The Oxidation–Reduction Potential of the Reaction-Centre Chlorophyll (P700) in Photosystem I

EVIDENCE FOR MULTIPLE COMPONENTS IN ELECTRON-PARAMAGNETIC- RESONANCE SIGNAL 1 AT LOW TEMPERATURE

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The oxidation–reduction potential of the reaction-centre chlorophyll of Photosystem I (P700) in spinach chloroplasts was determined by using the ability of the reaction centre to photoreduce the bound ferredoxin and to photo-oxidize P700 on illumination at 20 K as an indicator of the oxidation state of P700. This procedure shows that P700 is oxidized with E_m (pH 8.0) (mid-point redox potential at pH 8.0) $\approx +375$ mV. Further oxidation of the chloroplast preparations by high concentrations of $K_3Fe(CN)_6$ (10 mM) in the presence of mediating dyes leads to the appearance of a large radical signal with an apparent $E_m \approx +470$ mV. A second, light-inducible, radical also appears over the same potential range. We propose that these signals are due to bulk chlorophyll oxidation and not, as was previously thought [Knaff & Malkin (1973) *Arch. Biochem. Biophys.* **159**, 555–562], to reaction-centre oxidation. A number of optical techniques were used to determine E_m of P700. Dual-wavelength spectroscopy (697–720 nm) indicates $E_m \approx +460$ – $+480$ mV. The spectrum of the sample during the titration showed a large contribution to the signal by bulk chlorophyll oxidation, in agreement with the electron-paramagnetic-resonance results and those of Ke, Sugahara & Shaw [(1975) *Biochim. Biophys. Acta* **408**, 12–25]. The light-induced absorbance change at 435 nm, usually attributed to P700, showed a potential dependence similar to that of bulk chlorophyll oxidation. Determination of E_m of P700 on the basis of the appearance of the P700 signal in oxidized-versus-reduced difference spectra showed E_m (pH 8.0) $\approx +360$ mV. Measurements of the effect of potential on the irreversible photo-oxidation of P700 at 77 K showed that P700 became oxidized in this potential range. We conclude that the reaction-centre chlorophyll of Photosystem I has E_m (pH 8.0) $\approx +375$ mV.

The primary event in photosynthesis is thought to be the absorption of light, followed by the photo-oxidation of a chlorophyll molecule in a special environment, the reaction-centre chlorophyll. This photo-oxidation is coupled to the reduction of an electron acceptor. In O_2 -evolving organisms two photochemical reactions are involved in photosynthesis, each with a specialized reaction centre. Photosystem II is thought to be involved in the generation of a high-potential oxidant, for water oxidation, and a reductant at about 0 mV. Photosystem I is involved in the generation of the low-potential reductant for CO_2 fixation, by using electrons provided by Photosystem II as electron donor. The reaction-centre chlorophyll is thought to be part of a photosynthetic unit in which there is one reaction centre for each 400–600 chlorophyll molecules.

Kok (1956) proposed that an optical change observed at 703 nm on illumination or oxidation of

chloroplasts represented the oxidation of the reaction-centre chlorophyll of Photosystem I. This absorption change is widely accepted as representing the oxidation–reduction state of the Photosystem-I reaction centre. At about the same time as the discovery of P700, an e.p.r.*-detectable light-induced free-radical signal (signal 1) was observed in chloroplasts (Commoner *et al.*, 1956). Beinert *et al.* (1962) suggested that P700 and signal 1 represented optical and e.p.r. detection of the same component. At room temperature this identification has been confirmed by quantitative measurements (Warden & Bolton, 1973; Baker & Weaver, 1973). Both optically detected P700 and the species giving rise to e.p.r. signal 1 can be oxidized by illumination of chloroplasts at 77 K and below; however, at low temperatures Beinert & Kok (1964) were unable to find any quantitative correlation between the two. More recently it has

* Abbreviation: e.p.r., electron paramagnetic resonance.

been shown that the low-temperature photo-oxidation of the species giving signal 1 is coupled either irreversibly to the reduction of a bound iron-sulphur protein (Malkin & Bearden, 1971; Evans *et al.*, 1972), or, under reducing conditions, to the reversible reduction of a component X identified only by its e.p.r. spectrum (Evans & Cammack, 1975; Evans *et al.*, 1975, 1976a,b). Bearden & Malkin (1972) found a quantitative relationship between signal 1 and the bound ferredoxin reduced. However, it has become apparent from our present experiments that considerable variations in signal 1 may occur depending on the conditions of sample preparation.

