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CORRELATION OF REACTION-CENTER CHLOROPHYLL (*P*-700) OXIDATION AND BOUND IRON-SULFUR PROTEIN PHOTOREDUCTION IN CHLOROPLAST PHOTOSYSTEM I AT LOW TEMPERATURES

ALAN J. BEARDEN[a] and RICHARD MALKIN[b]

[a]*Donner Laboratory and* [b]*Department of Cell Physiology, University of California, Berkeley, Calif. 94720 (U.S.A.)*

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SUMMARY

The extent of *P*-700 photooxidation at 18 °K has been followed in three different chloroplast preparations (unfractionated chloroplasts and two preparations enriched in Photosystem I). More than 90 % of $P\text{-}700^+$ formation in all preparations was eliminated by the addition of sodium dithionite at pH 10. Photoreduction of a bound chloroplast iron-sulfur protein was also decreased by at least 90 % under similar conditions. Electron paramagnetic resonance spectra of the chloroplast preparations in the presence of dithionite showed chemical reduction of bound iron-sulfur protein under conditions where primary photochemistry is eliminated. These results indicate that *P*-700 photooxidation is concomitant with photoreduction of a bound iron-sulfur protein and that this iron-sulfur protein functions as the primary electron acceptor of Photosystem I.

INTRODUCTION

The photoreduction of a bound chloroplast iron-sulfur protein at cryogenic temperatures was first observed in 1971 [1]. Subsequent work, principally with chloroplast fragments enriched in Photosystem I, led to the proposal that a bound iron-sulfur protein is the primary electron acceptor of Photosystem I and that it accepts electrons from *P*-700, the reaction-center chlorophyll of Photosystem I [2–4]. Independently, Hiyama and Ke [5] identified by flash kinetic optical spectroscopy a component, designated *P*-430, that displayed properties consistent with its assignment as the primary electron acceptor of Photosystem I. A later study [6] indicated that *P*-430 and the bound iron-sulfur protein are probably the same chloroplast component. Recent reviews have summarized current thought concerning the primary electron acceptor of Photosystem I [7, 8].

An important finding in the assignment of the photoreducible iron-sulfur protein as the primary electron acceptor of Photosystem I is the observed stoichiometry between photoreduced iron-sulfur protein and photooxidized reaction-center

chlorophyll (*P*-700). Quantitative electron paramagnetic resonance (EPR) spectroscopic estimation of the number of photoreduced iron-sulfur protein molecules and photooxidized *P*-700 molecules in chloroplasts and Photosystem I subchloroplast fragments showed a 1 : 1 correspondence after illumination and measurement at 25 °K under conditions where the photoinduced charge separation is irreversible [4]. More recently, investigators studying the reversibility of the primary reaction of Photosystem I at cryogenic temperatures have attempted by other methods to verify the relationship between *P*-700 and the bound iron-sulfur protein. Bearden and Malkin [9], Visser et al. [10] and Ke et al. [11] have found that the decay of $P\text{-}700^+$ can be correlated with that of the reduced bound iron-sulfur protein at temperatures ranging from 10 °K to 225 °K. This finding supports the assignment of these two components as a donor-acceptor couple. However, McIntosh et al. [12] and Evans and Cammack [13] reported different decay kinetics for $P\text{-}700^+$ and the reduced bound iron-sulfur protein and suggested that the bound iron-sulfur protein functions only as a secondary electron acceptor in Photosystem I, by accepting electrons from an unknown primary electron acceptor.

Because a correlation of the kinetic response of *P*-700 and bound iron-sulfur protein is an important feature in the characterization of these components as the donor-acceptor pair in Photosystem I, we have examined the photoresponses of the two components under conditions where *P*-700 is reduced and the iron-sulfur protein is either chemically oxidized or chemically reduced prior to low-temperature illumination. Our results indicate that more than 90 % of the *P*-700 photooxidation observed at 18 °K in three different preparations is linked to the photoreduction of the bound iron-sulfur protein, a result that supports the previous assignment of these chloroplast components as the primary photochemical reactants of Photosystem I.

