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The Properties of the Primary Electron Acceptor in the Photosystem I Reaction Centre of Spinach Chloroplasts and its Interaction with P700 and the Bound Ferredoxin in Various Oxidation–Reduction States

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The properties of the component 'X' identified as the primary electron acceptor of Photosystem I in spinach was investigated by electron-paramagnetic-resonance spectroscopy and the complete spectrum obtained for the first time. Component 'X' has $g_x = 1.78$, $g_y = 1.88$ and $g_z = 2.08$; it can be observed only at very low temperatures (8–13K) and high microwave powers. Component X was identified in Photosystem I particles prepared with the French press or with Triton X-100. In samples reduced with ascorbate, illumination at low temperatures results in the photo-oxidation of P700 and reduction of a bound iron–sulphur protein; this is irreversible at low temperature. In samples in which the iron–sulphur proteins are reduced by sodium dithionite, illumination at low temperature results in the oxidation of P700 and the reduction of component 'X'; this is reversible at low temperature. The light-induced P700 signal is the same size with either ascorbate or dithionite as reducing agent, showing that all of the P700 involved in reduction of bound ferredoxin also functions in the reduction of component 'X'.

The primary photochemical event in Photosystem I in oxygen-evolving organisms is the photo-oxidation of the reaction-centre chlorophyll, P700, and the transfer of an electron to a primary electron-acceptor complex (Ke, 1973; Bearden & Malkin, 1975). Although P700 has been clearly identified as the electron donor of Photosystem I, the nature of the primary electron acceptor is still uncertain. It has been known for many years that the photo-oxidation of the reaction-centre chlorophyll in both bacteria and Photosystem I can be observed by e.p.r. (electron paramagnetic resonance) at cryogenic temperatures (i.e. temperatures below 77K) (Commoner *et al.*, 1956; Calvin & Sogo, 1957). In bacteria this photo-oxidation is reversible, whereas under most conditions it is irreversible in Photosystem I. Malkin & Bearden (1971) showed that the electron acceptor for the irreversible reaction in Photosystem I is a bound ferredoxin. This ferredoxin has clearly been shown to be associated with Photosystem I and to accept electrons from P700 at temperatures as low as 4K. We have presented evidence that it has two four-iron Fe–S centres, referred to as Centre A, with a midpoint potential of −550mV, and Centre B with a midpoint potential of −590mV (Evans *et al.*, 1972, 1974; Ke *et al.*, 1974; Cammack & Evans, 1975).

The membrane-bound ferredoxin was thought to be the primary electron acceptor. However, the fact that its photoreduction is irreversible at low temperature, whereas that of the primary electron acceptor in bacterial photosynthesis is reversible suggests that other components may be involved. This view was supported by results from a number of laboratories indicating that some part of the photo-oxidation of P700 may be reversible (Mayne & Rubinstein, 1966; Warden *et al.*, 1974; Lozier & Butler, 1974). We have presented preliminary evidence that this reversibility may depend on the redox state of the bound ferredoxin (Evans & Cammack, 1975) and that when this is fully reduced a previously unknown component accepts an electron reversibly from P700 (Evans *et al.*, 1975). We have now fully characterized the relationships between the redox state of the bound ferredoxin and the extent and reversibility of P700 photo-oxidation at low temperatures in Photosystem I preparations made by both mechanical and detergent disruption of chloroplasts. We have also investigated the e.p.r. properties of the new

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component and have obtained a complete e.p.r. spectrum of this component, which we propose is the primary electron acceptor of Photosystem I.