A number of workers have reported the oxidation-reduction potential of the ground state of the reaction-centre chlorophyll P700. These have shown considerable variation, and some reports have indicated a very high potential. Kok (1961), using ferricyanide oxidation of acetone-extracted chloroplasts, obtained a value of +420 mV. Rumberg (1964), measuring the loss of ability to photo-oxidize P700 (as determined by flash spectroscopy at 703 nm) on oxidation, obtained a value of +460 mV (± 100 mV). Ke *et al.* (1975), using dual-wavelength spectroscopy, obtained a value of +468 mV, but were unable to obtain a true end point for the titration. Calvin & Androes (1964), measuring the disappearance of the light-inducible signal 1 at room temperature, obtained a value of +420 mV [corrected from +450 mV for the E'_0 (mid-point redox potential at pH 7.0) of $K_3Fe(CN)_6$ used], and Knaff & Malkin (1973), using either dual-wavelength spectroscopy or measuring the ferricyanide-induced e.p.r. signal 1, obtained +520 mV. In view of the considerable variation in these values, and the fact that none of them measured a physiological function of the reaction centre independently of P700 signal, we have re-determined this potential on the basis of the ability of the reaction centre to photoreduce the bound ferredoxin at low temperature. We have also investigated the properties of the light- and ferricyanide-induced e.p.r. signal 1. The oxidation-reduction potential of P700 obtained by measuring the ability to reduce the bound ferredoxin is significantly different from the potentials previously reported. We have therefore also determined the potential of P700 by a number of optical techniques. A preliminary report of this work has been made elsewhere (Evans *et al.*, 1976b).

Materials and Methods

Washed broken spinach (*Spinacia oleracea*) chloroplasts were prepared by the procedure of Whately & Arnon (1963). Particles enriched in Photosystem I were isolated by using a French press as described by Sane *et al.* (1971) or with the anionic detergent Triton X-100. The Triton X-100 treatment

was performed as described by Vernon & Shaw (1971) to the stage where the Photosystem-II particles were removed by centrifugation; the Photosystem-I particles were then isolated by chromatography on hydroxyapatite. The supernatant from the centrifugation (approx. 200 ml) was applied to a column (4.5 cm $\times$ 15 cm) of hydroxyapatite equilibrated with 0.01 M-potassium phosphate buffer, pH 6.9, containing 0.1% Triton X-100 and 0.2 M-NaCl. The column was then washed with 0.02 M-Tris/HCl buffer, pH 7.5, containing 0.1% Triton X-100 and 0.2 M-NaCl to remove a chlorophyll-containing band. The Photosystem-I particles were then eluted with 0.09 M-potassium phosphate buffer, pH 6.9, containing 0.1% Triton and 0.2 M-NaCl. The eluate was concentrated by ultrafiltration by using a Diaflo XM 100 membrane (Amicon, High Wycombe, Bucks., U.K.). The particles were made 0.1 M with Tris/HCl buffer, pH 8.0, and stored frozen in liquid N_2 until required. If Tris buffer was not added the particles were inactivated by pH changes during freezing of the phosphate buffer. The preparation normally had a molar P700:chlorophyll ratio, determined as the ascorbate-reduced-versus-ferricyanide-oxidized difference spectrum in the 700 nm region, of between 1:80 and 1:120, assuming a molar extinction coefficient for P700 of 64 000 (Hiyama & Ke, 1972). This ratio appeared to be a good index of Photosystem-I reaction-centre concentration when compared with the size of the bound-ferredoxin signals seen by e.p.r. spectrometry. The hydroxyapatite was prepared by the procedure of Tiselius *et al.* (1956).