MATERIALS AND METHODS

Broken spinach chloroplast fragments were prepared as previously described [14]. Subchloroplast fragments enriched in the Photosystem I reaction center were prepared by the digitonin procedure of Anderson and Boardman [15] and by the dodecyldimethylamine oxide procedure of Malkin [16]. Just prior to use, the fragments were suspended in 0.1 M glycine buffer at pH 10. This pH is necessary for reproducible chemical reduction of the bound iron-sulfur proteins with sodium dithionite. Control experiments showed that the extent of *P*-700 photooxidation at 18 °K at pH 10 was approximately 80 % of that observed at pH 7 under similar conditions.

EPR spectra were recorded at 18 °K with an X-band JEOL EPR spectrometer incorporating a liquid helium cooling system, as previously described [4, 14]. The kinetic response (0.1 s time constant) of $P\text{-}700^+$ formation was monitored at a magnetic field setting corresponding to the positive peak of the usual first-derivative $P\text{-}700^+$ free-radical EPR spectrum with a g value of 2.0026 and a linewidth of 7.5 G (ΔH_{pp}); 100 kHz field modulation amplitudes were in the range of 2–5 G. With 5 G modulation amplitude (5 G in the rotating-frame coordinate system), under our conditions a kinetic response samples about 20 % of the spins contributing to the total $P\text{-}700^+$ signal. Measurement of the kinetic response of the reduced bound iron-sulfur protein signal is far more difficult because this signal extends over a magnetic

field range of about 350 G for spectra at X-band (9.22 GHz) while only 20 G modulation amplitude is available in the rotating-frame coordinate system. Therefore, for the iron-sulfur protein the kinetic response samples less than 1 % of the total spins contributing to the EPR signal, even when the magnetic field setting for kinetic experiments is optimized at the positive peak of the $g = 1.94$ resonance line. It is also important to note that, in addition to the small number of spins sampled, the usual EPR bridge and reflection cavity spectrometer arrangement used for the high-sensitivity EPR spectroscopy [17, 18], where the microwave absorption caused by the paramagnetic species is monitored by a semiconductor diode at room temperature, make it difficult to sense spin concentration changes corresponding to less than 5 % of the total spins affected in our experiments with these iron-sulfur proteins. This is because of the presence of thermal noise components, mostly from the diode microwave intensity detector. Selection of a detector with lowest noise was made from a number of Schottky-barrier silicon diodes manufactured by Microwave Associates (MA40075); independent measurements of other noise-producing spectrometer components indicated that klystron frequency modulation noise in the 100 kHz sidebands was the next most important noise source: a low-noise model VA-265 klystron (Varian Associates, Palo Alto, California) was selected for these experiments.

Samples were illuminated in the JEOL EPR spectrometer TE_{011} cylindrical mode microwave cavity ($Q_{\text{loaded}} = 5000$) at 18 °K with saturating 645 nm wavelength monochromatic light (10 nm full width at half-maximum). The light intensity incident on the sample was $5 \cdot 10^5$ ergs/cm^2 per s.

Digitonin and phenazine methosulfate were purchased from the Sigma Chemical Company; dodecyldimethylamine oxide (30 % solution of Ammonyx-Lo) was a gift from the Onyx Chemical Company.

RESULTS

The primary photochemistry of Photosystem I at 18 °K was monitored by following the formation of the EPR "Signal I" that originates from $P\text{-}700^+$ [g value, 2.0026; linewidth, 7.5 G (ΔH_{pp})] (see refs. 4, 19 and 20). As shown in Fig. 1, in unfractionated chloroplast fragments (P-700/chlorophyll = 1 : 400) which have been preincubated with sodium ascorbate plus phenazine methosulfate prior to freezing, there is a large P-700 photooxidation that is irreversible after the cessation of illumination when the temperature of the sample is held at 18 °K. If the extent of the reaction was measured on a sample as isolated, without the addition of ascorbate plus phenazine methosulfate, only about one-third as much P-700 photooxidation was observed, an indication that about 60 % of the P-700 was oxidized prior to illumination. In samples allowed to incubate for about 1 min before freezing with sodium dithionite (5 μmol), at least 90 % of the P-700 oxidation was eliminated. As shown in the traces of Fig. 1, approximately half of the residual $P\text{-}700^+$ formed in the light in the presence of dithionite is reversible.

