxidation of P700.

Photoreduction at 298 K of Ferredoxin and Bound Iron–Sulfur Centers with Water as Electron Donor. Because the photoreduction of ferredoxin and centers A and B is attributed solely to photosystem I, we first set out to determine whether 715-nm illumination which, at 298 K, severely impedes electron flow from water (photosystem II activity) but fully sustains such photosystem I activity as cyclic photophosphorylation (6, 28), can also support the photoreduction of the iron–sulfur centers at a physiological temperature in the presence of ascorbate to keep P700 in a reduced form prior to illumination. Under these conditions, 715-nm light was ineffective in inducing the photoreduction of either iron–sulfur centers A and B or soluble ferredoxin (Fig. 2). The small signals (top trace) were not affected by the addition of ferredoxin (middle trace) or ferredoxin plus $NADP^+$ (bottom curve). The same negative results (not

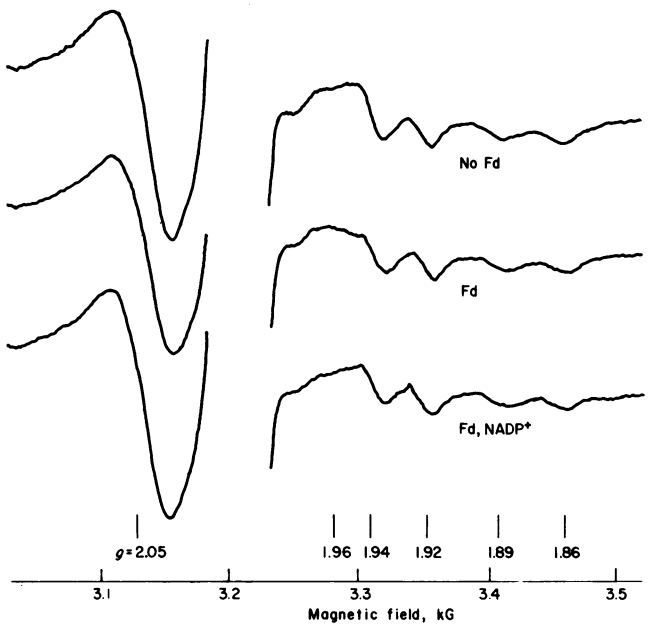

FIG. 2. Photoreduction of bound iron–sulfur centers and soluble ferredoxin in fraction C with 715-nm illumination (1.5×10^5 ergs·cm^{-2}·sec^{-1}) at 293 K. Electron donor, water. The reaction mixtures were as in Fig. 1 except that, where indicated, the following were added (per 1.0 ml): spinach chloroplast ferredoxin Fd, 10 nmol; saturating amounts of spinach ferredoxin–NADP reductase; and $NADP^+$, 2 μmol. EPR recording conditions as in Fig. 1.

shown) were obtained under 650-nm illumination when the inhibitor 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea of photosystem II was added to block the photooxidation of water.

Different results were obtained with 650-nm illumination which, in the absence of inhibitors, is known to support active electron flow from water at physiological temperatures in *Nostoc* membrane fragments (23) as well as in chloroplasts. As shown in Fig. 3 (top trace), in addition to a common resonance at g = 2.05, 650-nm illumination generated resonances at g = 2.05, 1.94, and 1.86 characteristic of reduced center A and at g = 1.92 and 1.89 characteristic of reduced center B. When soluble ferredoxin was added the resonances due to reduced centers A and B were not diminished but additional resonances at g = 1.96 and 1.89 characteristic of ferredoxin were photoinduced (middle trace). A part of the g = 1.96 resonance due to soluble ferredoxin probably accounts for the increased amplitude of the g = 1.94 resonance seen in the middle trace.

The photoreduction of ferredoxin by water in a photosynthetic system has previously been demonstrated by optical (29) but not by EPR spectroscopy. The EPR spectrum of isolated ferredoxin was determined after a chemical reduction by dithionite (7, 30, 31).

If centers A and B were acting as intermediate electron carriers between water and soluble ferredoxin, their steady-state reduction levels would be expected to decrease after the addition of ferredoxin and the decrease to be reflected in a diminution of amplitude of their EPR signals. However, no such diminution in the EPR signals of centers A and B was observed, even when the amount of added soluble ferredoxin was increased 10-fold.

