Quantitative Electron-Paramagnetic-Resonance Measurements of the Electron-Transfer Components of the Photosystem-I Reaction Centre

THE REACTION-CENTRE CHLOROPHYLL (P700), THE PRIMARY ELECTRON ACCEPTOR X AND BOUND IRON-SULPHUR CENTRE A

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An e.p.r. spectrum of the reduced form of the electron-transport component (X), thought to be the primary electron acceptor of Photosystem I, was obtained. By using line-shape simulations of this component and the free-radical e.p.r. signal I of the oxidized reaction-centre chlorophyll (P700), it was possible to determine the ratio of the number of electron spins to which these signals correspond in Photosystem-I particles under a variety of conditions. On illumination at cryogenic temperatures of Photosystem-I preparations, in which both bound iron-sulphur centres A and B were reduced, the measured ratio of free radical to component X varied between 1.04 and 2.23, with an average value of 1.54 ± 0.18 where a Gaussian line-shape is assumed for the component-X signal in the simulation. The error in this measurement is estimated to be up to 50%. In a similar way component X and centre A of the bound iron-sulphur protein were quantified, the ratio between these two components varying between 1.26 and 0.61 with an average value of 0.75 ± 0.06 . These results indicate that the quantitative relationship, in terms of net electron spins, between centre A, component X and P700 is of the order to be expected if component X is indeed the primary electron acceptor in Photosystem I and a component of the photosynthetic electron-transport chain.

The initial events in the conversion of light-energy into chemical energy (in the form of a low-potential reductant) in plants occurs in the Photosystem-I reaction centre. Absorption of light results in the photo-oxidation of a reaction-centre chlorophyll (P700) (Kok, 1956) and the transfer of an electron to an electron-acceptor complex. This initial reaction is temperature-independent and can be observed at temperatures as low as 4.2K. This reaction is most easily followed by e.p.r. spectrometry, which can detect the components of the electron-acceptor complex as well as the electron donor. The low-temperature photo-oxidation of P700 is coupled irreversibly to the reduction of an electron-acceptor complex, identified as a bound iron-sulphur protein (Malkin & Bearden, 1971; Evans *et al.*, 1972). This protein was shown to contain two four-iron iron-sulphur centres A and B (Evans *et al.*, 1974; Cammack & Evans, 1975). If both centres are oxidized, illumination at low temperatures results in the appearance of signal I and the reduction of centre A, and a quantitative transfer of electrons between these two components has been demonstrated (Bearden & Malkin, 1972; Williams-Smith *et al.*, 1978).

Although these reactions are irreversible at low temperature, partial reversal in the dark of the light-induced appearance of signal I was reported by Mayne & Rubinstein (1966) and Warden *et al.* (1974). Evans & Cammack (1975) showed that the light-induced appearance of signal I became completely reversible when both centres A and B of the bound iron-sulphur protein were reduced. This suggested that there was an intermediate electron carrier between the reaction-centre chlorophyll and the bound iron-sulphur centres. Kinetic evidence for the presence of this carrier (X) was obtained by McIntosh *et al.* (1975), who found kinetic changes in the spectrum at $g = 2.06$ and $g = 1.76$. Using more highly purified Photosystem-I particles prepared with Triton X-100, Evans *et al.* (1975) showed the presence of this component under steady-state illumination conditions, and demonstrated that it was a chemically stable component by preparing fully reduced samples in which the reduced component could be observed in the dark. The steady-state light-minus-dark difference spectrum of component X, observed at low temperature (8–12K) and high microwave power, has $g_z = 1.76$, $g_y = 1.86$ and $g_x = 2.06$ (M. C. W. Evans *et al.*, 1976). McIntosh & Bolton (1976) have also obtained a spectrum of

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component X by kinetic resolution methods with signals at $g=2.07$, 1.86 and 1.75. The e.p.r. spectrum of component X has also been observed in spinach chloroplast and blue-green algal membrane fractions made without detergent treatment (M. C. W. Evans *et al.*, 1976; E. H. Evans *et al.*, 1976).

Although the preceding evidence suggests that component X is a component in electron transfer in the Photosystem-I reaction centre, the quantitative relationship between component X and the other known electron-transfer components in Photosystem-I has not been established. In the present paper we report quantitative determinations of component X relative to the iron-sulphur centre A, and of the ratio of the number of electrons involved in the photoreduction of component X and the photo-oxidation of the reaction-centre chlorophyll giving rise to signal I.