Similar results were obtained with a digitonin subchloroplast preparation (D-144; P-700/chlorophyll = 1 : 150) (Fig. 2). In the presence of ascorbate plus phenazine methosulfate the magnitude of P-700 photooxidation is approximately three times as large as that observed with no additions. In the samples incubated with sodium dithionite, a small P-700 photooxidation (about 5 % of that observed in the

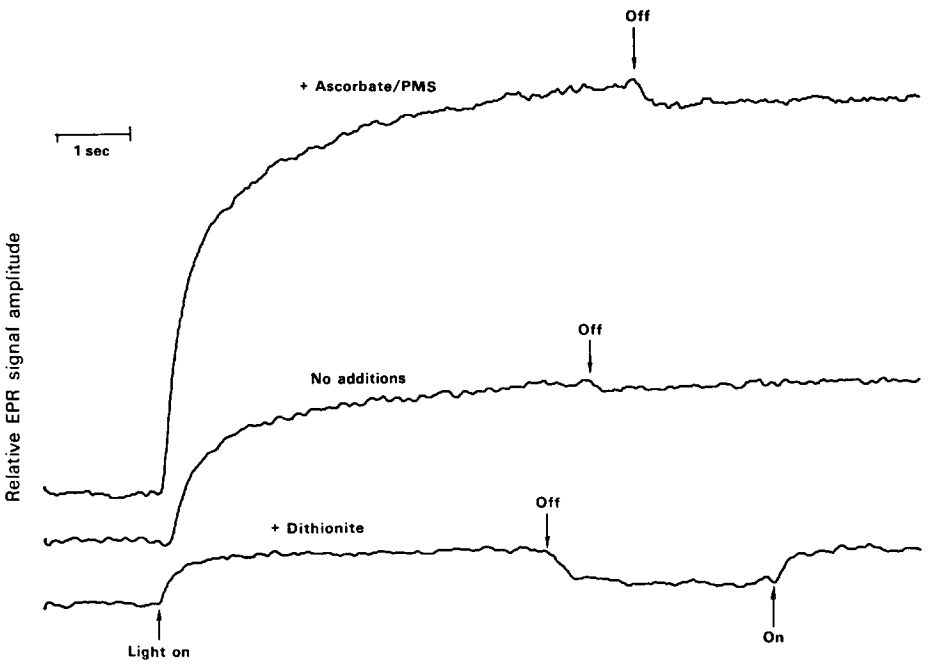

Fig. 1. Kinetics of $P\text{-}700^+$ formation at 18 °K in chloroplasts. The reaction mixture contained unfractionated chloroplast fragments (1.0 mg chlorophyll per ml), 0.1 M glycine buffer (pH 10) and, where indicated, sodium ascorbate (10 mM) and phenazine methosulfate (PMS) (20 μM). A small amount of solid sodium dithionite ($\sim$ 5 μmol) or 5 μmol of a solution of freshly prepared sodium dithionite (in 0.5 M Tris · HCl buffer, pH 8.8) was added under a stream of nitrogen gas. Samples were incubated in the dark for 1 min at 4 °C prior to freezing. The $P\text{-}700^+$ response was monitored at the low-field positive peak of the first-derivative absorption-mode free radical EPR signal at 18 °K with the following spectrometer conditions: frequency, 9.220 GHz; microwave power, 2 mW (to the TE_{011} cavity); cavity Q(loaded), $\sim$5000; modulation amplitude (rotating frame), 5 G; microwave detector current (Microwave Associates MA-40075 diode), 0.2 mA. All traces were recorded at the same amplifier gain of $120 \cdot 10^3$. The power saturation properties of $P\text{-}700^+$ were identical in all traces; under these spectrometer conditions, $P\text{-}700^+$ displays less than 20 % deviation from linearity.

presence of ascorbate plus phenazine methosulfate) was observed, and approximately half of this change was reversible.

In a third, more highly enriched, Photosystem I preparation (dodecyldimethylamine oxide Photosystem I subchloroplast fragments; $P\text{-}700$/chlorophyll = 1 : 40), the results on $P\text{-}700$ photooxidation are similar to those observed with the less enriched preparations. In the dodecyldimethylamine oxide preparation, incubation with ascorbate alone prior to freezing is sufficient to poise the system in a photochemically active state (see ref. 16). A large photooxidation of $P\text{-}700$ is observed under these conditions (Fig. 3), whereas in the absence of ascorbate the extent of the change is decreased by about 50 %. The addition of sodium dithionite prior to freezing results in the elimination of about 95 % of the $P\text{-}700^+$ formation in this preparation, and there is a small residual reversible change under these conditions.

