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THE ROLE OF THE MEMBRANE-BOUND IRON-SULPHUR CENTRES A AND B IN THE PHOTOSYSTEM I REACTION CENTRE OF SPINACH CHLOROPLASTS

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Summary

Photosystem I particles prepared from spinach chloroplast using Triton X-100 were frozen in the dark with the bound iron-sulphur Centre A reduced. Illumination at cryogenic temperatures of such samples demonstrated the photoreduction of the second bound iron-sulphur Centre B. Due to electron spin-electron spin interaction between these two bound iron-sulphur centres, it was not possible to quantify amounts of Centre B relative to the other components of the Photosystem I reaction centre by simulating the line-shape of its EPR spectrum. However, by deleting the free radical signal I from the EPR spectra of reduced Centre A alone or both Centres A plus B reduced, it was possible to double integrate these spectra to demonstrate that Centre B is present in the Photosystem I reaction centre in amounts comparable to those of Centre A and thus also signal I (*P*-700) and X.

Oxidation-reduction potential titrations confirmed that Centre A had $E_m \simeq -550$ mV, Centre B had $E_m \simeq -585$ mV. These results, and those presented for the photoreduction of Centre B, place Centre B before Centre A in the sequence of electron transport in Photosystem I particles at cryogenic temperatures. When both A and B are reduced, *P*-700 photooxidation is reversible at low temperature and coupled to the reduction of the component X. The change from irreversible to reversible *P*-700 photooxidation and the photoreduction of X showed the same potential dependence as the reduction of Centre B with $E_m \simeq -585$ mV, substantiating the identification of X as the primary electron acceptor of Photosystem I.

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Introduction

Illumination of chloroplasts results in the oxidation of the reaction centre chlorophyll *P*-700 [1] of Photosystem I and the transfer of the electron to an electron acceptor complex. An electron parameter resonance (EPR)-detectable light-induced free radical signal (signal I) was observed in chloroplasts [2]; and associated with *P*-700 [3—5].

Malkin and Bearden [6] observed an EPR signal characteristic of an iron-sulphur centre (Centre A with $g_x = 2.05$, $g_y = 1.95$, $g_z = 1.86$) when spinach chloroplasts were illuminated at 77 K and the spectra recorded at 25 K. They attributed this signal to a membrane-bound iron-sulphur centre and suggested that it might act as a primary low potential electron acceptor in chloroplast photosynthesis. Other workers confirmed this initial observation [7,8]. Evans et al. [9] demonstrated that this bound ferredoxin was enriched in Photosystem I subchloroplast particles and concluded that it was acting as primary electron acceptor for Photosystem I. However, They also found that the EPR spectra indicated the presence of a second iron-sulphur centre (Centre B $g_x = 2.05$, $g_y = 1.92$, $g_z = 1.89$). Both centres could be photoreduced at room temperature and were thought to represent two active centres of a single protein. Although Malkin and Bearden [6] had observed these other signals in their EPR spectra, they had attributed them to a damaged form of the initially reduced Centre A.

Bearden and Malkin [10] determined the relative amounts of Centre A and *P*-700 generated upon illumination of Photosystem I at 77 K, using simulations of the EPR spectra of these components, and concluded that the stoichiometric relationship between the two components was unity. These results suggested that Centre A was indeed acting as the primary acceptor of electrons from the reaction centre chlorophyll *P*-700.

Oxidation-reduction potential titrations [11,12] showed that both centres had very low mid-point potentials, Centre A $E_m = -550$ mV and Centre B $E_m = -590$ mV confirming that they were good candidates for the role of primary electron acceptors. In these titrations it was found that the $g = 1.86$ component of the Centre A signal dissappeared as Centre B was reduced. This had also been observed in photochemical reduction experiments. This change in the spectrum can be most satisfactorily explained as being due to electron spin-electron spin interactions between the two centres [12], rather than to the presence of multiple components as was originally proposed [11]. The identification of the components as iron-sulphur centres is confirmed by iron 57 substitution experiments [13] and the effect of dimethyl sulfoxide treatment on the spectra indicates the presence of two 4Fe-4S centres [14]. We have presented preliminary evidence for the low temperature photoreduction of Centre B [15] and have shown that the intermediary carrier X between *P*-700 and the iron-sulphur centres is only observed in the reduced state when both iron-sulphur centres are reduced [13,16]. The quantitative relationship of Centre B to other components of the reaction centre has not, however, been investigated. We have now confirmed the low temperature photoreduction of Centre B by *P*-700 and determined the quantitative relationship between Centres A and B. We have also determined the precise relationship between

the oxidation state of the iron-sulphur centres and the reversible photooxidation of *P*-700 coupled to the reduction of X.