Materials and Methods

Photosystem-I particles were prepared from spinach (*Spinacea oleracea*) by using the non-ionic detergent Triton X-100 as described in the preceding paper (Williams-Smith *et al.*, 1978), which also gives details of the computer simulations of e.p.r. spectra, preparation of e.p.r. samples and e.p.r. equipment used. For illumination of e.p.r. samples in the spectrometer cavity, a 1000 W projector was used, with a 1 cm water filter and a Schott KG3 heat filter (Schott; H. J. Skan, Solihull, Warwickshire, U.K.) in the light-path. Tris, Triton X-100 and sodium ascorbate were from Sigma (London) Chemical Co. (Kingston-on-Thames, Surrey, U.K.). Other chemicals were from BDH Chemicals (Poole, Dorset, U.K.), AnalaR-grade reagents being used where possible.

Results and Discussion

Quantification of component X and signal I

When Photosystem-I particles were illuminated in the presence of dithionite and then frozen after a short period (90 s) in the dark, the e.p.r. spectrum (Fig. 1a) showed signals at $g=2.04$, 1.94, 1.92 and 1.89 attributed to the two iron-sulphur centres A and B (Evans *et al.*, 1972, 1974; Cammack & Evans, 1975). If illumination of the particles was continued during freezing the e.p.r. spectrum (Fig. 1b) also has signals attributed to the electron-transfer component X (M. C. W. Evans *et al.*, 1975, 1976; E. H. Evans *et al.*, 1976). The difference between these two spectra yielded an e.p.r. spectrum for component X (Fig. 1c) with $g_x=1.76$, $g_y=1.87$ and $g_z=2.08$. These g values agree with those measured from the published light-minus-dark difference spectra for component X observed in spinach Photosystem-I particles (M. C. W. Evans *et al.*, 1976;

McIntosh & Bolton, 1976) and membrane fractions of the blue-green alga *Chlorogloea fritschii* (E. H. Evans *et al.*, 1976). The shape of the spectrum is essentially the same as those published by M. C. W. Evans *et al.* (1976) and E. H. Evans *et al.* (1976) but differs in some respects from that published by McIntosh & Bolton (1976). This may be due to the kinetic resolution methods used by McIntosh & Bolton (1976) to obtain a spectrum of component X, where this signal was small in comparison with the background noise. We have been able to simulate approximately the line-shape that results after spectral subtraction by using the computer program described in the preceding paper (Williams-Smith *et al.*, 1978). This program generates the line-shapes expected for dilute randomly oriented $S=\frac{1}{2}$ systems which show no electron-spin-electron-spin interactions. In contrast, the spectra generated by kinetic methods are not easily simulated in this way, as pointed out by McIntosh & Bolton (1976).

Fig. 1. E.p.r. spectrum of the electron-transport component X

Photosystem-I particles prepared with Triton X-100 (1.2 mg of chlorophyll/ml) were frozen with both bound iron-sulphur centres A and B reduced (a) or centres A and B and the component X reduced (b) as described in the text. Subtraction of spectrum (a) from spectrum (b) yielded a difference e.p.r. spectrum for component X (c). The spectra were recorded at 10 K with the following instrument settings: frequency 9.07 GHz; power 100 mW; modulation amplitude 1 mT; scan rate 250 mT/min; instrument gain 5×10^2 .

Fig. 2. Comparison of the experimental e.p.r. spectrum of component X with the Gaussian line-shape simulation

(a) Experimental spectrum; (b) Gaussian line-shape simulation; (c) superimposition of the experimental (—) and simulated (----) spectra. The experimental spectrum has $g_x = 2.077$, $g_y = 1.865$ and $g_z = 1.763$ with $\Delta H_x = 102.5$, $\Delta H_y = 75$ and $\Delta H_z = 77.5$. The simulated spectrum had input parameters $g_x = 2.084$, $g_y = 1.870$, $g_z = 1.763$, $\Delta H_x = 90$, $\Delta H_y = 79$ and $\Delta H_z = 63$ and output parameters $g_x = 2.082$, $g_y = 1.869$, $g_z = 1.763$, $\Delta H_x = 110.9$, $\Delta H_y = 102.5$ and $\Delta H_z = 83.3$. ΔH_y was broadened relative to the measured value from the experimental spectrum so that the height of this component in the simulated spectrum matched that observed in the experimental spectrum. This change decreased the height of the $g = 1.76$ component of the simulated spectrum (used in the quantification) by less than 10%.