Materials and Methods

Washed broken chloroplasts were prepared from market spinach (*Spinacea oleracae*) essentially as described by Whatley & Arnon (1963). Photosystem I particles were prepared by either French-press treatment as described by Sane *et al.* (1971) or treatment with the non-ionic detergent Triton X-100 (octylphenoxypolyethoxyethanol). Triton treatment was carried out as described by Vernon & Shaw (1971) to the stage where Photosystem II particles are removed by centrifugation. Photosystem I particles were further purified as previously described (Cammack & Evans, 1975). The preparations used in the e.p.r. experiments contained 0.1–0.3 M-NaCl in 0.02 M-Tris/HCl buffer, pH 7.5. French-press particles were suspended in 0.1 M-Tris/HCl, pH 8.0. Material for preparation of samples at pH 10.0 was made 0.1 M with glycine/KOH buffer, pH 10.0, and the pH further adjusted with KOH. The particle preparations were stored frozen in liquid N_2.

E.p.r. samples were prepared anaerobically in silica tubes (internal diameter 3 mm). Samples which were exposed to reducing agents for less than 5 min were prepared in the tubes under a stream of N_2. Samples exposed to dithionite for longer periods were prepared in an anaerobic vessel in which the pH could be continuously measured and adjusted. The samples were transferred anaerobically to the e.p.r. tubes immediately before freezing. Samples were prepared under normal room illumination unless otherwise specified. 'Dark' samples were prepared under a weak green safelight and frozen in complete darkness; 'illuminated' samples were illuminated for 2 min with a 250 W projector and frozen under the same illumination. E.p.r. spectra were obtained by using a Varian E4 spectrometer, as described previously (Evans *et al.*, 1972). Samples were illuminated in the spectrometer cavity with white light from a 1 kW projector filtered through a 1 cm water filter. Spectra were stored and averaged, and light-minus-dark difference spectra were obtained by using a Nicolet 1020A digital oscilloscope (Techmation, Edgware, Middx., U.K.). Chlorophyll was determined by the procedure of Arnon (1949).

Triton X-100, Tris and sodium ascorbate were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; DEAE-cellulose was from Whatman, Maidstone, Kent, U.K.; other chemicals were obtained from British Drug Houses, Poole, Dorset, U.K.

Results and Discussion

In our original experiments we showed that in samples in which the bound ferredoxin was fully reduced by exposure to dithionite at alkaline pH for 40 min, the photo-oxidation of P700 observed at cryogenic temperatures was largely reversible (Evans & Cammack, 1975). The extent of this photo-oxidation was much greater than that reported by earlier workers (Warden *et al.*, 1974); however, direct comparison with the total P700 signal size in samples without dithionite was difficult, because the latter samples often have large free-radical signals before illumination.

We have now compared the relative size of P700 and bound-ferredoxin signals induced by illumination at low temperature of samples in which the components giving rise to free radicals have been reduced by treatment in the dark, either with ascorbate or by short exposure to dithionite or by prolonged exposure to dithionite. Most of the samples used were prepared at pH 10.0, because at any pH more acid than 9.0, sodium dithionite solutions do not reduce the bound ferredoxin completely. Qualitatively similar results can be obtained at pH 7.5–8.5 by using various illumination procedures to reduce the bound ferredoxin. However, these are not fully reproducible or as easy to understand as the present experiment, which uses chemical reduction of the bound ferredoxin.

Fig. 1 shows the results of such an experiment with particles prepared by mechanical disruption with the French press. In these experiments the P700 (seen as the e.p.r. signal at $g = 2.00$) is fully reduced in the dark, and the samples show negligible free-radical signals before illumination. With these particles the results with ascorbate (Fig. 1*a*) or short exposure to dithionite (Fig. 1*b*) are essentially the same. In the dark, P700 is fully reduced and there is little of the bound ferredoxin (trace 1, Figs. 1*a* and 1*b*). Illumination results in the oxidation of P700 and reduction of the bound ferredoxin (trace 2, Figs. 1*a* and 1*b*); when the light is turned off there is no reversal of the light-induced signals (trace 3, Figs. 1*a* and 1*b*). Subsequent illumination cycles do not result in any further change in the signals; the signal size is proportional to chlorophyll concentration between 0.2 and 2.0 mg of chlorophyll/ml. The relative size of the light-induced signals is the same when either dithionite or ascorbate is the reductant.