To confirm the observation that the additional signals shown in Fig. 3 (middle trace) were due to the photoreduction of the added soluble ferredoxin, the same EPR tubes, with and without added ferredoxin, that gave the upper and middle traces at 20 K were scanned at 50 K, a temperature at which the EPR signals of centers A and B, but not those of soluble ferredoxin, broaden and cease to be detectable. At 50 K, the EPR spectrum of soluble ferredoxin was detected only when ferredoxin was added to the reaction mixture (compare Fig. 4 top and middle traces). The

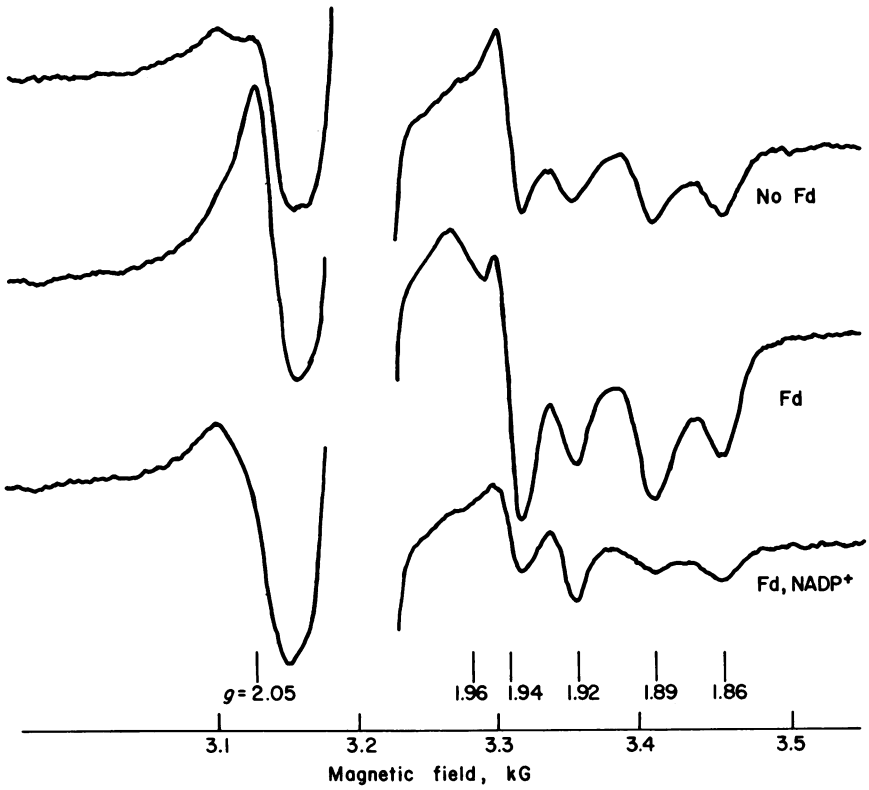

FIG. 3. Photoreduction of bound iron–sulfur centers and soluble ferredoxin in fraction C with water as electron donor and 650-nm illumination (5×10^5 ergs·cm^{-2}·sec^{-1}) at 293 K. Other experimental conditions were as in Fig. 2.

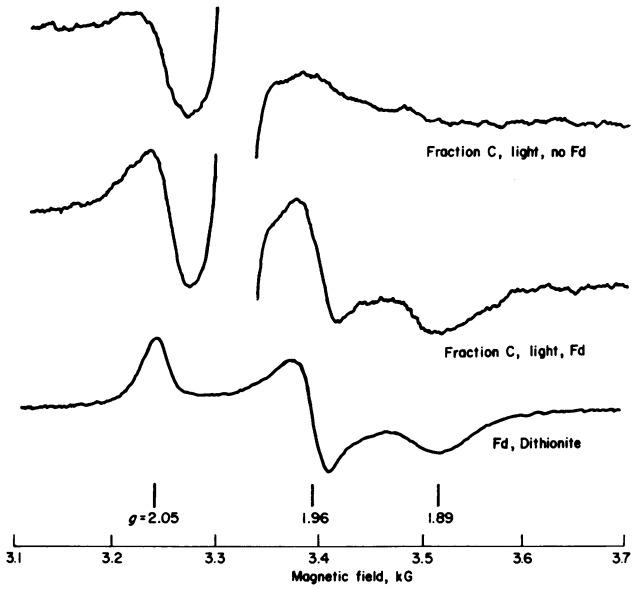

FIG. 4. Spectra of soluble ferredoxin. The top and middle spectra were recorded at a higher temperature (50 K) on the same samples that were used for the top two spectra in Fig. 3. The bottom spectrum was taken on a sample of 0.10 μM purified spinach chloroplast ferredoxin reduced with a few crystals of sodium dithionite; recording conditions were as in Fig. 1 with the exception of the amplifier gain which was 1×10^4.