In all three of the above-described preparations, the formation of $P\text{-}700^+$ in the presence of ascorbate or ascorbate plus phenazine methosulfate is accompanied by

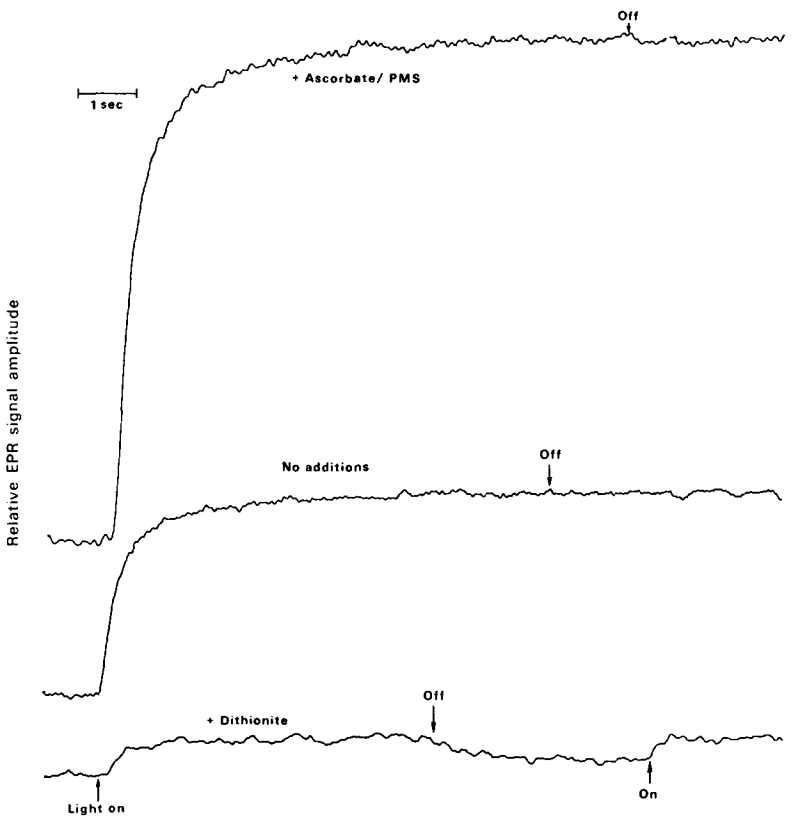

Fig. 2. Kinetics of $P\text{-}700^+$ formation at 18 °K in D-144 Photosystem I subchloroplast fragments. The conditions were the same as in Fig. 1 except that the D-144 fragments were present at a concentration of 0.2 mg of chlorophyll per ml. All traces were recorded at the same amplifier gain of $100 \cdot 10^3$.

the photoreduction of a bound iron-sulfur protein. The experiment with unfractionated chloroplast fragments (Fig. 4) shows that in the light minus dark difference spectrum an iron-sulfur protein with observed g-values of 2.05, 1.94 and 1.86 is photoreduced at 18 °K. This photoreduction has been described in detail in our previous work [1, 2, 4, 16] and in reports from other laboratories [3, 6, 10, 11]. In the presence of dithionite (Fig. 4), only a small residual light minus dark iron-sulfur signal is present, and an accurate estimation of the amount of residual light-induced change is difficult because of the noise level in this experiment. It appears that in this preparation, as well as in the two more enriched preparations, at least 90 % of the light-induced iron-sulfur signal is eliminated by the addition of dithionite.

The EPR spectra of the dithionite-treated samples prior to illumination at 18 °K are shown in Fig. 5. These spectra are characterized by five observed EPR spectral features that have been assigned to the g values of two different iron-sulfur components. One center has g values of 2.05, 1.94 and 1.86 and is the protein previously referred to that has been demonstrated to undergo low-temperature photoreduction (see Fig. 4). The second component has observed g values of 2.05, 1.92 and

