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QUANTITATIVE EPR STUDIES OF THE PRIMARY REACTION OF PHOTOSYSTEM I IN CHLOROPLASTS

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SUMMARY

Quantitative electron paramagnetic resonance studies of the primary event associated with Photosystem I in chloroplasts have been carried out at 25 °K. After illumination of either whole chloroplasts or Photosystem I subchloroplast fragments (D-144) with 715-nm actinic light at 25 °K, equal spin concentrations of oxidized P700 and reduced bound iron–sulfur protein (bound ferredoxin) have been measured. Quantitative determination of the concentration of these two carriers by EPR spectroscopy after illumination at low temperature indicates that Photosystem I fragments are enriched in P700 and the bound iron–sulfur protein as compared with unfractionated chloroplasts. These results indicate that P700 and the bound iron–sulfur protein function as the donor–acceptor complex of chloroplast Photosystem I.

INTRODUCTION

Although many of the photoreactions of the electron carriers in the chloroplast photosynthetic electron transport chain have been studied in great detail, the nature of the primary reactants, *i.e.* the chemical species formed directly as a result of photon capture, is less well understood. In Photosystem I, P700, a specialized form of chlorophyll *a*, has characteristics compatible with a role as the primary electron donor[1,2], while in Photosystem II a new component, C550, has been suggested to be the primary electron acceptor[3,4].

In previous communications[5,6] we reported the discovery of a new electron carrier involved in primary chloroplast photosynthetic reactions. This carrier was identified on the basis of its low-temperature electron paramagnetic resonance (EPR) spectrum and has been referred to as a bound iron–sulfur protein, or bound ferredoxin, to distinguish it from the previously characterized soluble ferredoxin[7]. The existence of this new bound iron–sulfur protein has recently been confirmed in other laboratories[8–10].

The bound iron–sulfur protein in chloroplasts can be photoreduced at a temperature where chemical reactions do not commonly occur (77 °K or 25 °K), a finding

Abbreviations: DPPH, 2,2′-diphenyl-1-picrylhydrazyl; TEMPOL, 2,2,6,6-tetramethyl-4-hydroxypiperidine.

which suggested the association of this carrier with a primary photochemical reaction[5]. Further studies[6] showed this carrier could be photoreduced as effectively with far-red light ($\lambda > 700$ nm), associated with Photosystem I, as with red light. In addition, the bound iron–sulfur protein was found in subchloroplast fragments enriched in Photosystem I (refs 6, 10). These findings led to the proposal[6] that this bound iron–sulfur protein serves as the primary electron acceptor of Photosystem I in chloroplast photosynthesis.

In this communication we report quantitative EPR studies of the primary event associated with Photosystem I in chloroplasts. We have measured the concentrations of the presumed electron donor (P700) and acceptor (the bound iron–sulfur protein) of Photosystem I by EPR spectroscopy after illumination of chloroplast preparations at 25 °K with far-red light. If these two components function as donor–acceptor pair, the charge separation produced by each absorbed photon would yield two unpaired electrons, one associated with P700 and the second with bound ferredoxin. Our findings are discussed in terms of the nature of the primary electron donor and acceptor of Photosystem I in chloroplasts.

MATERIALS AND METHODS

Materials

Whole chloroplasts were prepared from greenhouse spinach. The leaves were harvested 1–2 h before each experiment, washed with distilled water, and stored at 4 °C to retain their turgidity. Approx. 25 g of leaves were freed of midribs and disrupted in a Waring blendor for approx. 15 s (at 4 °C) in 75 ml of the following solution: 0.3 M sucrose, 50 mM Tris–HCl buffer (pH 7.8), 10 mM NaCl, 10 mM sodium ascorbate, 1 mM sodium-EDTA (pH 7.8), and 2 mg/ml bovine serum albumin. The slurry was squeezed through four layers of filtering silk and the filtrate centrifuged at $2500 \times g$ for 1 min. The precipitate, which contained whole chloroplasts, was resuspended in a minimum volume of the blending solution immediately before use to give a final chlorophyll concentration of 3–5 mM. The chloroplasts were used within 1 h of preparation.

Subchloroplast fragments enriched in Photosystem I were prepared by the digitonin procedure described by Hauska *et al.*[11]. The $144000 \times g$ precipitate (D-144) was suspended in a solution containing 0.3 M sucrose, 50 mM Tris–HCl buffer (pH 7.8), 10 mM NaCl, and 2 mM sodium-EDTA (pH 7.8). The final chlorophyll concentration was 3–4 mM. The fragments were stored at −20 °C prior to use.