E.p.r. spectra were obtained by using a Varian E4 spectrometer as described previously (Evans *et al.*, 1972, 1976a). E.p.r. samples were prepared anaerobically in silica tubes (3 mm internal diameter). The oxidation-reduction-potential titrations were done in a vessel similar to that described by Dutton (1971) and samples transferred anaerobically to e.p.r. tubes through a stainless-steel tube. The oxidation-reduction potential was measured with a platinum electrode (Radiometer, Copenhagen, Denmark; type P101) and a standard calomel electrode (Radiometer; type K401). The electrodes were standardized with saturated quinhydrone, pH 7.0. All potential measurements were made at 25°C. The titrations were done under a dim green safelight after preincubation of the material in the dark for 20 min. After removal from the titration vessel the samples were kept in complete darkness for 30 s before freezing in liquid N_2 and then stored in liquid N_2 in the dark. The samples were illuminated in the e.p.r. spectrometer at the temperature used for the measurements (15–20 K) with a 1000 W projector. The titrations were done in the oxidizing direction by using $K_3Fe(CN)_6$ (0.2 or 1 M) as oxidizing agent or in the reducing direction by using 0.2 M- $Na_2S_2O_4$ in 0.1 M-Tris/HCl buffer, pH 9.0, as reducing agent. The material was

initially reduced to about 0mV with $\text{Na}_2\text{S}_2\text{O}_4$ or oxidized to about +500mV with $\text{K}_3\text{Fe}(\text{CN})_6$ and equilibrated for 20min before starting the titration. The reaction mixture also contained 100 μM -benzoquinone ($E'_0 = +280\text{mV}$) and in some cases 100 μM -*NN*-dimethyl-*p*-phenylenediamine ($E'_0 = +380\text{mV}$), which together with the ferricyanide function as mediators between the electrode and the electron-transport-chain components.

Absorption spectroscopy was done with an Aminco DW2 spectrophotometer operating in the split-beam or dual-wavelength mode as appropriate. Liquid- N_2 -temperature (77K) spectra were measured by using the standard Aminco accessory. In this system the reference-sample and experimental-sample cuvettes are attached to each other and it is virtually impossible to illuminate one without illuminating the other. We have therefore used the following procedure to obtain light-minus-dark difference spectra of P700. A fully oxidized sample was placed in the reference cuvette and a sample poised at a known potential in the sample cuvette. The split-beam difference spectrum was recorded and stored in one memory of a Nicolet 1020A Signal Averager (Techmation, Edgware, Middx., U.K.). Both samples were then illuminated at 77K. After illumination the difference spectrum was again recorded and stored in the second memory of the Nicolet instrument. A light-minus-dark spectrum for the sample was then obtained by subtracting one spectrum from the other. A number of assumptions are involved in this procedure. It must be assumed that in the oxidized sample (+480mV) P700 is fully oxidized; we could not detect any light-induced difference between samples at +420mV and the oxidized sample, suggesting that this assumption is reasonable. It must also be assumed that any light-induced changes other than those due to P700 will be the same in both samples. Our results suggest that this is the case, at least in the 685–720nm region. Samples for 77K spectroscopy were prepared in 60% (v/v) glycerol to form a glass on freezing. The oxidation-reduction potentials were poised as described below.

Oxidation-reduction-potential titrations were performed either by the procedure described by Dutton (1971), in which the potential of the reaction mixture is measured continuously and adjusted by addition of oxidant [0.2M- $\text{K}_3\text{Fe}(\text{CN})_6$] or reductant (0.2M- $\text{Na}_2\text{S}_2\text{O}_4$) in the presence of the mediators used in the e.p.r. experiments, or by the addition to the sample of known mixtures of $\text{Fe}(\text{CN})_6^{2+}/\text{Fe}(\text{CN})_6^{3+}$. In the latter case the potential of the sample was also measured with a platinum electrode.

The light-induced absorbance change at 430nm attributed to P700 was measured as the change in absorbance of the sample on illumination with 649nm light obtained by using a Balzers (Berk-

hamsted, Herts., U.K.) B40 interference filter. The spectrophotometer was operated in the split-beam mode with identical samples in the reference and measuring beams. The photomultiplier was protected from the actinic light by a Schott BG12 filter (H. V. Skan, Solihull, West Midlands, U.K.). The reference sample was kept dark by shielding it from the actinic beam while the measured sample was illuminated. The extent of the reversible light-induced absorbance change was determined at different applied oxidation-reduction potentials. The potential was measured before and after illumination and it was found that illumination did not cause any significant change in potential of the solution. This procedure was used rather than the more conventional dual-wavelength method because of interference by the absorption of the $\text{K}_3\text{Fe}(\text{CN})_6$ used as oxidant. The absorbance change observed was of a size predicted for the amount of P700 present in the sample on the basis of the extinction coefficient for the changes at 697 and 430nm (Hiyama & Ke, 1972).