A comparison of experimental and simulated line-shapes for the spectrum of component X is shown in Fig. 2. The simulation is based on Gaussian line-shapes. A reasonable agreement between the spectra is obtained, except for the shape of the high-field side of the $g_x = 2.08$ component of the experimental spectrum. This is probably due to the difficulties inherent in subtraction of spectra from two different e.p.r. samples at a region ($g = 2.04$) where the reduced iron-sulphur centres present in both samples have a very sharp and strong e.p.r. signal (see Figs. 1a and 1b). Because of these experimental difficulties in generating the spectrum of the component X alone, we have used simulations based in both Gaussian and Lorentzian line-shapes in performing the quantification, and from these and other simulations we would conservatively estimate a maximum error of $\pm 50\%$ in quantifying component X in this way.

The ratio of the intensities of the low-field g_z peak

Fig. 3. Saturation curve for the $g = 1.76$ component of component X

Photosystem-I particles prepared with Triton X-100 (1.84 mg of chlorophyll/ml) were illuminated and frozen in the presence of sodium dithionite (0.2%, w/v) under anaerobic conditions, freezing component X in the reduced state. The signal intensity of the $g = 1.76$ component was recorded at 9.5 K at various microwave powers with the following instrument settings: frequency 9.165 GHz; modulation amplitude 1 mT; scan rate, 250 mT/min; instrument gain 5×10^2 .

in the first-derivative simulation of the spectrum of component X and the peak-to-trough height for the simulated P700 radical (see Williams-Smith *et al.*, 1978) was used in quantifying the relative spin concentrations of the two species in the experimental spectra, since the program generated in each case first-derivative intensities for the same total number of spins. Considerable care was taken to ensure that the experimental signals were measured at non-saturating microwave power, measurement conditions being 20 mW, 9.5 K for the signal of component X and 77 K, $5 \mu\text{W}$ for the P700 free radical. The saturation plots for these two species are presented in Figs. 3 and 4; the signals are considered to be unsaturated at those microwave powers where the first-derivative intensity divided by the square root of the power ($Y_m'/P^{1/2}$) remains constant as the power varies. The ease of saturation of the free radical was such that at 9.5 K the signal was saturated at the

Fig. 4. Saturation curve for the light-induced reversible free radical associated with the photoreduction of component X

Photosystem-I particles prepared with Triton X-100 (1.37mg of chlorophyll/ml) were illuminated under anaerobic conditions in the presence of sodium dithionite (0.2%, w/v), and then frozen after a dark period of 60s. The peak-to-peak signal intensity of signal I during illumination was recorded under various microwave-power conditions at 77K and the following instrument settings: frequency 9.125GHz; modulation amplitude 0.1mT; scan rate 12.5mT/min; instrument gain 10^4 .

lowest microwave powers attainable with our equipment (50nW), and, since the electron-spin-lattice relaxation times of component X are too short for this signal to be observable above 12K, this necessitated separate measurements of the spectra of X and the free radical at two temperatures. To relate these measurements a Cu/EDTA sample was run immediately after each measurement of component X and of the free radical and the intensity of the copper signals used to predict the hypothetical experimental intensity of the free radical under the measuring conditions of component X (if no power saturation had occurred). The copper standard corrects for both the intensity increase, on lowering the temperature, from a greater population difference between ground and excited states and the differing microwave magnetic fields incident on the samples in two cryostats of different geometry. A non-saturating microwave power of 0.1mW was used for measuring this standard at 9.5K, and for convenience we used a power of 5μW at 77K. By comparing the experimental intensities of component X and free-

Table 1. Ratios between the reversible light-induced signal I (P700) and the associated reversible light-induced $g = 1.76$ signal (X)

Photosystem-I particles prepared with Triton X-100 (1.37mg of chlorophyll/ml) were illuminated in the presence of sodium dithionite (0.2%, w/v) for 2min, kept in the dark for 60 or 120s, and then frozen. The peak-to-peak height of the light-induced reversible signal I, and the signal intensity of the light-induced reversible $g = 1.76$ component of component X, were measured and used together with the simulated spectra to obtain these results. Signal I was measured during and after illumination at 77K with the following instrument settings: frequency 9.17GHz; microwave power 5μW; modulation amplitude 0.1mT; scan rate 12.5mT/min. Component X was measured during and after illumination at 10K with the following instrument settings: frequency 9.165GHz; microwave power 20mW; modulation amplitude 1mT; scan rate 250mT/min.