It should be stressed that these measurements are of the apparent signal size under specified conditions. Changes in signal size are proportional to the amount of a component but do not give an absolute measure of the amount of the component. E.p.r. can be used to determine the absolute number of free electron spins present in any component. However, different saturation behaviour of different components means that it is not possible to make absolute quantitative measurements of different components under a single set of conditions. We have been unable to quantify the

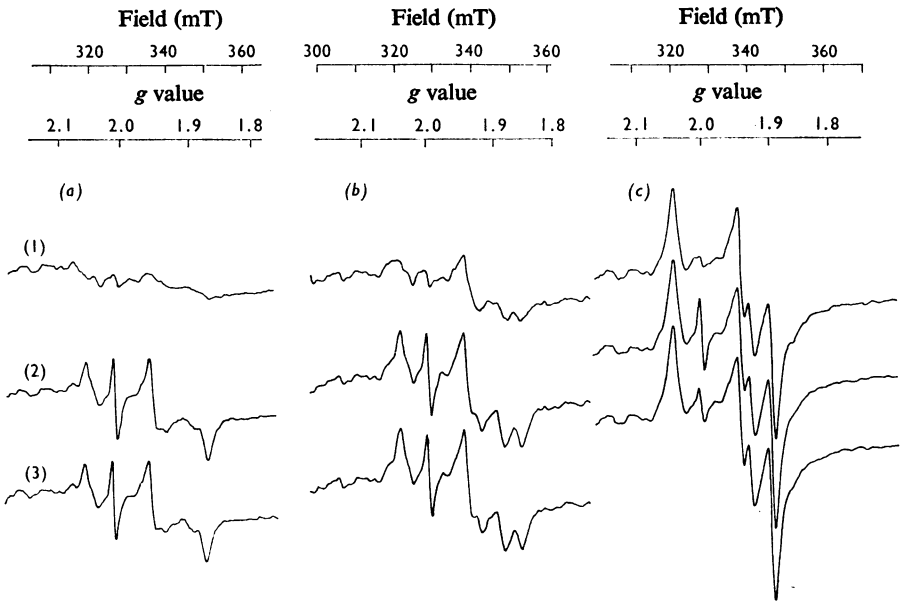


Fig. 1. *Effect of illumination on the e.p.r. spectra in the g = 2.00 region of Photosystem I particles, made with the French press*

The samples, which were prepared in the dark, contained Photosystem I particles made with the French press (4.0mg of chlorophyll/ml) in 0.1 M-glycine/KOH, pH 10.0, with the addition of: (*a*) 0.02M-sodium ascorbate, 5min before freezing; (*b*) 0.01M-sodium dithionite, 2min before freezing; (*c*) 0.01M-sodium dithionite, 40min before freezing. The spectra were recorded at 20K with the following instrument settings: frequency 9.25 GHz; power 20mW; modulation amplitude 1mT; scan rate 100mT/min; gain 1000. Trace (1), in the dark; trace (2), during illumination of the sample; trace (3), in the dark after illumination.