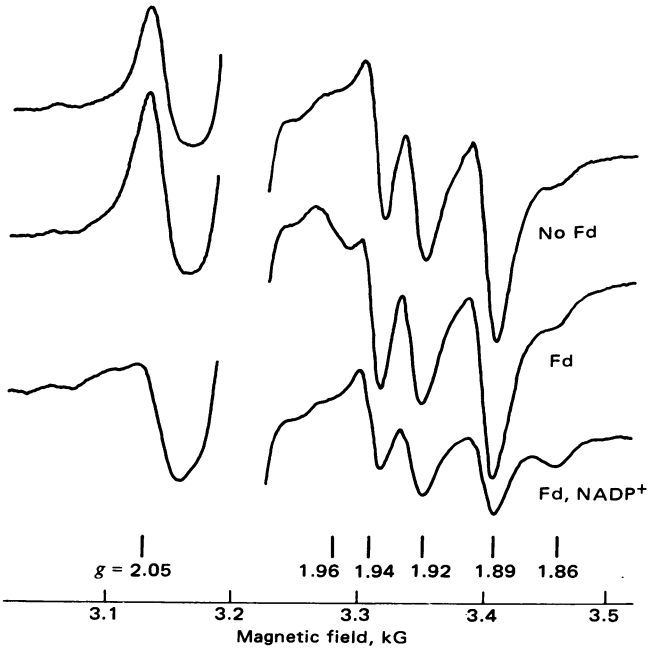

FIG. 5. Photoreduction of bound iron–sulfur centers and soluble ferredoxin in fraction C with 715-nm illumination at 293 K and $DCIPH_2$ as the electron donor. Experimental conditions were as in Fig. 2 except that 0.20 μmol of $DCIPH_2$ (per 1.0 ml) was present throughout.

EPR spectrum of the photoreduced ferredoxin (middle trace) was comparable to the EPR spectrum (also recorded at 50 K) of chemically reduced purified ferredoxin (bottom trace), included here for comparison.

The photoinduced EPR signals of centers A and B that were not diminished by the addition of soluble ferredoxin were markedly affected by the further addition of $NADP^+$ (and ferredoxin–$NADP^+$ reductase) (Fig. 3 bottom trace). The addition of ferredoxin plus $NADP^+$ diminished the EPR signals of all three iron–sulfur components, centers A and B and ferredoxin. These results provide, so far as we are aware, the first direct evidence that the photoreduction of $NADP^+$ by water affects the oxidation-reduction state of the bound iron–sulfur centers in photosynthetic membranes. An interpretation of these observations is given in the *Discussion*.

Photoreduction at 298 K of Ferredoxin and Bound Iron–Sulfur Centers with $DCIPH_2$ as Electron Donor. The *Nostoc* membrane fragments were capable of $NADP^+$ reduction at high rates with either water or an artificial electron donor, $DCIPH_2$ (23). $DCIPH_2$ is an effective electron donor for $NADP^+$ reduction by these membrane fragments at 715 nm (32); its effect on the photoreduction of bound iron–sulfur centers and ferredoxin under 715-nm illumination is shown in Fig. 5.

The pattern of photoreduction of centers A and B by $DCIPH_2$ was very different from that of photoreduction by water. As indicated by the large changes at g = 1.89 and 1.92, $DCIPH_2$ was a far more effective reductant for center B but produced a barely detectable signal at g = 1.86 identified with center A (compare top traces of Figs. 5 and 3). When soluble ferredoxin was added, the g = 1.96 signal, characteristic of soluble ferredoxin, appeared without causing a decrease in the amplitude of the center B signal (Fig. 5 middle trace).