Preparation	Sample	Signal-I/component-X ratio	
		Gaussian simulation	Lorentzian simulation
A	1	1.04	1.22
	2	1.81	2.11
	3	2.23	2.59
	4	1.84	2.16
	5	2.03	2.37
B	1	1.11	1.30
	2	0.82	0.96
	3	1.44	1.67
		1.54 ± 0.18	1.80 ± 0.21

radical signals with the computed intensities the ratio of spins observed in the free radical compared with those in component X was obtained.

Table 1 shows the result of measurements of the relative spin concentrations of free-radical signal I and component X in eight samples from two different batches of Photosystem-I particles prepared with Triton X-100. These signals are reversibly generated by illumination at low temperatures (M. C. W. Evans *et al.*, 1975, 1976) under the conditions used. The signal intensity taken to be that of signal I was the difference between measurements during and after illumination, as an irreversible component of the free-radical signal (approx. 20% of the total signal) was always induced by the first illumination of the sample (M. C. W. Evans *et al.*, 1976). On illumination of the copper standard there was no change in signal intensity, the temperature of the sample remaining constant. The ratio of signal I to component X was between 0.82 and 2.23 for the Gaussian simulation, and 0.96 to 2.59 for the Lorentzian simulation, with averages of 1.54 ± 0.18 and 1.80 ± 0.21 respectively. Any errors introduced

by the simulations would make the ratio consistently above or below the real value.

The wide variation in these values on both sides of the predicted value of 1 can therefore probably be attributed to two factors. Firstly, the signal of component X and the reversible signal I were measured in different cryostats, making it difficult to ensure that there were no small variations in intensity of illumination of the samples. Secondly, the size of the $g = 1.76$ signal seen on illumination at low temperature is normally smaller than that which can be observed in samples frozen after photoreduction at room temperature. This is in part due to the fact that some part of the reaction-centre chlorophyll (usually about 20%) is always irreversibly photo-oxidized at low temperatures because of the difficulty of preparing samples with both iron-sulphur centres fully reduced. It is also due to the competing back-reaction transferring electrons back from component X to the reaction-centre chlorophyll. This might be overcome by the use of higher light-intensities; however, we could not do this because of problems of sample heating.

Besides possible uncertainties in the true line-shape of the signal of component X, there could be two other sources of consistent error. Firstly, we have assumed that component X, signal I, and the simulation of centre A used below, have a paramagnetism corresponding to one unpaired electron. For centre A, which is believed to be a four-iron iron-sulphur centre (Cammack & Evans, 1975), this would appear to be a reasonable assumption. Magnetic-susceptibility measurements indicate at low temperatures a net spin of $\frac{1}{2}$ for two-iron iron-sulphur proteins, e.g. reduced spinach ferredoxin (Moss *et al.*, 1969) and reduced adrenodoxin (Kimura *et al.*, 1970), whose susceptibilities obey the Curie-Weiss law below 77K, and in oxidized *Chromatium vinosum* high-potential iron protein (Moss *et al.*, 1969), a four-iron iron-sulphur protein. We also expect signal I to behave ideally on the basis of the properties of bacteriochlorophyll radicals at low temperature (McElroy *et al.*, 1974); however, we cannot be sure that these assumptions are valid for component X, since its chemical identity is still unknown. A second possible source of consistent error is that the rates of the forward and back reactions may have different temperature-dependencies. In this case, even under identical illumination conditions different amounts of signal I and component X would be produced at 77K and 9.5K. Although more variable than measurements of the signal-I/centre-A ratio (Williams-Smith *et al.*, 1978), these results show a reasonable equivalence between the number of electrons leaving the reaction-centre chlorophyll on photo-oxidation (as indicated by signal I) and the number of electrons in the photoreduced compound X, suggesting that a quantitative transfer of elec-

trons between these two components is indeed possible.