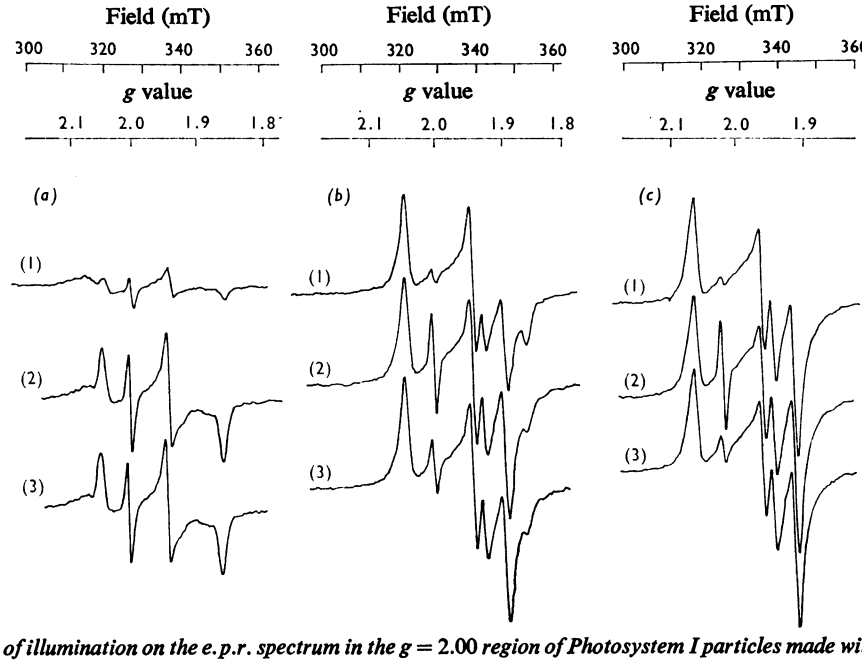


Fig. 2. *Effect of illumination on the e.p.r. spectrum in the g = 2.00 region of Photosystem I particles made with Triton X-100*

The samples, which were prepared in the dark, contained Photosystem I particles made with Triton X-100 (1.0mg of chlorophyll/ml) with the addition of (*a*) 0.02M-sodium ascorbate, 5min before freezing; (*b*) 0.01M-sodium dithionite, 2min before freezing; (*c*) 0.01M-sodium dithionite, 40min before freezing. Spectra were recorded at 20K as in Fig.1. Trace (1), in the dark; trace (2), during illumination of the sample; trace (3), in the dark after illumination of the sample.

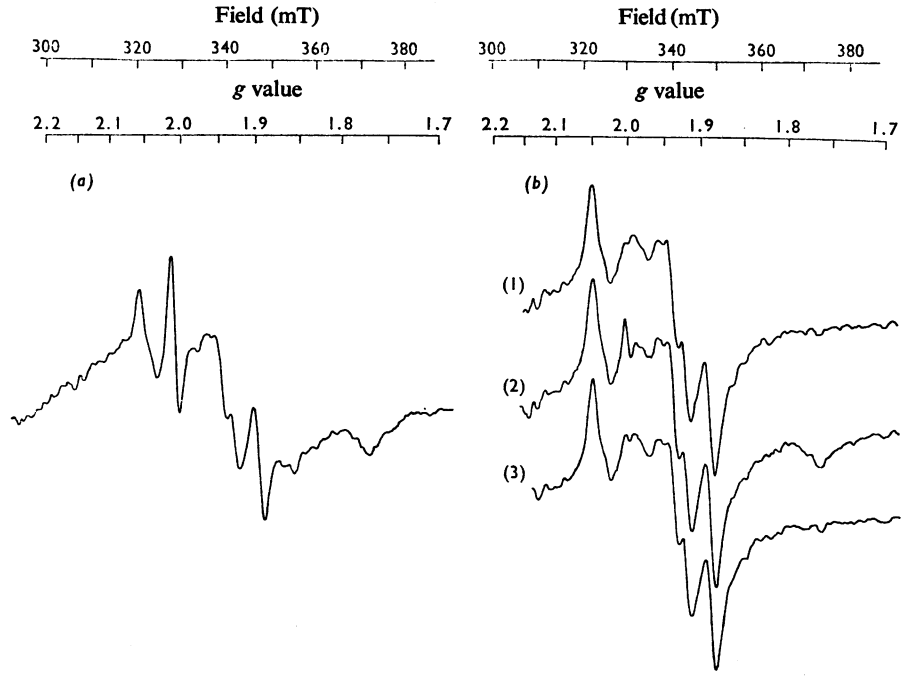


Fig. 3. *E.p.r. spectrum of the primary electron acceptor in Photosystem I particles made with the French Press*