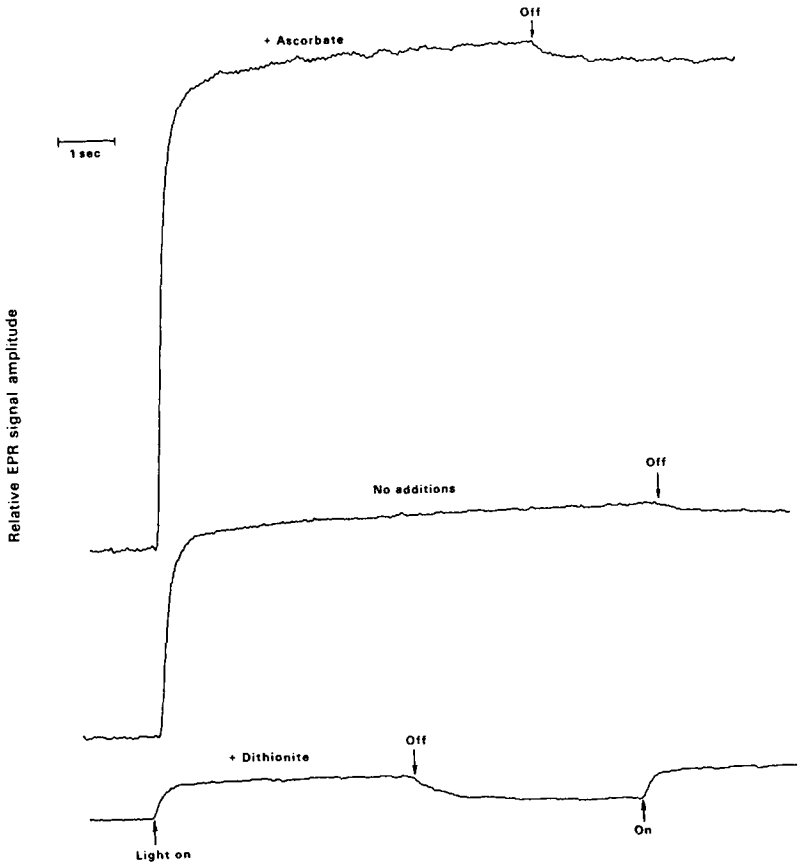

Fig. 3. Kinetics of $P\text{-}700^+$ formation at 18 °K in dodecyldimethylamine oxide Photosystem I subchloroplast fragments. The conditions were as in Fig. 1 except that the dodecyldimethylamine oxide fragments were present at a concentration of 0.12 mg of chlorophyll per ml. All traces were recorded at the same amplifier gain of $100 \cdot 10^3$.

1.89. The oxidation-reduction properties of these two reduced iron-sulfur centers have been shown to be quite distinct: One center (g values of 2.05, 1.94 and 1.86) has a half-reduction potential (E_m) of about −530 mV at pH 10, and the second center (g values of 2.05, 1.92 and 1.89) has an E_m of about −600 mV at pH 10 [21, 22]. Based on the relative intensity of the resonance lines at $g = 1.86$ and 1.89, it appears that the center with $E_m = -530$ mV is almost fully reduced in the dark in all three preparations and that the extent of reduction of the second center is less complete and shows more variation (cf. refs. 21 and 22). Because the loss of photochemical activity in all of these preparations is similar, even though the extent of reduction of the center with $E_m = -600$ mV is variable, it appears that both P-700 oxidation and bound iron-sulfur protein photoreduction decrease as the center with $E_m = -530$ mV becomes chemically reduced. A small photoreduction of the second center has also been observed in the presence of dithionite after illumination at 18 °K (see Fig. 4), but the extent of this reaction is almost negligible when compared with the photo-