Materials and Methods

Photosystem I particles were prepared from spinach (*Spinacea oleracea*) using the non-ionic detergent Triton X-100 as described previously [17]. EPR spectra was recorded on a Varian E4 spectrometer operating at X band frequencies, and stored on cassette tape using a Hewlett-Packard 9830A (Hewlett Packard Ltd., London, U.K.) interfaced to a Nicolet 1020A (Nicolet Instrument Corp., Warwick, U.K.). Deletion of the free-radical component of the EPR spectrum of bound iron-sulphur centres was performed using this system, as was double integration of the resulting spectra. The EPR samples were cooled to 20.0K in an Oxford Instruments Company Ltd. (Oxford, U.K.) liquid helium cryostat. The oxidation-reduction potential titrations were carried out as described by Evans et al. [18], samples being illuminated in the EPR cavity using a 1000 W projector with a water filter and a Schott KG3 heat filter (Schott, H.J. Skan Ltd., Solihull, Warwicks., U.K.) in the light path. Tris, Triton X-100 (octylphenoxypolyethoxyethanol) were from Sigma (London) Chemical Co. (Kingston-upon-Thames, U.K.). Other chemicals were from BDH Ltd., (Poole, Dorset, U.K.) using AnalaR-grade reagents where possible.

Results

The ability of the Photosystem I reaction centre to photoreduce Centre B, and the involvement of Centre B in electron transport in the photosystem reaction centre has not been widely accepted. Fig. 1 shows the photoreduction of Centre B at 20 K in a Photosystem I preparation in which Centre A was largely reduced before freezing. Photosystem I particles prepared using the detergent Triton X-100 were incubated in the dark with dithionite for a short period and frozen in liquid nitrogen in the dark. These samples showed the EPR spectrum of Centre A with signals at $g_x = 2.05$, $g_y = 1.95$ and $g_z = 1.86$, only a very small free radical signal, and a small fraction of the EPR spectrum of Centre B with components at $g_x = 2.05$, $g_y = 1.92$ and $g_z = 1.89$ (Fig. 1a). Illumination of this sample in the EPR cavity increased the size of the components of the EPR spectrum of Centre B, and produced a free radical signal (Fig. 1b). Subtraction of these spectra yielded a difference spectrum for the light-induced Centre B (Fig. 1c). This spectrum does not have the lineshape expected for a bound four-iron iron-sulphur centre. The $g = 1.89$ component is disproportionately large. We think that this is due to electron spin-electron spin interaction with Centre A resulting in the movement of the $g = 1.86$ component of Centre A to $g = 1.89$ first observed by Evans et al. [12].

We have previously quantified the relative amounts of *P*-700, Centre A and X in Photosystem I particles using computer simulations of their spectra. This has enabled us to compare the light-induced signals of these components and show stoichiometric relationship between *P*-700 oxidation and reduction of the accepters. [17,19]. We had hoped to do this with Centre B. However,