(*a*) The $g = 1.78$ signal frozen in the reduced state. The sample, containing Photosystem I particles made with the French press (4mg of chlorophyll/ml) in 0.1M-Tris/HCl buffer, pH 8.0, 0.01M-sodium dithionite and 15μM-Methyl Viologen, was illuminated for 2 min before freezing in the light. The spectrum was recorded in the dark at 11K with the following instrument settings: frequency 9.25 GHz; power 100mW; modulation amplitude 1mT; scan rate 100mT/min; gain 1000. (*b*) The light induced $g = 1.78$ signal. The sample, containing Photosystem I particles produced in the French press (4.0mg of chlorophyll/ml) in 0.1M-glycine/KOH, pH 10.0, and 0.01M-sodium dithionite, was incubated in the dark for 40 min at 25°C before freezing. The spectra were recorded as in (*a*). Trace (1), in the dark; trace (2), during illumination of the sample; trace (3), in the dark after illumination.

spectra because the P700-radical signal is saturated at all microwave power levels obtainable with our spectrometer.

After prolonged exposure of Photosystem I particles to dithionite at alkaline pH, the bound ferredoxin is almost fully reduced, with both Centre A and Centre B reduced, and P700 is also reduced (Fig. 1*c*). Illumination of this sample again results in the photo-oxidation of P700, with the generation of a free-radical signal equal in size to that seen when ascorbate or short exposure to dithionite is used as reductant. This photo-oxidation is, however, 75–80% reversible when the light is turned off. Further light–dark transitions result in photo-oxidation of P700, giving the same free-radical signal as in Fig. 1(*c*) (trace 2). This photo-oxidation is completely reversible in the dark. There is no change in the bound ferredoxin signal associated with the reversible photo-oxidation of P700.

Fig. 2 shows an experiment similar to that shown in Fig. 1, except that Photosystem I particles made with the non-ionic detergent Triton were used. These have a larger ratio of reaction centre to chlorophyll than do particles made with the French press. When these particles are reduced with ascorbate, illumination results in the irreversible oxidation of P700 and reduction of Centre A of the bound ferredoxin, as in particles made with the French press. In these particles the iron–sulphur centres are more readily reduced by dithionite than in those made with the French press, presumably reflecting a greater accessibility to the solvent. Even short exposure to dithionite results in extensive reduction of the iron–sulphur centres. Frequently, as in Fig. 2(*b*), Centre A is almost fully reduced and Centre B partially reduced. Illumination of such samples results in the oxidation of P700 to the same extent as in ascorbate-reduced samples; however, this oxidation is partly reversed in the dark. In parallel with the irreversible component of P700 oxidation, there is an irreversible reduction of Centre

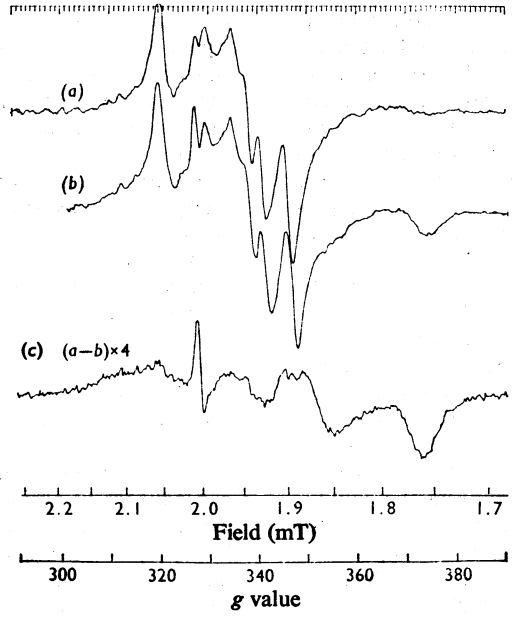


Fig. 4. *Complete e.p.r. spectrum of the primary electron acceptor of Photosystem I in Photosystem I particles made with Triton X-100*