Tris, Triton X-100 and sodium ascorbate were from Sigma (London) Chemical Co. (Kingston-upon-Thames, U.K.). Other chemicals were from BDH (Poole, Dorset, U.K.). AnalaR-grade reagents were used when available; for other chemicals the supplier's purest grade was used.

Results and Discussion

If chloroplasts or Photosystem-I particles are equilibrated in the dark, frozen, and their e.p.r. spectra examined at 20K there are normally essentially no signals in the $g = 2.00$ region. If the sample is then illuminated, at 20K, a free-radical signal ($g = 2.0026$), corresponding to the oxidation of the reaction-centre chlorophyll, and signals at $g = 2.05$, 1.94 and 1.86, corresponding to centre A of the bound ferredoxin, appear (Fig. 1). This reaction will occur over a wide initial oxidation-reduction-potential range. At very low potentials, when the iron-sulphur centres are chemically reduced, reaction-centre chlorophyll oxidation becomes reversible (Evans & Cammack, 1975) and the reversible reduction of a component thought to be the primary electron acceptor is observed (Evans *et al.*, 1975). It might be expected that at the oxidized end of the oxidation-reduction-potential range oxidation of the reaction centre would mean that it is no longer possible to donate electrons to centre A on illumination. Fig. 1 shows that this is indeed the situation and that the ability to photoreduce centre A is lost between +300 and +400mV. This loss of activity is paralleled by the appearance of a chemically oxidized free radical in the sample in the dark. The spectra shown in Fig. 1(a) were obtained by using particles obtained by mechanical disruption of chloroplasts with the French

press. Similar results are obtained for the reduction of the iron-sulphur centre A in particles prepared with the detergent Triton X-100 (Fig. 1b) and also in unfractionated chloroplasts. However, in these preparations the changes in the free radical are more difficult to see, because a very 'large' ferricyanide-induced free radical overlaps the potential range of the reaction-centre radical. The spectrum above $g = 2.00$ is also obscured by a large signal, which has been attributed to plastocyanin (Knaff & Malkin, 1973), with an oxidation-reduction potential in a similar range to that of the reaction centre. This signal is absent from most preparations made by French press. The experiment may also be difficult to do in unfractionated chloroplasts because very high chlorophyll concentration must be used and often large e.p.r. signals corresponding to manganese obscure the spectrum. We have used this change in ability to photoreduce the bound iron-sulphur centre and oxidize the reaction centre to determine the mid-point oxidation-reduction potential (E_m) of the reaction-centre chlorophyll. Fig. 2 shows a titration in the +300–+400 mV range of Photosystem-I particles prepared with the French press; it shows the

changes in the light-induced iron-sulphur signal (Fig. 2a) and the light-induced free-radical signal (Fig. 2b). Both signals decrease in size as the potential becomes more oxidized, with $E_m \approx +375$ mV. This experiment clearly shows that the reaction centre that donates electrons to the iron-sulphur centre is oxidized with $E_m = +375$ mV, and the parallel behaviour of the light-induced free radical suggests that the component giving rise to it is the electron donor to the bound ferredoxin. We have also done this experiment with Photosystem-I particles prepared with Triton X-100, in which the P700:chlorophyll ratio is 1:80–1:100. Fig. 3 (a) shows that the ability to photoreduce the iron-sulphur centre is again lost, with $E_m \approx +375$ mV. However, in these particles (Fig. 3b) there is no parallel loss of the light-inducible free-radical signal. The apparently random behaviour of the light-induced radical is due to an overlapping of the potential range in which the reaction-centre radical and a second light-induced radical described below are observed in these preparations. The second light-induced radical, which appears under the conditions used to be much larger than the reaction-centre radical, is observed at

Fig. 2. Oxidation-reduction-potential titrations of the light-inducible e.p.r. signal of the Photosystem-I reaction centre in Photosystem-I particles prepared with the French press

(a) The $g = 1.94$ signal of the reduced bound ferredoxin; (b) the $g = 2.00$ signal of the oxidized reaction-centre chlorophyll. The samples were prepared and spectra recorded as in Fig. 1. The light-induced increase in height of the signal at $g = 1.94$ was taken as a measure of the extent of reduction of the bound ferredoxin, and the light-induced increase in the free-radical signal at $g = 2.00$ as a measure of the extent of photo-oxidation of the reaction-centre chlorophyll. The curves drawn are the theoretical curves for a one-electron-accepting centre with $E_m = +375$ mV.