Total chlorophyll concentrations and chlorophyll *a*:*b* ratios were determined by the method of Arnon[12].

Digitonin and crystalline bovine serum albumin were purchased from the Sigma Chemical Company. 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Eastman Organic Chemicals and potassium nitrosodisulfonate from Alfa Inorganics. 2,2,6,6-Tetramethyl-4-hydroxypiperidine (TEMPOL) was a gift of Professor A. D. Keith.

EPR techniques

(1) *Instrumentation.* X-band EPR spectra were recorded using a modified JEOL ME-1X spectrometer with a TE_{011}-mode cylindrical cavity operating at a

frequency of 9.26 GHz. First-derivative spectra were recorded using 100 kHz modulation of the magnetic field and phase-sensitive detection at that frequency. A 3-dB improvement in signal-to-noise ratio, as measured by observations on "weak pitch", was obtained by substituting a "hot-carrier" diode (Hewlett–Packard 5082-2757) for the 1N23F detector diode and by selecting low-noise transistors (2N929, especially selected for favorable noise characteristics) in the input stages of the 100-kHz preamplifier. g-values were measured as previously described[5].

Samples in standard X-band quartz EPR tubes (3 mm internal diameter) were cooled to 25 °K by a stream of ^{4}He gas boiled in a storage dewar and passed through a quartz dewar assembly placed in the cavity. The sample temperature was controlled to within ± 1 °K at 25 °K by the use of a carbon resistance thermometer and by the control of power to the boiloff resistor in the liquid-helium storage dewar.

(2) *Microwave power, modulation amplitude, and amplifier settings.* In performing quantitative EPR measurements it is important that the lineshape not be perturbed by overly large microwave power (saturation effect) or by use of a modulation amplitude that is too large (modulation broadening). Since the EPR signal of P700 was found to be easily saturated at 25 °K, auxiliary power-saturation studies were carried out at this temperature. To overcome the variation in the detection diode characteristic ("linear" *vs* "square-law"), saturation studies were done by introducing a mechanical microwave attenuator in the diode arm of the microwave bridge and keeping the power incident on the diode constant while the microwave power to the cavity was increased. Microwave power to the cavity was monitored by a −20-dB directional coupler and a Hewlett–Packard 432-A microwave power meter. The free-radical signal associated with P700 in whole chloroplasts and D-144 subchloroplast fragments had slightly different power-saturation properties at 25 °K; 0.1 mW is a suitable nonsaturating power for studies with whole chloroplasts but less power, 0.02 mW, was necessary in the experiments with D-144 subchloroplast fragments. The bound ferredoxin signal is less susceptible to power saturation than is the P700 signal; a power of 15 mW (to the cavity) was used in all cases as no difference in saturation properties could be seen between the bound ferredoxin signal in whole chloroplasts and in subchloroplast fragments.

To preserve accurate lineshapes and to permit accurate quantitation of the first-derivative EPR signals, the modulation amplitude must be small compared to the linewidth. Preliminary measurements and comparison to Gaussian and Lorenztian lineshapes showed that the P700 radical had a Gaussian lineshape, well-fitted by a peak-to-peak linewidth of 8.0 gauss. When compared to a simulated spectrum in which the computer program inputs are the three g-values, the microwave frequency, the field settings of the spectrometer, and the linewidth, the signal of the bound ferredoxin is best fitted with a peak-to-peak linewidth of 20 gauss. Therefore, a modulation amplitude of 2.0 gauss was chosen for the quantitative EPR studies. The spectra of the P700 radical taken with modulation amplitudes of 1.0 or 2.0 gauss could be superimposed after scaling to compensate for the increased signal due to the larger modulation amplitude. The use of the same modulation amplitude for both the P700 radical and the signal from the reduced bound ferredoxin removed the necessity for corrections due to differing modulation amplitudes.

The stability of the preamplifier in the 100-kHz channel, the accuracy of the

gain controls (a variable attenuator placed between the preamplifier and the main amplifier), and the linearity of all components of the 100-kHz channel from the detector to the analog-to-digital converter of the computer were checked and found to be well within 1 % in accuracy and resetability. A 2^9-bit resolution analog-to-digital conversion with 500 data points per 25 s scan (for the signals of both P700 and the bound ferredoxin) allowed "signal-averaging" for 10 to 50 scans. Digitized spectral data could be numerically integrated, heights measured, scaling done, spectra subtracted from or added to one another, and data plotted or stored for retrieval in magnetic tape cassettes. A versatile program written by Mr H. Holmes provided this important system, without which this quantitation experiment would have been nearly impossible.