The sample, containing Photosystem I particles made with Triton X-100 (1.0mg of chlorophyll/ml) in 0.1 M-glycine/KOH, pH 10.0, and 0.01 M-sodium dithionite, was incubated for 40 min in the dark at 25° C before freezing. Spectra were recorded at 10.5K as in Fig. 3. Four scans were recorded and averaged. Trace (*a*) in the dark; trace (*b*), during illumination of the sample; trace (*c*) shows the light-minus-dark difference spectrum expanded by a factor of four showing the light-induced signal due to P700 ($g = 2.00$) and the primary electron acceptor ($g = 2.08$, 1.88 and 1.78).

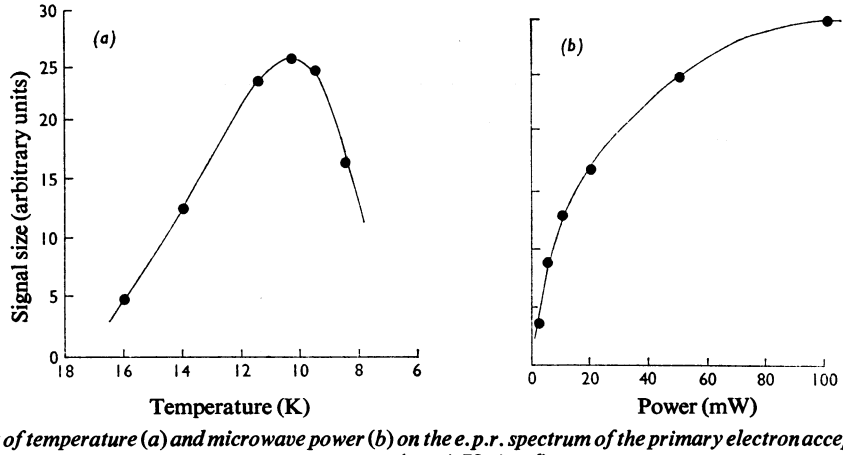


Fig. 5. *Effect of temperature (a) and microwave power (b) on the e.p.r. spectrum of the primary electron acceptor of Photosystem I ($g = 1.78$ signal)*

The spectrum of a sample of Photosystem I particles made with Triton X-100 frozen under illumination in 0.1 M-glycine/KOH (pH 10.0) (1.0mg of chlorophyll/ml), 0.01 M-sodium dithionite, was recorded as in Fig. 4. The peak height of the $g = 1.78$ signal was taken as a measure of signal size. (*a*) Power 100mW; (*b*) temperature 10.5K.

B, seen as an increase in the $g = 1.89$ signal (Fig. 2*b*). In samples exposed to dithionite for longer periods (Fig. 2*c*), both Centres A and B are reduced and the P700 photo-oxidation is reversible. The size of the P700 signal is the same under all these conditions.

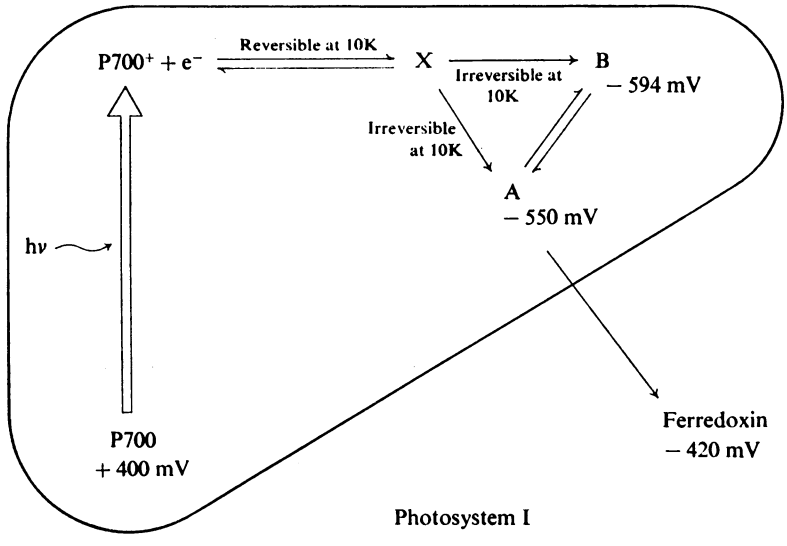
These experiments show clearly that the extent of P700 photo-oxidation is independent of the redox state of the iron–sulphur centres; the redox state of these centres does, however, appear to determine whether the photo-oxidation is reversible or not.