Fig. 1. Effect of the oxidation-reduction potential on the light-inducible changes in Photosystem I at low temperature (a) Photosystem-I particles prepared with the French press (2 mg of chlorophyll/ml); (b) Photosystem-I particles prepared with Triton X-100 (1 mg of chlorophyll/ml). The samples were poised at the different potentials as described in the Materials and Methods section. The spectra of the samples was recorded in the dark and under illumination. There was no further change if the light was turned off. The spectra were recorded at 18 K with the following instrument settings: frequency, 9.2 GHz; power, 20 mW; modulation amplitude, 1 mT; scan rate, 100 mT/min; instrument gain, 1000.

Fig. 3. Oxidation-reduction-potential titrations of the light-inducible e.p.r. signals of the Photosystem-I reaction centre in Photosystem-I particles prepared with Triton X-100

(a) The $g = 1.94$ signal of the photoreduced bound ferredoxin; (b) the light-induced signal at $g = 2.00$. The experiments were performed as in Figs. 1 and 2. The curves drawn are the theoretical curves for a one-electron-accepting centre with $E_m = +375$ mV.

more oxidized potentials. This radical appears at a potential about 20 mV more reduced in Photosystem-I particles made with Triton X-100 than in particles made with the French press. An experiment with unfractionated chloroplasts also showed $E_m \approx +380$ mV for iron-sulphur centre A photoreduction, but no variation in the light-inducible free radical. In all of these preparations the E_m for photoreduction of the iron-sulphur centres is more reduced than +400 mV and is some 40 mV more negative than any reported potential for P700. We have obtained E_m more oxidized than +400 mV for only one type of preparation, that made by treatment with digitonin and Triton X-100 by the procedure of Bengis & Nelson (1975). For these particles we found $E_m = +415$ mV; however, the membrane system is presumably modified in these particles and we consider that the value of $E_m = +375$ mV found in particles made by French press is likely to be the best indicator of the potential *in vivo*. We have confirmed that our technique is giving a correct value for the oxidation-reduction potential in the region being studied by titrating a solution of pure plastocyanin. This is reported to have $E_m = +370$ mV (Kato, 1971). In two titrations we obtained values of $E_m = +375$ and +370 mV. This result confirms that our technique is providing accurate results.

The large difference between our value for the E_m of the reaction-centre chlorophyll of Photosystem I and those reported by other workers for P700 raises the question whether we are in fact measuring P700. If we are not, we should have to conclude either that P700 is not the reaction-centre chlorophyll of Photosystem I or that the system we are studying is not Photosystem I at all. If, on the other hand, we are

measuring P700, which is the reaction-centre chlorophyll of Photosystem I, it would appear that the techniques used by other workers have given values for the E_m of P700 that are consistently too oxidized. In an attempt to resolve this problem we have further investigated the effect of oxidation-reduction potential on the 'signal 1' free radical seen at low temperature and have used a number of optical techniques in an attempt to obtain equivalent results for P700.

Even at potentials more reduced than +400 mV we have found that the free-radical signal induced in the dark by chemical oxidation does not correlate very well with the reaction-centre free radical, and that in the particles prepared with Triton X-100 the size of the light-induced free radical also appears to be unrelated to the extent of photoreduction of the iron-sulphur proteins. If the titration is continued into the +400–+500 mV range with particles prepared either with the French press (Fig. 4a) or with Triton X-100 (Fig. 5a), a very large free-radical signal appears, which under the conditions of measurement used in these experiments may be up to 100 times larger than the reaction-centre signal. This signal continues to increase in size up to the most oxidized potential that we have been able to obtain by using ferricyanide as oxidant ($\approx +510$ mV). This signal is presumably the signal measured by Knaff & Malkin (1973) and assumed by them to be due to P700. They report it to have $E_m = +520$ mV. We were unable to obtain the very oxidized potentials that they reported; if it is assumed that a single one-electron oxidation is involved, our results suggest a potential between +450 and +500 mV. We do not think that this signal is due to a reaction-centre chlorophyll. It is probably due to the oxidation of bulk chlorophyll, a conclusion