(3) *Use of standards.* Two different types of EPR standards were used: a primary set of standards for determinations of abolute spin concentrations and g-values and an internal secondary standard for comparison of the number of P700 spins with the number of bound ferredoxin spins. For determination of spin concentrations, "weak pitch", $3.3 \cdot 10^{-4}$ % pitch in KCl (Varian Associates, Palo Alto) and DPPH were employed. Weak pitch, as supplied by the manufacturer, contains 10^{13} spins per cm of length ($\pm$ 15 %). The vertical-axis sensitivity of the cylindrical microwave cavity was mapped with a small single crystal of DPPH; then, by using the relative vertical-axis sensitivity, the effective number of spins ($1.0 \cdot 10^{13}$) presented by the standard could be determined. The spin concentration with DPPH in benzene solution was calculated on the basis of the extinction coefficient at 525 nm (11.9 $mM^{-1} \cdot cm^{-1}$, ref. 13) and by the use of double-numerical integration.

Potassium nitrosodisulfonate and TEMPOL were used to calculate the g-value for the light-induced P700 free-radical EPR signal. Potassium nitrosodisulfonate dissolved in a saturated solution of sodium carbonate has a g-value of 2.0057 (ref. 14) and a hyperfine splitting of 13.0 gauss; TEMPOL in n-butanol has a g-value of 2.0059 and a hyperfine splitting of 16.1 gauss (ref. 15).

The placement of an aligned spherical single-crystal ruby (2 mm diameter) near the cavity wall and the observation of the Cr(III) EPR signals from this room-temperature standard[16–18] were used to relate the spin concentrations in P700 to bound ferredoxin and to relate either of these spin concentrations to the primary standards (weak pitch and DPPH). When the c-axis of a magnetically dilute ruby is aligned, by optical means, parallel to the steady magnetic field axis of the spectrometer (H_0), resonances occur at low and high fields, with the region from 2000 to 4000 gauss free of absorption. A resonance at 4300 gauss displayed Gaussian lineshape with a peak-to-peak linewidth of 36 gauss; no ruby resonances interfered with signals produced in the g-regions of interest. The 4300 gauss ruby resonance has the advantage that it may be recorded under the same instrumental conditions as those of the chloroplast samples simply by changing the intensity of the magnetic field. The ruby signal will then reflect any changes in instrumental conditions, *i.e.* detector nonlinearity, cavity Q filling factor, power, *etc.* The advantage of this type of standard, always in place, is readily apparent in these experiments where two different EPR signals are to be compared, each signal having different EPR-instrumentation requirements: microwave power, amplifier gain, field scan. Use of the ruby standard in the sequence of measurements will be discussed more fully below.

(4) *Experimental procedure.* In a typical experimental run, nine different EPR

spectra are recorded in sequence, all at 25 °K and all with the ruby and the sample tube containing either whole chloroplasts or subchloroplast fragments in place. Five spectra are of the ruby standard under experimental conditions identical with the conditions of measurement for (1) P700 and (2) bound ferredoxin signals, respectively.

The ruby standard is run (Run 1) at the settings (power, amplifier gain, modulation, field scan) used subsequently for the ferredoxin measurement. In addition, a ruby spectrum at an indicated modulation amplitude of 20 gauss (the actual 100-kHz modulation amplitude at the ruby position is less than 20 gauss by a factor (approx. 4) dependent only on the cavity geometry) (Run 2) is also taken. Run 3 is a record of the region of the bound ferredoxin signal prior to illumination. The only variables between Runs 1 and 3 are the magnetic field setting and the amplifier gains; the modulation and power are kept constant. Next, the field scan is changed to a narrower region ($\pm$ 50 gauss) to display the P700 signal, the power is reduced to the level necessary to avoid power saturation of the P700 signal, and the dark (Run 4) and light (Run 5) data are collected. Run 6 involves returning the magnetic field intensity to the ruby value and changing the modulation amplitude to 20 gauss. This higher modulation is required to observe the ruby signal at the lower power required for the P700 measurement. Run 7, the light-bound ferredoxin run, involves resetting the magnetic field intensity to the ferredoxin region (resetting accuracy about 0.5 gauss) and then bringing the microwave power back to 15 mW, a level which can be monitored to $\pm$ 1 % with the power meter. Runs 8 and 9 are repeats of Runs 2 and 1 as a check on instrument drifts during the course of the experiment. As each run is "signal- averaged" for 10–50 scans, the entire experimental procedure requires about 2 h. The order of the runs is carefully chosen so that "reset" operations are minimized and those that are required may be done with the desired accuracy. To standardize the illumination procedure[6], 1 min of 715 nm illumination (saturating light of intensity $2 \cdot 10^{-4}$ ergs $cm^{-2}s^{-1}$) was provided immediately before each of the "light" runs; as the decay of the photoinduced signals is of the order of several hours, the signal level may be considered constant during the runs. Each run is made under optimal conditions of amplifier gain to maximize the signal-to-noise ratio and to fit the "window" of the analog-to-digital conversion. The amplifier gain factors were carefully calibrated to within $\pm$ 1 %. Runs 1, 2, 8, and 9 provide a measure of the constancy of conditions during the experiment; those runs agreed to within 2 % of each other. This procedure allows use of the ruby standard runs to correct for any spectrometer adjustment necessary to the running of the P700 or bound ferredoxin spectra.