Quantification of component X and centre A

If the component X is playing a significant role in electron transport in Photosystem I, acting as an intermediate in electron transfer between the reaction-centre chlorophyll and the eventual electron-acceptor centre A, it is necessary to demonstrate that component X is present in amounts comparable with those of the bound iron-sulphur centre A. By comparison of the simulation of centre A obtained previously (see Williams-Smith *et al.*, 1978) with the Gaussian simulation of the signal of component X, quantification of component X and centre A was performed in a similar way to that used for signal I and component X. Here the $g_y = 1.94$ signal of centre A and the $g_z = 1.76$ signal of component X were used. The best reduced spectrum of component X is seen in particles fully reduced before and during freezing by illumination in the presence of dithionite (Fig. 1b). Although ideally both component X and centre A should be measured in the same sample, it is known that there is electron-spin-electron-spin interaction between the iron-sulphur centres A and B, which affects the $g = 1.86$ component of centre A (Evans *et al.*, 1974) and could affect the $g = 1.94$ component. It was therefore considered best to measure the signal intensity of the $g = 1.94$ component when only centre A is reduced in the Photosystem-I particles. Duplicate samples of a Photosystem-I particle preparation were frozen in matched e.p.r. tubes, half the samples being fully reduced and the remainder being incubated in the dark at pH8.0 with ascorbate before freezing and illumination at cryogenic temperatures. The signal intensities at $g = 1.76$ (component X) and at $g = 1.94$ (centre A) were measured. Since the spectra of component X were recorded at 9.5K, whereas those of centre A were recorded at 20K, a Cu/EDTA standard was measured after each sample. Power values of 20mW (component X), 5mW (centre A) and 0.1mW (Cu/EDTA, at 9.5 and 20K) were used. The average of nine separate determinations (Table 2) yields a ratio of net electron spins in centre A to those in component X of 0.75 ± 0.06 (mean $\pm$ s.e.m.). The variation in these measurements is small, most of the measurements giving a ratio slightly below 1. Although this low value may be due to uncertainties in the exact line-shape of the signal of X, we would have expected that the relative quantifications of centre A to component X and signal I to component X would then err in the same direction, since the ratio of centre A to signal I is close to unity (see Williams-Smith *et al.*, 1978). It could be argued that illumination at cryogenic temperatures was not reducing centre A completely, owing to concentration problems, but

Table 2. Ratios of bound iron-sulphur centre A to component X

Photosystem-I particles, prepared by using Triton X-100, were either (a) incubated in the dark with ascorbate before freezing or (b) illuminated in the presence of dithionite (0.2%, w/v) and frozen in the light. Illumination of these samples yielded spectra of centre A or of centre A plus centre B and component X respectively. The sizes of the $g = 1.95$ component of centre-A spectra and the $g = 1.76$ component of component-X spectra were measured at 15K and 10K respectively and with the following instrument settings: centre A, frequency 9.165 GHz; microwave power 5mW; modulation amplitude 0.4mT; scan rate 100mT/min; component X, frequency 9.165 GHz; microwave power 20mW; modulation amplitude 1mT; scan rate 250mT/min.

Chlorophyll concn. (mg/ml)		Centre-A/com- ponent-X ratio
1.18	Sample 1	0.68
	2	1.18
	3	0.75
	4	0.79
	5	0.76
	6	0.62
	7	0.70
		0.78 ± 0.07
0.59	Sample 1	0.68
	2	0.61
	Average of all results	0.75 ± 0.06

diluting the particles by a factor of 2 did not increase the ratio of centre A to component X (Table 2). The lesser degree of variability in all of these results compared with those presented in Table 1 suggests that the problems of illumination during measurement mentioned above could well have been a contributing factor in the large variability of the signal-I/component-X ratios.

It has been established in the preceding paper (Williams-Smith *et al.*, 1978) that signal I associated with the photoreduction of centre A represents a stoichiometric electron transfer from the reaction-centre chlorophyll to centre A. In the present paper it has been demonstrated that the reversible signal I and the reversible component X seen on illumination at cryogenic temperatures of Photosystem-I particles with both centres A and B reduced could represent a close-to-stoichiometric transfer of an electron from the reaction-centre chlorophyll to the transient

electron acceptor X. This reaction has been established as an integral step in electron transport in Photosystem I by the demonstration that component X is present in amounts comparable with those of the eventual electron acceptor, centre A.

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