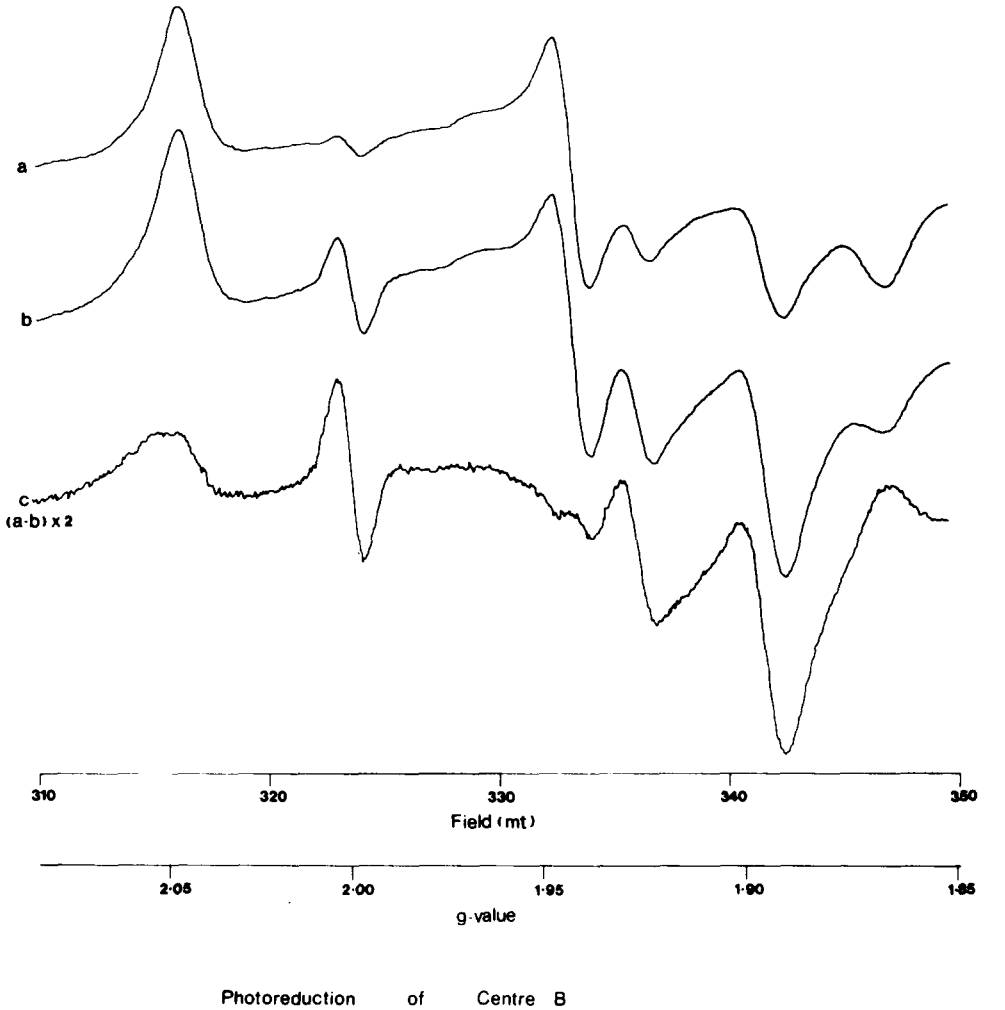

Fig. 1. EPR spectra of Photosystem I particle showing the photoreduction of the bound iron-sulphur Centre B. Photosystem I particles prepared using the detergent Triton X-100 (1.2 mg chlorophyll/ml) were pre-incubated at pH 8.0 in the dark for 2 min in the presence of dithionite (0.2%, w/v) and frozen in the dark. (a) Spectrum in the dark. (b) After illumination for 30 s at 20 K. (c) (a — b) X 2. The spectra were recorded at 20 K and the following instrument settings: frequency, 9.06 GHz; microwave power, 20 mW; modulation amplitude, 1 mT; scan rate, 20 mT/min; instrument gain, $5 \cdot 10^2$.

we have been unable to simulate either the spectrum of Centre B or of Centre A plus B because of the electron spin-electron spin interaction between the two centres: we have not, therefore, been able to quantify the relationship between the light-induced *P*-700 and Centre B, However, the relative amounts of Centre A and Centre A plus B can be determined. The EPR spectra of Centre A alone and Centre A plus B from the same preparation could be double integrated, to give an indication of the relative quantity of electrons that they represented. Centre A spectra (Fig. 2a), produced by illuminating ascorbate-reduced particles at low temperature, show a free radical signal due to oxidised *P*-700 as well as the reduced Centre A signal. This is also seen in spectra of Centres A