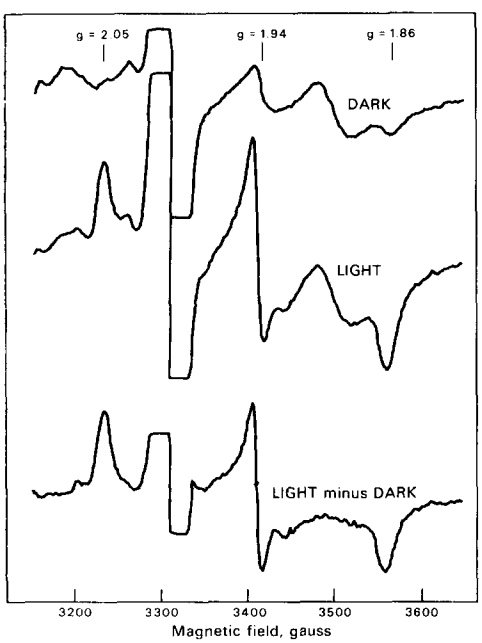
(5) *Double integration of EPR signals and calculation of ferredoxin: P700 ratio.* Light *minus* dark spectra were generated by subtraction of the appropriate digitized spectra suitably scaled to account for any difference in amplifier gain settings. These were then stored on magnetic tape for future comparisons.

The area of the P700 light *minus* dark signal was calculated in two different ways: by numerical double integration of the 500-point digitized data and by formula integration[19] for the determination of the spin concentration as represented by a first-derivative Gaussian curve

$$\text{Area} = (1.0332) y'_m (\Delta H_{pp})^2$$

The maximum amplitude of the first derivative (y'_m) is one-half the peak-to-peak height read directly from the digitized spectra. The peak-to-peak linewidth in gauss was determined by comparison of calculated Gaussian curves to experimental P700 spectra. These two methods of computing the area for the P700 spectra gave results which agreed to within $\pm$ 10 %.

Because of the interfering signals at $g = 2$ from chloroplast free radicals the area of the bound ferredoxin light *minus* dark signal had to be calculated by numerical integration of a fitted simulated spectrum. Once the doubly integrated areas for the signals of P700 and the bound ferredoxin were measured, intercomparison was made, assuming both were $S = 1/2$ states, by relation of these areas through the signal heights recorded with the ruby run under each set of conditions. Also included in this final calculation for each experiment was a factor $g^2_{\text{ferredoxin}}/g^2_{\text{P700}}$, the experimental amplifier gains and integration constants which adjusted the double numerical integrations to formula integration methods. A computer program was written to ensure that these corrections were uniformly made in all experiments.