Fig. 4. Oxidation-reduction-potential titrations of the 'large' free-radical signals observed in Photosystem-I particles prepared with the French press

(a) The $g = 2.00$ radical signal induced by chemical oxidation in the dark; (b) the light-induced increment to the $g = 2.00$ radical signal after 30s illumination at 20K. The experiments were performed as in Figs. 1 and 2. The units of signal size are the same as those used in Figs. 2 and 3. The curves drawn are the theoretical curves for a one-electron-accepting centre with $E_m = +490$ mV. They are shown only as an approximation. The titrations do not show an end point, so that the true mid-point potential cannot be determined.

Fig. 5. Oxidation-reduction potential titration of the 'large' free-radical signal observed in Photosystem-I particles prepared with Triton X-100

(a) The $g = 2.00$ radical signal induced by chemical oxidation in the dark; (b) the light-induced increment to the $g = 2.00$ radical signal after 30s illumination at 20K. The experiments were done as in Figs. 1 and 2. The units of signal size are the same as those in Figs. 2, 3 and 4. The curves drawn are the theoretical curves for a one-electron-accepting centre with $E_m = +475$ mV. They are shown only as an approximation. The titrations do not show an end point, so the true mid-point potential cannot be determined.

supported by the optical spectra reported below. The appearance of this 'large' radical due to chemical oxidation is accompanied by the appearance of a second 'large' radical induced by illumination at low temperature (Figs. 4b and 5b). The signal size shown in Figs. 4 and 5 is after 30s illumination. This signal in fact has a very slow rise time and the signal con-

tinues to increase in size during illumination for 5 min or longer. Although the conditions under which this signal is seen are similar to those described by Bearden & Malkin (1973) for a signal which they suggest is related to the Photosystem-II reaction centre, it seems unlikely that the signal seen in these experiments is closely related to a reaction centre.

Fig. 6. Oxidation-reduction-potential titrations of Photosystem-I particles, prepared with Triton X-100, by dual-wavelength spectroscopy (698–720 nm)

(a) Oxidative titration; (b) reductive titration. The reaction mixture contained 50 mM-Tris/HCl buffer, pH 8.0, 66 μ M-benzoquinone and Photosystem-I particles prepared with Triton X-100 (15 μ g of chlorophyll/ml). The sample was initially reduced to about 0 mV with sodium ascorbate and titrated by addition of $K_3Fe(CN)_6$ (a) or oxidized by addition of 10 mM- $K_3Fe(CN)_6$ (about +550 mV) and titrated with sodium ascorbate (b). The solid lines drawn are the theoretical curves for a one-electron-accepting centre with $E_m = +460$ mV (a) and +480 mV (b). They are shown as an approximation. The titrations do not show an end point, so the true mid-point potential cannot be calculated. The broken line indicates the maximum signal size that would have been expected for the amount of P700 in the sample. The experiment was done at 298 K.

The slow rise time of the signal on illumination, together with its appearance in parallel with the large dark chemically induced free radical, suggest it is more likely to be a bulk chlorophyll reaction.

We conclude from these experiments that there are three components that may be seen as signal 1 at low temperature, only one of which is due to oxidation of reaction-centre chlorophyll. This is different from the situation at room temperature, where there is very good evidence that only one free radical induced by illumination or chemical oxidation is observed, which is due to the reaction centre (Warden & Bolton, 1973; Calvin & Androes, 1964). This difference is presumably due to differences in the saturation characteristics of the signals. All of the signals show saturation effects under the conditions used for the present experiments and are saturated at the lowest microwave energy available with our spectrometer at both 20 and 77 K. The 'small' free radical due to the reaction-centre chlorophyll appears to be some five times larger at 0.5 than at 20 mW.