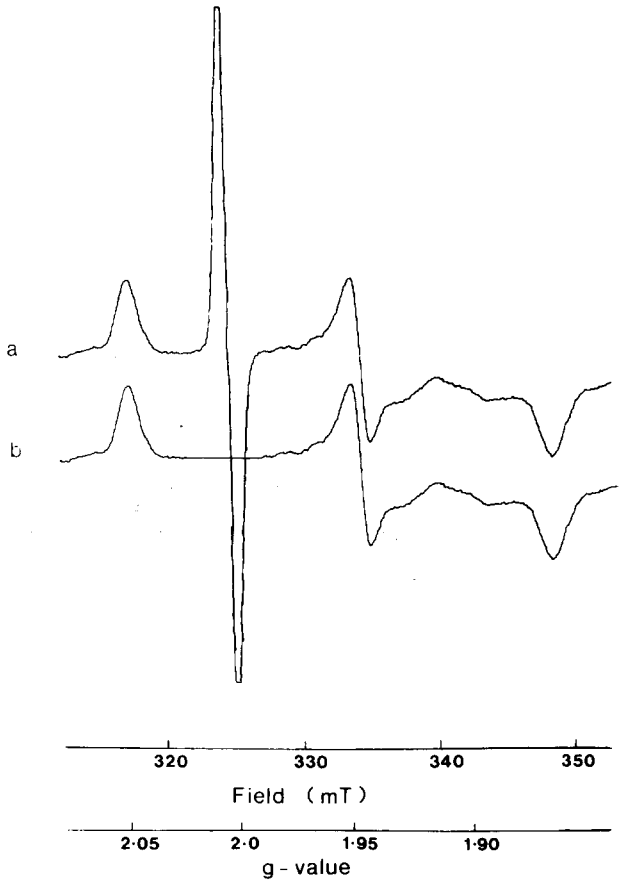

Fig. 2. The deletion of the free radical component of a recorded bound iron-sulphur Centre A EPR spectrum. Photosystem I particles prepared using the detergent Triton X-100 (1.52 mg chlorophyll/ml) were preincubated in the dark at pH 8.0 with 10 mM ascorbate and frozen in the dark. The measured EPR spectrum of the sample (after illumination at 77 K in a finger dewar) is shown before (a) and after (b) the deletion of the free radical signal I. The EPR spectrum was recorded at 20 K and the following instrument settings: frequency, 9.11 GHz; microwave power, 5 mW; modulation amplitude, 0.4 mT; scan rate, 25 mT/min; instrument gain, $1 \cdot 10^3$.

plus B (Fig. 3a) produced by illuminating particles at room temperature in the presence of dithionite, turning the light off to allow the signal of X to decay, and then freezing the sample to liquid nitrogen temperatures. The samples were reilluminated at 77 K to ensure reduction of any small proportion of the iron-sulphur centre which might have become oxidised. To allow comparison of the double integrated intensities of the bound iron-sulphur centre spectra alone, the Hewlett-Packard 9830A was programmed to delete the region of the spectrum containing the free radical and substitute an artificial baseline as shown in Fig. 2b and 3b. The signal size of a standard copper-EDTA sample was recorded after each measurement of a bound iron-sulphur centre spectra, to ensure that there were not changes in temperature during the experiment. In each of two experiments using different Photosystem I particle preparations, the double integrated intensity of 8 Centre A spectra and 8 Centre A + B

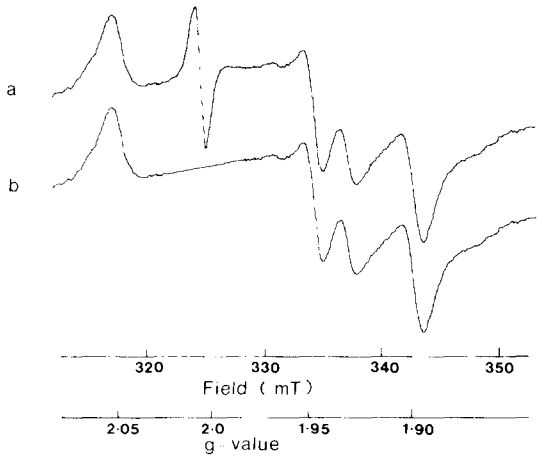

Fig. 3. The deletion of the free radical signal I from the recorded EPR spectrum of the bound iron-sulphur Centres A and B. Photosystem I particles prepared using the detergent Triton X-100 (1.52 mg chlorophyll/ml) were illuminated in the presence of sodium dithionite (0.2%, w/v) for 2 min at room temperature, left in the dark for 90 s and then frozen in the dark. The samples were later illuminated in a finger dewar at 77 K to ensure that the irreversible reduction of Centre B was complete. The recorded EPR spectrum before (a) and after (b), the deletion of the free radical signal I are shown above. The EPR spectrum was recorded at 20 K and the following instrument settings: frequency, 9.11 GHz; modulation amplitude, 0.4 mT; microwave power, 5 mW; scan rate, 25 mT/min; instrument gain, $1 \cdot 10^3$.