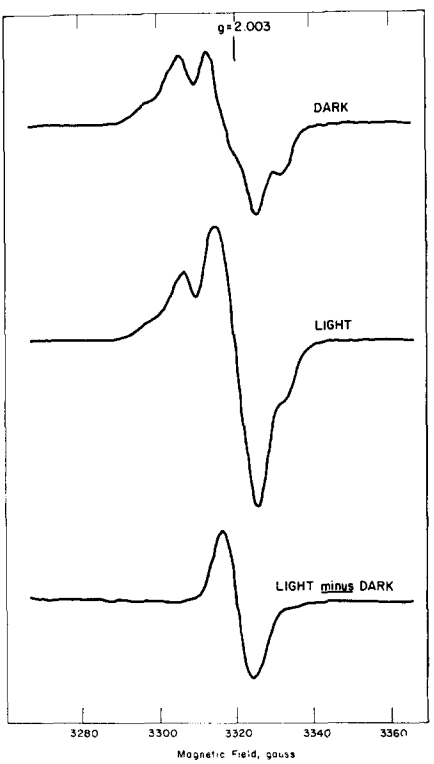


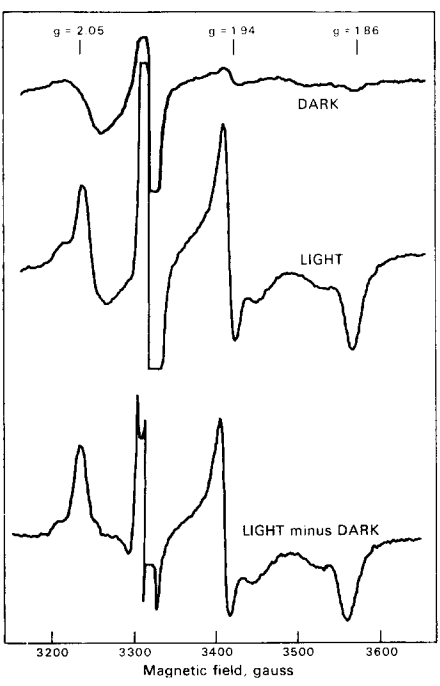
Fig. 1. Light-induced EPR changes of bound ferredoxin in whole chloroplasts after illumination at 25 °K with 715 nm actinic light. Chlorophyll concentration, 4.1 mM. First-derivative EPR spectra recorded at 25 °K with the following instrument settings: frequency, 9.26 GHz; power, 15 mW; modulation amplitude, 2 gauss; scan rate, 10 gauss/s. Data in all figures are photoreproductions of original chart-recorder traces.

Fig. 2. Light-induced EPR changes of P700 in whole chloroplasts after illumination at 25 °K with 715 nm actinic light. The same sample used for the study in Fig. 1 was used for the study of P700 changes. Conditions of EPR spectroscopy: frequency, 9.26 GHz; power, 0.1 mW; modulation amplitude, 2 gauss; scan rate, 4 gauss/s.

RESULTS

In this study we have confined our investigation to the primary photochemical event associated with Photosystem I in chloroplasts. Because far-red light is known to activate primarily Photosystem I in chloroplasts[20–23] and our light-induced studies are carried out at 25 °K (a temperature which precludes ordinary chemical reactions), we can effectively isolate the light-induced changes associated solely with the primary photochemical reaction of Photosystem I.

Illumination of whole chloroplasts with 715 nm actinic light at 25 °K produced light-induced changes at g-values of 2.05, 1.94, and 1.86, as indicated in Fig. 1. The light *minus* dark spectrum, shown in the lower portion of the figure, indicates most clearly the light-induced changes, although the region at $g = 2.00$ is obscured by the large (off-scale) free-radical signals observed at this gain setting. The free-radical region of the same sample is shown in the EPR spectra in Fig. 2. A rather complicated signal is present in the dark in whole chloroplasts; the nature of the



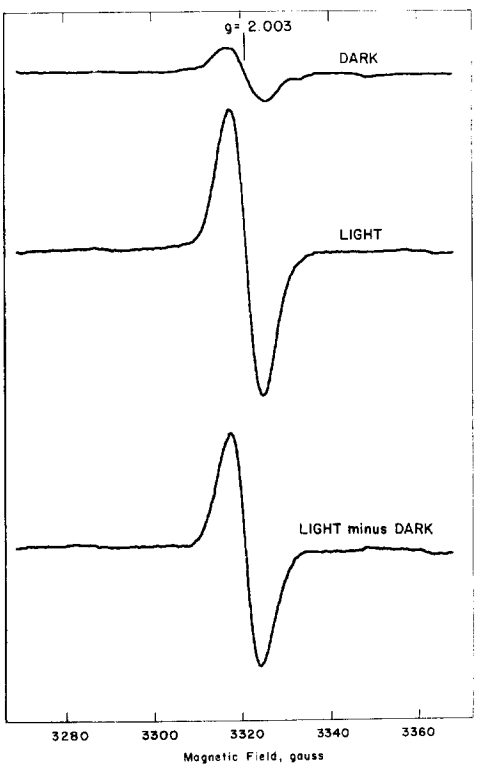


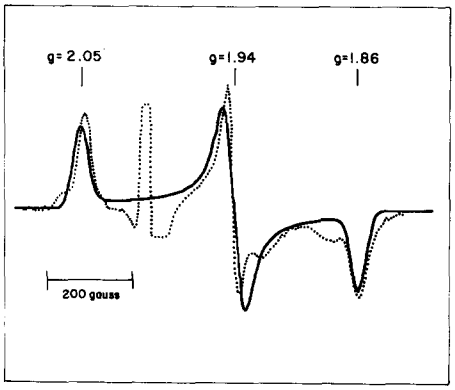
Fig. 3. Light-induced EPR changes of bound ferredoxin in Photosystem I subchloroplast fragments (D-144) after illumination at 25 °K with 715 nm actinic light. Chlorophyll concentration, 3.7 mM. Prior to freezing, 10 μmoles of sodium ascorbate were added to the chloroplasts. Conditions of EPR spectroscopy as in Fig. 1.