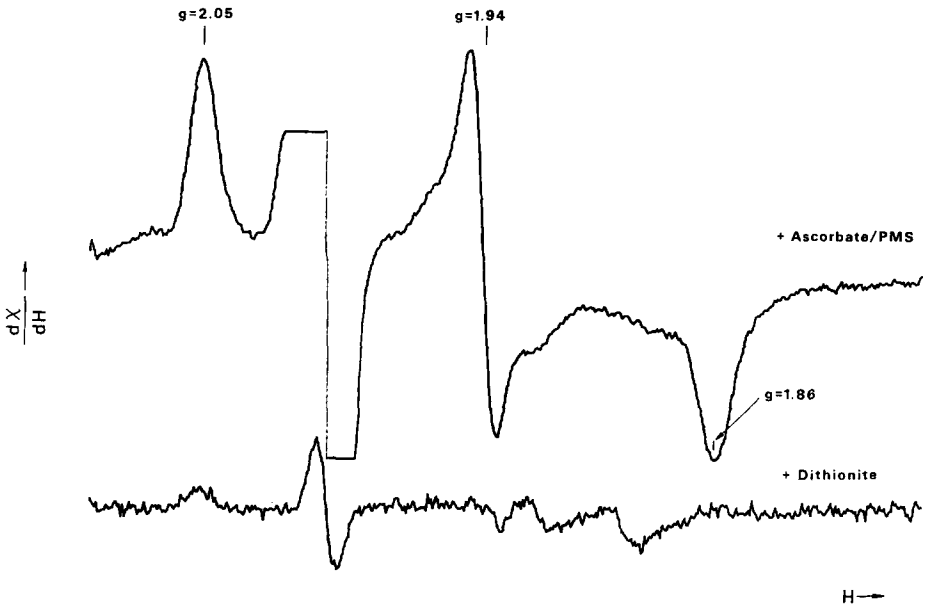

Fig. 4. Photoreduction of bound iron-sulfur protein at 18 °K in chloroplasts. The reaction mixture was as in Fig. 1, the light minus dark difference spectra were obtained by computer subtraction of the dark spectrum from the spectrum obtained after illumination in the presence of either ascorbate plus phenazine methosulfate (PMS) or dithionite (see ref. 4). EPR conditions: frequency, 9.220 GHz; microwave power, 10 mW; modulation amplitude, 10 G.

reduction of the center with $E_m = -530$ mV in the absence of dithionite; the significance of this small photoreduction in relation to the Photosystem I primary reaction is not clear.

DISCUSSION

We have attempted to verify the proposed role of a bound chloroplast iron-sulfur protein as the Photosystem I primary electron acceptor. Consistent with this view, the primary photochemistry of Photosystem I, as determined by the extent of *P*-700 photooxidation at low temperature, would be lost when the bound iron-sulfur protein was chemically reduced. However, if an electron acceptor functions prior to the bound iron-sulfur protein, chemical reduction of the iron-sulfur protein would not be expected to affect significantly the extent of low-temperature *P*-700 photooxidation. Our results indicate that the loss of Photosystem I primary photochemistry at 18 °K in the presence of dithionite in the three different preparations tested can be correlated with the chemical reduction of one bound iron-sulfur protein before freezing. We found that over 90 % of the *P*-700 photooxidation in photochemically active preparations correlated with the photoreduction of a bound iron-sulfur protein and that prior chemical reduction of this iron-sulfur protein resulted in a concomitant loss of *P*-700 photooxidation. These results differ from those of Evans and Cammack [13], who compared only the extent of *P*-700 photooxidation in the presence of dithionite with the extent of *P*-700 oxidation with no additions. With all of our preparations it