These experiments suggest that the bound ferredoxin centres are in fact secondary acceptors and that some other component is the primary electron acceptor. McIntosh *et al.* (1975) obtained preliminary kinetic evidence for the presence of a component undergoing reduction in parallel with the photo-oxidation of P700. We were able to confirm (Evans *et al.*, 1975) the presence of this component in Photosystem I particles made by using Triton and identify conditions under which it could be frozen in the reduced state. We have now shown that this component can be identified in particles made with the French press (Fig. 3). These experiments show that the component may be frozen in the reduced state after illumination of the sample in the presence of dithionite and Methyl Viologen (Fig. 3*a*) or may be photoreduced at low temperature in samples in which the bound ferredoxin is reduced in the dark before freezing (Fig. 3*b*). These experiments show that the component is not a detergent-induced artifact. We have also observed the signal in unfractionated chloroplasts.

Fig. 4 shows the effect of illumination on the e.p.r. spectrum of Photosystem I particles made with Triton X-100 and the light-minus-dark difference spectrum. This shows the P700 free-radical signal and the broad signal of the component ('X') which we think is the primary electron acceptor. The approximate g values of this component are $g_x = 1.78$, $g_y = 1.88$, $g_z = 2.08$, with $g_{av.} = 1.91$. The spectrum can be observed only under very closely defined conditions. Fig. 5 shows the effect of temperature and of microwave power on the intensity of the $g = 1.78$ signal. This shows that the signal can only be easily observed at temperatures between 8 and 13K and at high microwave power. The spectrum is, however, quite large and although we have used the average of four scans in the light and dark to improve the signal-to-noise ratio of the spectra shown in Fig. 4, a recognizable spectrum can readily be obtained from single scans of the spectrum in the light and in the dark by using simple illumination procedures. This spectrum is similar to that of the primary electron acceptor in bacterial photosynthesis (Leigh & Dutton, 1972).

The properties of the component 'X' are similar to those of the iron–sulphur proteins; the e.p.r. signal is observed in the reduced state, and has an average g value less than the free-electron value of 2.0023. These properties of the iron–sulphur proteins are a consequence of antiferromagnetic coupling between iron atoms (Gibson *et al.*, 1966). For compound 'X', however, the average g value ($g = 1.91$) is lower than that of any iron–sulphur protein so far reported, so its identity must at present remain uncertain. However, attempts to demonstrate the presence of iron by e.p.r. spectroscopy of Photosystem I particles from algae grown on ^{57}Fe failed to show any effect, though this experiment cannot be regarded as conclusive (Evans *et al.*, 1976).

It has been proposed that the bacterial acceptor, which has a similar spectrum, is a quinone complex (Feher *et al.*, 1972). We have attempted to extract the



Scheme 1. *Functional relationships of the electron-transport components of the Photosystem I reaction centre*

The potentials indicated are the measured mid-point oxidation–reduction potentials of the components.

quinones from Photosystem I particles by extraction of freeze-dried particles with heptane. This procedure has been shown to deplete plastoquinone in chloroplasts and inhibit electron transport (Arnon & Horton, 1963). This treatment had no effect on the low-temperature photochemical activity of the Photosystem I reaction centre as observed by e.p.r. spectroscopy. The electron-transfer reactions do not therefore depend on any readily extractable quinone or other lipids.