Fig. 4. Light-induced EPR changes of P700 in Photosystem I subchloroplast fragments (D-144) after illumination at 25 °K with 715 nm actinic light. The same sample used for the study in Fig. 3 was used for the measurement of P700 changes. Conditions of EPR spectroscopy as in Fig. 2 except for microwave power of 0.02 mW.

component responsible for this signal is not understood, although some of this signal may be the same as the "Signal II" previously observed in EPR studies of free radicals in chloroplasts at room temperature[24–16]. The chloroplast sample also shows a light-induced change in this field region after illumination with 715 nm actinic light, and the light *minus* dark difference spectrum shown in the lower portion of the figure indicates the photoproduction of a free-radical signal. Since this free radical is photoinduced at low temperature after illumination with far-red light and has EPR properties identical to those previously reported for P700 (refs 24, 27 and 28), the reaction-center chlorophyll of Photosystem I, we are confident of our assignment of this signal to the oxidized form of P700.

The results of a study of light-induced changes in subchloroplast fragments enriched in Photosystem I (D-144) are shown in Figs 3 and 4. The changes in the ferredoxin region (Fig. 3) are similar to those observed with unfractionated chloroplasts. The EPR spectrum of the free-radical region (Fig. 4) shows one significant difference, however, when compared with the spectrum of unfractionated chloroplasts. The dark signal at $g = 2.00$ is almost completely absent in the D-144 preparation (Fig. 4, top spectrum), indicating that the component(s) responsible for this signal is most likely associated with Photosystem II. After illumination at 25 °K, a large light-induced free-radical change may be observed, similar to the change induced in whole chloroplasts.

A comparison of the light *minus* dark spectra and computer-simulated spectra of the bound ferredoxin and P700 (obtained in an experiment with D-144 subchloroplast fragments) is shown in Figs 5 and 6. The simulated ferredoxin spectrum was



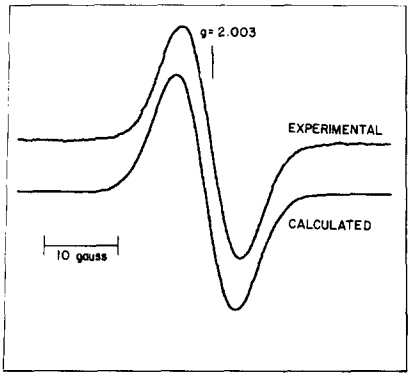


Fig. 5. Comparison of experimental (light *minus* dark) and calculated bound ferredoxin EPR spectra. The light *minus* dark bound ferredoxin spectrum (·····) was obtained with D-144 fragments at 25 °K. The calculated spectrum (———) was obtained using the following parameters: $g_x = 1.86$, $g_y = 1.94$, $g_z = 2.05$; linewidth, 20 gauss; $S = 1/2$ state.

Fig. 6. Comparison of experimental (light *minus* dark) and calculated P700 EPR spectra. The light *minus* dark P700 spectrum was obtained with D-144 fragments at 25 °K. The calculated spectrum was generated from a Gaussian curve with a linewidth of 8.0 gauss; $S = 1/2$ state.

generated using the following experimentally derived parameters: $g_x = 1.86$, $g_y = 1.94$, $g_z = 2.05$; linewidth, 20 gauss. As shown in Fig. 5, the agreement between the calculated and experimental spectra is good over most of the spectral region although some minor components of $g = 1.89$ and 1.93 are present in the light

minus dark spectrum. The origin of these components is not known, but they contribute only a small amount to the total integrated intensity of the experimental ferredoxin spectrum. The P700 spectrum (Fig. 6) was generated using a Gaussian curve with a linewidth of 8.0 gauss. The *g*-value of the P700 signal was found to be 2.003 ± 0.001 using potassium nitrosodisulfonate and TEMPOL as *g*-value markers.