spectra were compared. In the first experiment using 1,58 mg chlorophyll/ml, the intensities (in arbitrary units) were Centre A 5754 ± 172 and Centre A + B 10 960 ± 367 giving an A + B : A ratio of 1.905. In the second experiment, using 1.18 mg chlorophyll/ml, the intensities were Centre A 3135 ± 83, and Centre A + B 6351 ± 163 giving an A + B : A ratio of 2.03. These results strongly suggest that there are equal amounts of Centre A and Centre B, and that Centre B can act as a component in electron transfer in Photosystem I. The Centre A spectrum does contain small signals between $g = 1.94$ and $g = 1.86$, which could be attributed either to the Rieske protein observed by Malkin and Aparicio [20], or a small amount of Centre B. In fact, it seems likely that it is due to the presence of a small amount of Centre B, since the Rieske protein has been shown to have a mid-point potential of +290 mV [20], and should have been reduced during the dark incubation with ascorbate. Double integration of the measured Centre A spectrum, and a simulated EPR spectrum of Centre A [17] adjusted to the same size, indicated that this component contributed less than 10% of the double integrated intensity of the measured Centre A spectrum.

We have previously presented results [13,15,16] which showed that *P*-700 photooxidation becomes reversible at low temperature when Photosystem I particles are prepared under extreme reducing conditions so that both Centres A and B are reduced. The conditions used did not however show specific relationships of the iron-sulphur centres to this reversibility, or exclude the possibility that reduction of other components was involved. We have therefore determined the redox potential dependence of the change from irreversible photooxidation of *P*-700 to reversible *P*-700 photooxidation at low temperatures. Fig. 4 shows that the oxidation-reduction potential titration curves