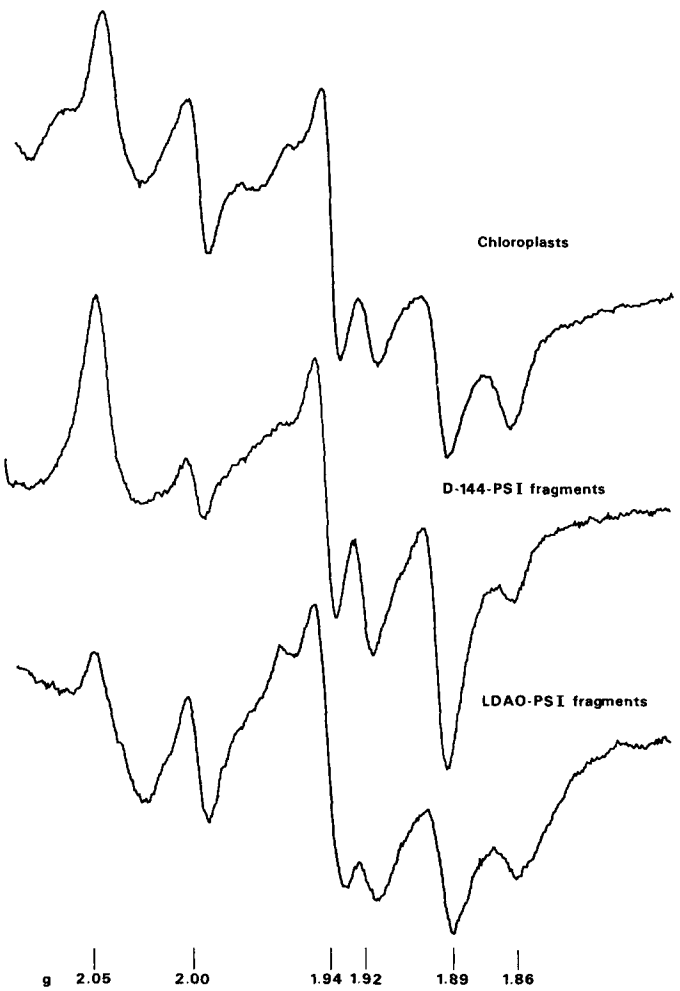

Fig. 5. EPR spectra at 18 °K of dark-adapted dithionite-reduced chloroplast and Photosystem I (PS I) preparations. The reaction mixture was as in Fig. 1 except for the following concentrations: chloroplasts, 1.0 mg of chlorophyll per ml; D-144 fragments, 0.5 mg of chlorophyll per ml; dodecyldimethylamine oxide (LDAO) fragments, 0.25 mg of chlorophyll per ml. EPR conditions were as described for Fig. 4. Amplifier gain settings for the upper two spectra were $250 \cdot 10^3$; the lower spectrum was recorded at a gain of $100 \cdot 10^3$.

was necessary to incubate samples with a mild reductant (ascorbate) to obtain a fully active Photosystem I since a large percentage of the *P*-700 was found to be in the oxidized form even in the dark-adapted samples with no additions.

McIntosh et al. [12] have recently reported the observation in Photosystem I subchloroplast fragments, prepared by Triton treatment, of a small (1–5%) reversibility of the *P*-700 change at 6 °K. According to these workers, this reversible *P*-700 change did not correlate with any changes in the oxidation-reduction state of the bound iron-sulfur proteins under comparable conditions. It should be re-emphasized

that, because of the nature of the EPR signal associated with the reduced form of iron-sulfur proteins and the limited modulation amplitudes that can be applied to samples at cryogenic temperatures, the detection of small (5–10 %) changes in the concentration of reduced iron-sulfur centers is improbable. This problem therefore precludes the correlation of a reversible iron-sulfur signal with the small reversible *P*-700 change (about 5 %) that we observed in the presence of dithionite in all of our preparations, and this problem could have prevented a similar detection by other workers who concluded that there is not a kinetic correlation between *P*-700 and the iron-sulfur protein [12, 13]. In nonbiological systems [23] and in experiments with cytochrome *c* [24] it has been possible to increase the sensitivity of the EPR spectrometer by using "rapid passage" techniques in place of the usual methods; the application of a variety of these techniques to the EPR spectroscopy of the bound iron-sulfur protein did not improve the signal-to-noise ratio for the kinetic measurements reported herein.

McIntosh et al. [12] and Evans et al. [25] have also reported the detection of an EPR signal in Photosystem I chloroplast fragments at low temperatures with *g* values of 2.06, 1.86 and 1.78. These workers propose that this species is the primary electron acceptor of Photosystem I. We have been unable to confirm by EPR measurements, under conditions similar to those reported in ref. 25, the existence of this species in any of the preparations used in our studies. In addition, it has been reported [25] that this new signal can be observed only in the presence of dithionite, methyl viologen and light. According to Evans et al. [25], little or none of the signal is observed in samples treated only with dithionite. Thus, one would expect the addition of dithionite to have no effect on the extent of *P*-700 photooxidation if this new component functions as the primary electron acceptor of Photosystem I. The results presented in this work clearly do not agree with those predictions and indicate that the primary electron acceptor of Photosystem I can be reduced by dithionite.

To summarize, it is our conclusion, based on the observation that, at pH 10, at least 90 % of $P\text{-}700^+$ formation can be correlated with bound iron sulfur protein ($E_m = -530$ mV) photoreduction and the observation that chemical reduction of this iron-sulfur protein prevents $P\text{-}700^+$ formation, that this iron-sulfur protein serves as the primary electron acceptor of Photosystem I. Our findings on the role of this iron-sulfur protein are also in agreement with recent measurements of the extent of *P*-700 photooxidation at low temperatures as a function of oxidation-reduction potential established prior to freezing [26, 27]. These studies revealed a half-reduction potential of about −530 mV, a value which is consistent with the proposed role of this iron-sulfur protein as the primary electron acceptor of Photosystem I.

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