The simulated light *minus* dark ferredoxin spectrum and the light *minus* dark P700 spectrum were used in the calculation of the relative amounts of ferredoxin and P700. The results of eight different experiments, five with whole chloroplasts and three with chloroplast Photosystem I fragments, are summarized in Table I. In

TABLE I

AMOUNTS OF P700 AND BOUND FERREDOXIN IN CHLOROPLASTS AND PHOTOSYSTEM I FRAGMENTS (D-144) DETERMINED BY EPR SPECTROSCOPY AFTER ILLUMINATION AT 25 °K WITH 715 nm ACTINIC LIGHT

The ratio of reduced bound ferredoxin to oxidized P700 at 25 °K was measured as described in Materials and Methods. These ratios are the experimental values without error considerations. The probable errors are of the order of ±10–20% (see Discussion). The P700 concentration was determined using weak pitch and DPPH as spin standards. The ratios of chlorophyll to P700 and bound ferredoxin were calculated from these measurements.

	Bound ferredoxin/P700	*Chlorophyll (mM)*	*P700 (μM)*	*Chlorophyll/P700*	*Chlorophyll/bound ferredoxin*
Whole chloroplasts					
(1)	1.37	4.0	6.8	590	430
(2)	1.25	5.0	13.1	380	300
(3)	1.11	3.7	9.8	380	340
(4)	1.22	2.5	5.4	460	380
(5)	1.20	4.1	12.1	340	280
Average	1.23			430 ± 75	350 ± 50
D-144 subchloroplast fragments					
(1)	1.11	3.0	15.8	190	170
(2)	0.78	3.3	20.6	160	210
(3)	0.94	3.7	26.4	140	150
Average	0.91			160 ± 15	175 ± 20

each of these experiments, the areas of the light *minus* dark ferredoxin and P700 spectra were calculated as described in Materials and Methods. In the studies with whole chloroplasts, a ratio of 1.2:1 for photoreduced ferredoxin:photooxidized P700 was obtained, and a ratio of 0.9:1 was obtained in the experiments with D-144 fragments. These experimental results, considered in the context of the possible errors in this type of quantitation (see Discussion), indicate that after illumination at 25 °K with far-red light the amounts of reduced bound ferredoxin and oxidized P700 are identical.

In addition to the relative amounts of photoreduced ferredoxin and photooxidized P700 shown in Table I, the concentration of these components in different preparations is given. In measurements with weak pitch and DPPH as spin concentration standards, the concentration of P700 was found to average 1 per 430 ± 75 chlorophyll molecules in whole chloroplasts; in the Photosystem I fragments

the average was 1 P700 per 160 ± 15 chlorophyll molecules. Similar values for the concentration of bound ferredoxin were also found in the two preparations.

The estimation of the P700 concentration of chloroplasts by EPR spectroscopy does not necessitate the knowledge of an extinction coefficient for the pigment and therefore has one advantage over the more widely used optical technique (see refs 29 and 30). By optical techniques, in unfractionated chloroplasts approximately one P700 per 400 chlorophyll molecules has been determined and in digitonin Photosystem I subchloroplast fragments one P700 per 200 chlorophyll molecules has been found[1, 31–33]. Our estimations based on spin concentrations, assuming no secondary reactions of P700 occur at 25 °K, are similar to the above-reported values, a result which indicates the photoreaction has gone to completion at 25 °K.

DISCUSSION

Before considering the significance of the values obtained in our estimates of P700 and bound ferredoxin at 25 °K, we must consider in some detail the accuracy and errors involved in our quantitative EPR procedures.

As described in Materials and Methods, because the estimations are based primarily on amplitudes, the accuracy of comparison via our internal ruby standard is within 1–2 %. In the case of the P700 light-induced signal, an additional factor besides the amplitude in the area determination is the square of the linewidth, but the results in Fig. 6 with a simulated spectrum indicate that the linewidth is known to within ± 0.2 gauss. Therefore, our formula integrations for the area of the P700 signal may result in an error of approx. 5 %. However, we have also calculated the light *minus* dark P700 area by a numerical double-integration procedure; the values obtained by this method were within 10 % of those by the formula method. We believe the formula method permits greater precision in the area determination since only the amplitude and linewidth of the signal must be known and any slight baseline drift may be ignored.

The estimation of the area of the light *minus* dark ferredoxin signal most likely was the source of the largest error in our quantitative measurements. We calculated this area by two different methods, and the results agree to within ± 10 %. One method uses the simulated spectrum procedure described in detail in Materials and Methods, while the second is a numerical double-integration of the light *minus* dark spectrum. In the latter procedure it was necessary to correct the $g = 2.00$ region because of the large off-scale free-radical signals. This type of correction introduced some uncertainty and, in general, we may have overestimated the ferredoxin area in the calculations based on this method. These considerations lead us to believe that our ferredoxin areas may contain an error as great as 10 % but that a larger error is unlikely.