The oxidation-reduction potential of P700 has been determined optically by at least three different procedures. Knaff & Malkin (1973) and Ke *et al.* (1975) used a dual-wavelength technique similar to that which is frequently used for oxidation-reduction potential titrations of cytochromes. Knaff & Malkin (1973) observed essentially the same E_m (+520 mV) as in their e.p.r. experiments (+525 mV). Ke *et al.* (1975) were unable to obtain an end point for the titration because of oxidation of bulk chlorophyll; however,

they found $E_m = +468$ mV by comparison of the maximum light-inducible signal with the ferricyanide-induced signal. We have repeated this experiment (Fig. 6) and obtained essentially the same result as Ke *et al.* (1975). We have done this experiment in both the oxidative (Ke *et al.*, 1975) and reductive directions (Knaff & Malkin, 1973). Our results suggest an $E_m \approx 475$ mV and do not agree with the E_m (+520 mV) reported by Knaff & Malkin (1973). However, in agreement with Ke *et al.* (1975), the observed signal size is much larger than we would have expected on the basis of the P700 concentration of the samples. If the spectrum of the samples at different potentials was measured, we found that there was a very large contribution from bulk chlorophyll oxidation to the $A_{697} - A_{720}$. We consider that the dual-wavelength titration is largely measuring the same change as that represented by the 'large' free-radical signal with $E_m \approx +475$ mV, and that this change is due to oxidation of bulk chlorophyll.

Kok (1961) determined the mid-point oxidation-reduction potential, E_m , of P700 to be +420 mV [corrected for E_m of $K_3Fe(CN)_6$ used] or +400 mV in aged particles followed either by measuring the difference spectrum of samples at different potentials against a reduced control or by measuring the light-induced change in A_{700} at different potentials. We have measured the E_m on the basis of a titration in which we measured the difference spectrum between the poised sample and a reduced sample to obtain a measure of the extent of oxidation of P700. Fig. 7

Fig. 7. Oxidized-minus-reduced difference spectra of Photosystem-I particles prepared with Triton X-100 at selected oxidation-reduction potentials

The reaction mixture contained 50 mM-Tris/HCl buffer, pH 8.0, 60 μ M-*NN*-dimethylphenylenediamine, 10 μ M-sodium ascorbate and Photosystem-I particles prepared with Triton X-100 (15 μ g of chlorophyll/ml). The initial potential of the reaction mixture was about 0 mV. The reaction mixture was divided in two; one half was placed in the reference cuvette and one half in the titration cuvette. The sample was then titrated to the required potential by addition of $K_3Fe(CN)_6$ and, after equilibration, the difference spectra were recorded. The experiment was done at 298 K. Wavelengths are shown in nm.

shows spectra at selected potentials and Fig. 8 shows an oxidation-reduction-potential titration based on these curves. Again it was difficult to define the end point of the titration because of bulk chlorophyll oxidation; however, it is clear that P700 oxidation commences at +320 mV and it is probably complete by +420–+440 mV. If it is assumed that a single-electron oxidation is involved, these experiments indicate that $E_m \approx +360$ mV, in reasonable agreement with our e.p.r. data. Kok (1961) apparently avoided most of the problems with bulk chlorophyll by acetone extraction of the chloroplasts before use. He did not show spectra of the samples used in the titration, so it is not possible to tell to what extent he corrected for this problem. It seems likely that our results are the same as his, within the experimental error of this procedure.

P700 photo-oxidation may be followed by measuring the change in A_{703} upon illumination. Rumberg (1964) has determined the mid-point potential of

Fig. 8. Oxidation-reduction-potential titration of P700 in Photosystem-I particles made with Triton X-100

Spectra were recorded as in Fig. 7. The ΔA_{698} was taken as a measure of P700 oxidation. The line drawn shows the theoretical curve for a one-electron-accepting centre with $E_m = +360$ mV.

P700 by using this method with samples poised at set redox potentials; he found $E_m = +460$ mV, although he gave an error of ± 100 mV. For comparison with this experiment we have measured the extent of photo-oxidation of the 435 nm change attributed to P700 (Hiyama & Ke, 1972). We used this wavelength to avoid fluorescence artifacts associated with measurements at 700 nm. We have measured the potential dependence of this light-induced change (Figs. 9 and 10), and in agreement with Rumberg (1964) observed $E_m = +464$ mV. This agrees with the E_m that we have found for the 'large' free radical, not for the reaction-centre chlorophyll.