A striking effect was observed upon the further addition of $NADP^+$. The large signals due to center B and soluble ferredoxin were greatly diminished without diminishing (seemingly even increasing) the signal at g = 1.86 indicative of center A. It appears, therefore, that photoreduction of $NADP^+$ by $DCIPH_2$ entails a photoreduction by photosystem I of center B, followed by electron transfer from center B to soluble ferredoxin and thence to $NADP^+$.

DISCUSSION

In past investigations of iron–sulfur components in chloroplasts and other photosynthetic membrane systems, optical and EPR spectrometry each had a limited sphere of applicability. Optical spectrometry has been used to measure the photoreduction of soluble ferredoxin by water (29) and by $DCIPH_2$ (33), whereas EPR spectrometry has been used to measure the photoreduction of bound iron–sulfur components, usually after illumination at cryogenic temperatures or with the aid of a strong reductant like dithionite, but it has not hitherto been used for measuring the photoreduction of soluble ferredoxin.

In this investigation, EPR spectrometry was used to measure both the photoreduction of soluble ferredoxin and the photoreduction of bound iron–sulfur centers A and B by an algal membrane preparation that contained the full complement of electron carriers needed for photosynthetic electron transport (23). All three of these iron–sulfur components were photoreduced at a physiological temperature (20°) with either water or $DCIPH_2$ as the electron donor. The simultaneous monitoring of these three components by EPR spectrometry made it possible to detect a relationship between the photoreduction of centers A and B, soluble ferredoxin, and $NADP^+$.

Insofar as current concepts of photosynthetic electron transport are concerned, a question of great interest is whether the electron-transport pathway from the bound iron–sulfur centers to ferredoxin and $NADP^+$ is the same whether water or a nonphysiological substitute, $DCIPH_2$, serves as the electron donor. According to the currently popular concept, the identity of the electron donor should make no difference, because in each case ferredoxin and $NADP^+$ are reduced by the same photosystem I. However, according to an alternative concept—

one that assigns the photoreduction of ferredoxin and $NADP^+$ by water to two photoacts that are distinct from the conventional photosystem I—the photoreduction of ferredoxin and $NADP^+$ by $DCIPH_2$ via photosystem I is an artifact that might indeed involve electron transfer steps different from those in the photoreduction of ferredoxin and $NADP^+$ by water. (For a further discussion of this point, see refs. 1 and 34.)

A comparison of Figs. 3 and 5 discloses certain similarities as well as differences among the iron–sulfur components, depending on whether water or $DCIPH_2$ served as the electron donor. The similarities were (*i*) with either electron donor the photoreduction of added soluble ferredoxin elicited signals characteristic of that protein (cf., g = 1.96) without diminishing the signals of centers A and B and (*ii*) the addition of $NADP^+$ (i.e., its photoreduction) resulted in a marked diminution of the amplitude of signals characteristic of soluble ferredoxin and center B.

$DCIPH_2$ as the electron donor differed from water in producing, upon illumination, much larger signals at g = 1.89 and 1.92, indicative of reduced center B, and a smaller signal at g = 1.86, indicative of reduced center A (Fig. 5). The decrease in the amplitude of ferredoxin and center B signals that resulted from the addition of $NADP^+$ leads to the conclusion that the photoreduction of $NADP^+$ by $DCIPH_2$ involves electron transfer via photosystem I to center B and thence to soluble ferredoxin and $NADP^+$.

A similar electron-transport pathway from center B to soluble ferredoxin and $NADP^+$ was less clear from the EPR spectra obtained when water was the electron donor (Fig. 3). Here, the photoreduction of $NADP^+$ was accompanied by a marked decrease in the amplitude of all EPR signals, those of centers A and B as well as those of soluble ferredoxin. These findings may be explained in two ways, the relative merits of which are being explored in experiments now underway. One explanation is that the photoreduction of soluble ferredoxin and $NADP^+$ by water involves electron transport through bound iron–sulfur centers A and B; the other is that the photoreduction of $NADP^+$ by water proceeds by a separate electron-transport pathway but that the formation of NADPH activates a concurrent parallel cyclic electron flow that involves one or more bound iron–sulfur centers. Recent work with chloroplasts has yielded evidence for a cyclic electron flow (and photophosphorylation) that is regulated by NADPH and that can operate concurrently with a noncyclic electron flow from water to $NADP^+$ (28, 35, 36).

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