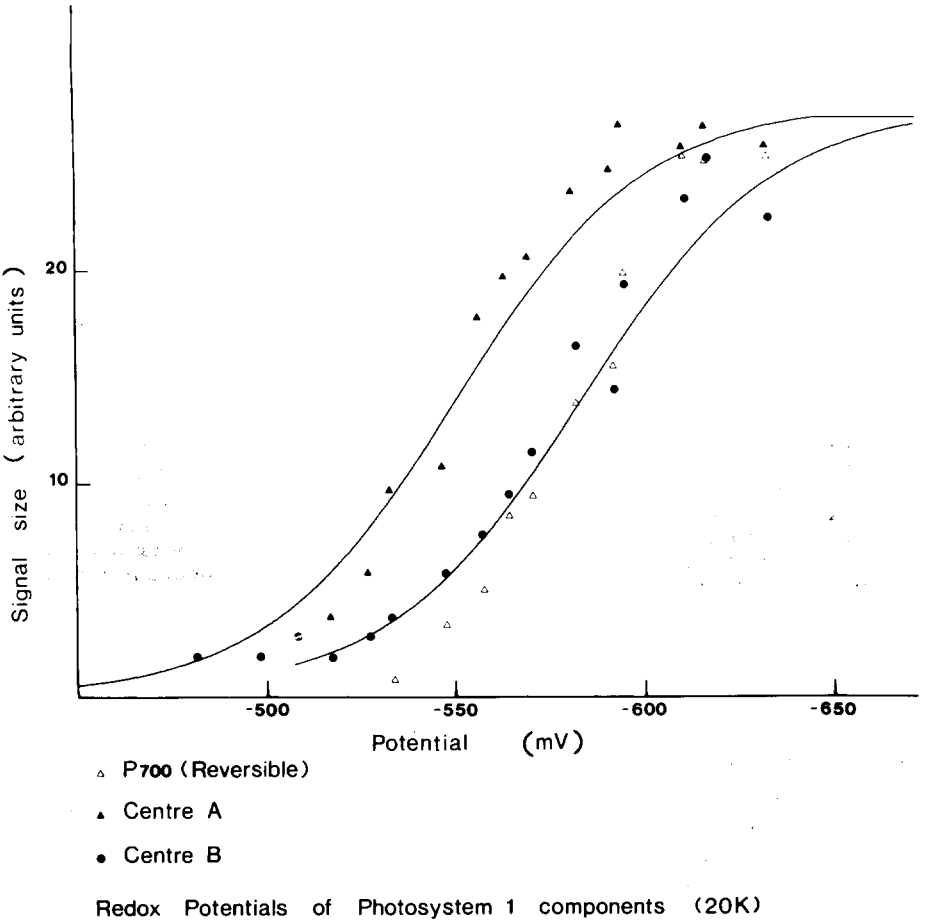

Fig. 4. Oxidation-reduction potential titration of reversible *P*-700 and the bound iron-sulphur centres. Photosystem I particles prepared using the detergent Triton X-100 (1.0 mg/ml) were suspended in an equal volume of 0.1 M glycine/KOH buffer (pH 10.0)/0.2 M NaCl with the following redox mediators: methyl viologen, Triquat and Tetraquat all at a concentrarion of 20 μM. The sample was reduced to —450 mV for 30 min and then titrated by standard procedures using sodium dithionite (2%, w/v) in 0.1 M Tris · HCl, pH 9.0, as reducing agent. The titration was performed under a green safelight and the samples frozen in complete darkness. The EPR spectra were recorded in the dark to determine the extent of reduction of the iron-sulphur centres, and then during and after illumination to determine the size of the light-induced reversible *P*-700 signal. The signal height of the $g = 1.95$ (Centre A), $g = 1.89$ (Centre B) and $g = 2.00$ signal (free radical signal I *P*-700) were measured for the data presented and normalised for convenience of presentation. EPR spectra were recorded at 20.0 K and the following instrument settings: microwave power, 20 mW; modulation amplitude, 1 mT; instrument gain, $5 \cdot 10^2$. ▲, Centre A; ●, Centre B; △, reversible *P*-700.

for the reduction of Centre A, Centre B and for the appearance of the reversible *P*-700 radical. At potentials more oxidised than —500 mV *P*-700 photooxidation is irreversibly coupled to the reduction of Centre A. Between about —500 and —550 mV *P*-700 photooxidation remains irreversible but as Centre A is reduced the extent of electron transfer to Centre B increases. Below —550 mV as the extent of Centre B reduction increases *P*-700 photooxidation becomes reversible, the increase in reversibility paralleling the reduction of

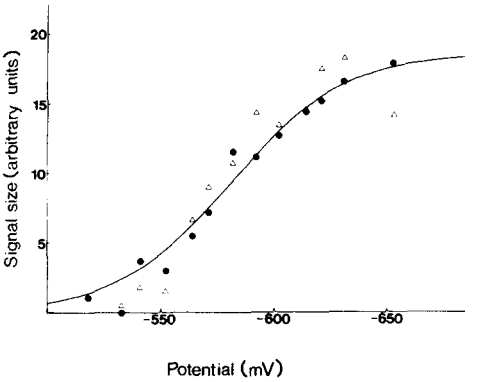

Fig. 5. The potential dependence of the light-induced appearance of X and *P*-700. Photosystem I particles prepared using the detergent Triton X-100 (1.75 mg/ml) were titrated by the procedure described in the legend for Fig. 4. The EPR spectra were measured before, during and after illumination to determine the size of the light-induced reversible X signal and reversible *P*-700. The signal height of the $g = 1.76$ component of X was recorded at 10 K and the following instrument settings: microwave power, 20 mW; modulation amplitude, 2 mT; instrument gain, 1000. The signal height of the reversible *P*-700 was recorded at 77 K and the following instrument settings: microwave power, 1 mW; modulation amplitude, 1 mT; instrument gain, 1000. •, component X; △, reversible *P*-700.