All the spectroscopic determinations of P700 potential discussed above have been done at room temperatures (25°C), whereas the e.p.r. determinations were done at cryogenic temperature; it therefore seemed desirable to confirm that the effect of potential on P700 determined by optical spectroscopy was the same at 77 K as at room temperature. P700 photo-oxidation can be observed at liquid-N₂ temperature (77 K) (Witt, 1971) and is irreversible at potentials more oxidized than –500 mV (Lozier & Butler, 1974). We have determined the extent of P700 photo-oxidation in the +300–+420 mV range as the light-minus-dark difference spectrum at 77 K. The design of our apparatus is not ideal for this experiment, as we had to obtain the spectra as the difference between two difference spectra (see the Materials and Methods section) and have had to assume that P700 is fully

Fig. 9. Light-induced absorption changes at 435 nm at different oxidation-reduction potentials

The reaction mixture contained 50 mM-Tris/HCl buffer, pH 8.0, and Photosystem-I particles prepared with Triton X-100 (18 μ g of chlorophyll/ml); a mixture of potassium ferri- and ferro-cyanide in the correct proportions was added to poise the potential, with a total concentration of 1 mM. The experiment was done at 298 K. $\downarrow$, Light on; $\uparrow$, light off. Experimental procedure was as described in the Materials and Methods section.

oxidized at +480 mV. In view of these problems, and the difficulties involved in preparing identical frozen samples for spectroscopy owing to cracking of the glycerol glass, we have attempted only a semi-quantitative determination of the P700 potential in this experiment. Fig. 11 shows the P700 spectrum induced by illumination of samples frozen at three different potentials. In the sample frozen at +312 mV,

Fig. 10. Oxidation-reduction-potential titration of the light-induced change at 435 nm

The extent of the light-induced change in A_{435} was determined as in Fig. 9. The line drawn is the theoretical curve for a single-electron-accepting centre with $E_m = +460$ mV.

Fig. 11. Light-induced absorption changes of P700 at 77 K at different oxidation-reduction potentials

Light-minus-dark difference spectra were obtained as described in the Materials and Methods section. The samples were poised at the potentials shown before freezing in the dark. Wavelengths are shown in nm.

a large P700 signal is induced by illumination at 77 K; this signal is in fact as large as that seen in an ascorbate-reduced-minus-ferricyanide-oxidized spectrum of the same material. At +387 mV the light-induced signal is significantly smaller and at +420 mV no light-induced signal is seen. These results suggest that P700 is fully oxidized at or above +420 mV and fully reduced at or below +312 mV, indicating a mid-point potential between +350 and +400 mV.

These experiments clearly show that the determination of the oxidation-reduction potential of the

reaction-centre chlorophyll of Photosystem I is extremely difficult. Determinations by optical spectroscopy at room temperature are subject to severe interference by bulk chlorophyll changes. Dual-wavelength techniques based on chemical oxidation appear to measure a change in the bulk chlorophyll with $E_m \approx +475$ mV; determinations based on the light-induced change at 435 nm give similar results. Techniques in which the spectral changes around 700 nm are determined as difference spectra at room temperature or at 77 K seem likely to give a better measure of the reaction-centre potential and indicate $E_m \approx +360$ mV, in agreement with the e.p.r. results. E.p.r. experiments based on the measurement of the chemically induced free radical indicate $E_m \approx +480$ mV, but show no correlation with the photochemical function of the reaction centre, and we attribute this signal to bulk chlorophyll. Measurement of the light-induced free radical at low temperatures is also misleading, as two such signals may be seen, and under the e.p.r. conditions normally used to investigate Photosystem I the reaction-centre signal appears very small.

We therefore conclude that the best measurement of the oxidation-reduction potential of the reaction centre is determined by measuring its ability to photo-reduce centre A of the bound ferredoxin at low temperature. This ability is lost with $E_m = +375$ mV. In particles made by French press the photo-oxidation of the 'small' free radical is also lost with $E_m = +375$ mV. We consider that this 'small' radical is due to the oxidized reaction-centre chlorophyll, whereas the very much 'larger' radicals seen under other conditions are due to bulk chlorophyll. Our results support the view that the reaction centre shows an extinction change around 700 nm on oxidation, which could be attributed to P700, but clearly show that it is unwise to attribute all changes in this region to the reaction-centre chlorophyll.

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