The above discussion is taken to indicate that we believe the ratios we have obtained are accurate to ± 10–20 %. Other errors, such as those due to variation in temperature, are also present, but these contribute relatively smaller amounts. Since our experimental results indicate a ratio of ferredoxin: P700 of 1.2:1 in whole chloroplasts and 0.9:1 in Photosystem I fragments, it is clear that within experimental error this value may be considered to be one.

The ratio of bound ferredoxin to P700 determined in these experiments after

low-temperature illumination differs significantly from the results of Yang and Blumberg[9]. These workers observed a ferredoxin signal with an area only 10% of that of the free-radical signal produced at low temperature. However, in that report, details of the method of quantitation of the EPR signals and conditions of EPR spectroscopy are not presented. The latter are particularly important because the free-radical signal saturates very readily at low temperatures and the measurements of Yang and Blumberg[9] were carried out at 1.5 °K. It is clear that Yang and Blumberg did not use monochromatic light for illumination of their chloroplast samples and that no attempt was made to exclude possible contributions from primary events associated with Photosystem II. We feel the experimental design used in our measurements eliminates any photoinduced changes associated with Photosystem II and allows study of primary reactions associated solely with Photosystem I

The quantitative estimates based on spin determination of bound ferredoxin and P700 in unfractionated chloroplasts and Photosystem I fragments confirm our previously published qualitative data that the bound ferredoxin is associated with Photosystem I and previous reports from other laboratories that D-144 fragments are enriched in P700 (refs 31–33). The quantitative determinations of the bound ferredoxin and P700 concentrations in these preparations indicate that the reaction under investigation at 25 °K has essentially gone to completion. Thus, our results are not based on a small percentage of the total possible change in either of these carriers.

A comparison of the light *minus* dark ferredoxin spectrum with calculated spectra indicates that the experimental spectrum can be reasonably fit with only three g-values: $g_x = 1.86$, $g_y = 1.94$, and $g_z = 2.05$. Additional absorptions are, however, present in the experimental light *minus* dark spectrum at $g = 1.89$ and 1.93. It has been suggested[10] that these two absorptions indicate a reduced iron–sulfur protein signal and that this component represents a second electron-accepting site in the primary electron acceptor. Our quantitative measurements indicate these two absorptions represent relatively minor components and do not contribute significant amounts to the total integrated intensity of the light *minus* dark ferredoxin signal, a result which does not support a multielectron-accepting center as the primary electron acceptor.

In chloroplasts, P700 has been considered to be the reaction-center chlorophyll of Photosystem I since the discovery and characterization of this pigment by Kok and co-workers[1,2]. Although this pigment was initially characterized on the basis of a photobleaching at 700 nm, the association of the oxidized form of P700 with a free-radical signal detectable by EPR spectroscopy has also been demonstrated: by oxidation–reduction behavior[34,35], light behavior[36–38], temperature-insensitivity of the photoreaction[25,39,40], and studies with algal mutants lacking P700 (ref. 41). Recent kinetic measurements have supported the assignment of this free radical to the oxidized form of P700 (ref. 42).

On the basis of the above-mentioned findings, there is widespread agreement that P700 functions as the primary donor molecule of Photosystem I but the nature of the primary acceptor is not clear. The first measurements of the ratio of the light-induced free-radical signal to photooxidized P700 gave values of 1:4 (ref. 28), while more recent measurements have indicated a ratio near one at room temperature[42,43]. No EPR signal resulting from any possible acceptor was identified on the basis of

these room-temperature studies. We have, however, recently discovered an EPR signal of a reduced iron–sulfur protein that can only be detected at temperatures below 40 °K (refs 5 and 6). The previously described properties of this new component are consistent with a role as the primary electron acceptor of Photosystem I (refs 5 and 6).

The results of our quantitative estimations of bound ferredoxin and P700 at 25 °K are consistent with these carriers functioning as an acceptor–donor complex in Photosystem I. According to this interpretation, the primary light act would consist of a charge separation resulting in reduced ferredoxin and oxidized P700; equivalent amounts of oxidized P700 and reduced iron–sulfur protein would be expected to accumulate at a temperature where further chemical reactions cannot occur.

Using the method of low-temperature (25 °K) EPR spectroscopy, where both the oxidized P700 free-radical signal and the reduced iron–sulfur protein signal can be observed, we have experimentally verified this expectation by the determination of equal spin concentrations of P700 and bound ferredoxin. This result lends the strongest support to our previously proposed role of bound ferredoxin as the primary electron acceptor of Photosystem I (ref. 6).

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