These experiments clearly show that the low-temperature photochemical reactions in Photosystem I involve a number of electron-transfer processes. These are summarized in Scheme 1. The photo-oxidation of P700 results in the photoreduction of a component (X in Scheme 1) which we consider to be the primary electron acceptor of Photosystem I. This reaction is reversible, and component 'X' must be very closely linked to P700. Electrons may then be transferred from component 'X' to either of the Fe–S centres of the bound ferredoxin. It is not clear at present whether these function sequentially or in parallel. This reaction is irreversible at low temperature, indicating a significant energy gap between component 'X' and the bound ferredoxin.

As the light-induced free-radical signal is the same size in ascorbate- and dithionite-reduced samples, although with ascorbate the bound ferredoxin is initially fully oxidized and with dithionite it is fully reduced, we consider that all of the P700 involved in photoreduction of the bound ferredoxin in the ascorbate samples is involved in the reversible photoreduction of the component 'X' in dithionite-reduced samples.

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References

Arnon, D. I. (1949) *Plant Physiol.* **24**, 1–15

Arnon, D. I. & Horton, A. A. (1963) *Acta. Chem. Scand.* **17**, 135–139

Bearden, A. J. & Malkin, R. (1975) *Q. Rev. Biophys.* **7**, 131–177

Calvin, M. & Sogo, P. (1957) *Science* **125**, 499–500

Cammack, R. & Evans, M. C. W. (1975) *Biochem. Biophys. Res. Commun.* **67**, 544–549

Commoner, B., Heise, J. J. & Townsend, J. (1956) *Proc. Natl. Acad. Sci. U.S.A.* **42**, 710–718

Evans, M. C. W., Telfer, A. & Lord, A. V. (1972) *Biochim. Biophys. Acta* **267**, 530–537

Evans, M. C. W., Reeves, S. G. & Cammack, R. (1974) *FEBS Lett.* **49**, 111–114

Evans, M. C. W. & Cammack, R. (1975) *Biochem. Biophys. Res. Commun.* **63**, 187–193

Evans, M. C. W., Sihra, C. K., Bolton, J. R. & Cammack, R. (1975) *Nature (London)* **256**, 668–670

Evans, E. H., Cammack, R. & Evans, M. C. W. (1976) *Biochem. Biophys. Res. Commun.* **68**, 1212–1218

Feher, G., Okamura, M. Y. & McElroy, J. D. (1972) *Biochim. Biophys. Acta* **267**, 222–226

Gibson, J. F., Hall, D. O., Thorneley, J. H. M. & Whatley, F. R. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 987–990

Ke, B. (1973) *Biochim. Biophys. Acta* **301**, 1–33

Ke, B., Hansen, R. E. & Beinert, H. (1973) *Proc. Natl. Acad. Sci.* U.S.A. **70**, 2941–2945

Leigh, J. S. & Dutton, P. L. (1972) *Biochem. Biophys. Res. Commun.* **46**, 414–421

Lozier, R. H. & Butler, W. L. (1974) *Biochim. Biophys. Acta* **333**, 460–464

Malkin, R. & Bearden, A. J. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 16–19

Mayne, B. C. & Rubinstein, D. (1966) *Nature (London)* **210**, 734

McIntosh, A. R., Chu, M. & Bolton, J. R. (1975) *Biochim. Biophys. Acta* **376**, 308–314

Sane, P. V., Goodchild, D. J. & Park, R. B. (1971) *Biochim. Biophys. Acta* **216**, 162–178

Vernon, L. P. & Shaw, E. R. (1971) *Methods Enzymol.* **23A**, 277–288

Warden, T., Mohanty, P. & Bolton, J. R. (1974) *Biochem. Biophys. Res. Commun.* **59**, 822–828

Whatley, F. F. & Arnon, D. I. (1963) *Methods Enzymol.* **6**, 308–313