Centre B. At —630 mV when Centre B is almost fully reduced *P*-700 photooxidation is essentially completely reversible. The mid-point oxidation-reduction potentials measured at pH 10 in these experiments agree well with those published previously, Centre A $E_m \simeq -550$ mV and Centre B $E_m \simeq -585$ mV. The reversibility of *P*-700 increases with $E_m \simeq -585$ mV the same as that of Centre B. The data shown in Fig. 4 were all obtained by measurements at 20 K. We have also measured the appearance of the reversible *P*-700 at 77 K and again find $E_m = -585$ mV at this temperature indicating that the photochemical events in the reaction centre are the same at 20 K and 77 K. All the measurements shown were made under saturating microwave power conditions. We have previously shown that the saturation characteristics of the *P*-700 radical vary with redox state of the preparation. However, there are no variations over the potential range used here [17] and the total *P*-700 signal size was constant over the potential range of the titration. We made measurements at 77 K at 1 mW (saturating) and 5 μW which is non-saturating [19] for the reversible free radical signal. The mid-point potential under these conditions was the same. We have also measured the size of the $g = 1.76$ signal of X induced by illumination at 10 K as a function of redox potential (Fig. 5). The appearance of the signal shows a dependence on potential and appears in parallel with the reduction of Centre B and appearance of the reversible *P*-700 photooxidation. At the very lowest potential obtained by this procedure (—650/—660 mV) there is a slight decrease in the size of the *P*-700 radical and light-induced X while a small part of X is chemically reduced. The method we are using does not allow reliable measurement of potential below about —640 mV but the results suggest a mid-point potential for X of about —700 mV in agreement with the results of Ke et al. [21].

Discussion

No determination of the amount of B has previously been made, although Bearden and Malkin [10] did show a small (less than 10%) contribution of B to the reduced iron-sulphur signals in an experiment equivalent to that shown in Fig. 2 for the photoreduction of Centre A.

The results presented here show that the two iron-sulphur centres A and B of the Photosystem I reaction centre are present in equivalent amounts. This result, together with our previous measurements of the relationship of *P*-700 Centre A and X [17,19], show that the four electron-carrying components identified in the reaction centre are present in essentially equivalent amounts, and are quantitatively involved in electron transport. We have clearly shown that iron-sulphur Centre B can be photoreduced at low temperature as well as at room temperature. This result, together with the oxidation-reduction potential titration data which show that *P*-700 photooxidation becomes reversible only when Centre B is fully reduced, suggest very strongly that Centre B is a functioning electron acceptor in Photosystem I. The very close agreement between the titration curves for Centre B and the change to reversibility of *P*-700 photooxidation and the appearance of X as electron acceptor, shows that it is very unlikely that there are any intermediates between X and Centre B. We found a mid-point potential $E_m \simeq -585$ mV for the change from irreversible to reversible *P*-700 photooxidation at both 77 K and 20 K. Ke et al. [21] have reported measurements on this change using EPR measurements of the free radical at 90 K and 15 K. At 90 K they observed $E_m = -530$ mV, they do not report a mid-point for the 15 K measurements but their published data suggest a value around −550 mV. They conclude that *P*-700 photooxidation becomes reversible when Centre A is reduced, but do not show parallel titrations of *P*-700 and the iron-sulphur centres. Our results show that this is no the case but that the reduction of Centre B is the controlling factor at temperatures up to 77 K.

The results presented here, together with those in our previous papers [17,19], confirm our model of the photochemical electron transport reactions in the Photosystem I reaction centre at low temperatures. A single reaction centre chlorophyll (*P*-700) is associated with an electron acceptor complex containing two iron-sulphur centres (A and B) and an intermediary, primary electron carrier X.

In the absence of kinetic data, photochemical reactions occurring in the picosecond and nanosecond time range, it is not possible to assign an absolute electron transport sequence. However, it seems reasonable to presume on the basis of the reversibility of its photoreduction, and its apparently very low redox potential, that X is the first electron acceptor and the the electron can then be transferred to the iron-sulphur centres. The oxidation reduction potential of the two iron-sulphur centres are very close and in titrations extensive reduction of Centre B occurs before Centre A is fully reduced. However, at low temperatures when only a single electron is supplied to the complex, essentially complete reduction of Centre A is seen with less than 10% of B reduced. Obviously at low temperatures Centre A functions as the terminal acceptor in the sequence between X and A. At room temperature, however,

it seems likely that photoreduction would result in a situation more likely that seen on chemical reduction with the extent of reduction of the two centres equilibrating on the basis of the oxidation-reduction potentials.

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