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EVIDENCE THAT THE LOW-POTENTIAL (−700 mV) ELECTRON ACCEPTOR (X) IN PHOTOSYSTEM I HAS TWO IRON-SULPHUR CENTRES

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The Photosystem I reaction centre contains two groups of iron-sulphur centres: Fe-S_A and Fe-S_B with redox potentials between −510 and −590 mV, and Fe-S_X with redox potential about −700 mV. Spin quantitation (Heathcote, P., Williams-Smith, D.L. and Evans, M.C.W. (1978) Biochem. J. 170, 373–378) and Mössbauer spectroscopy (Evans, E.H., Dickson, D.P.E., Johnson, C.E., Rush, J.D. and Evans, M.C.W. (1981) Eur. J. Biochem. 118, 81–84) did not show unequivocally whether Fe-S_X has one or two centres. Experiments are described which support the proposal that Fe-S_X has two centres. Fe-S_X can be photoreduced irreversibly by 210 K illumination of dithionite-reduced samples or reversibly by 7.5 K illumination of these samples. The amplitude of the Fe-S_X signal reversibly induced by illumination at 7.5 K is never more than 50% of the amplitude of the signal when Fe-S_X is prereduced by room temperature illumination or by 210 K illumination. Approx. half of the Fe-S_X is rapidly reduced by 210 K illumination, the remainder more slowly. The extent of reversible Fe-S_X reduction and P-700 photooxidation is little affected by the fast reduction of about half of the Fe-S_X. Subsequent reduction of the remaining Fe-S_X is paralleled by loss of the reversible photoreaction.

Introduction

Photosystem I (PS I) catalyses the light-induced transfer of electrons from P-700 to soluble ferredoxin. Several bound electron acceptors have been identified in the PS I reaction centre by electron paramagnetic resonance and optical spectroscopy. In addition to two intermediary acceptors A_0 and A_1 [1,2], three membrane-bound iron-sulphur centres have been identified [3,4]. Illumination of spinach PS I samples at cryogenic temperatures results in the photooxidation of the primary donor P-700 and transfer of the electron from P-700 to iron-sulphur centre A:

$$\text{P-700 FeS}_A \rightarrow \text{P-700}^+ \text{FeS}_A^-$$

If centre A is chemically reduced in the dark before the sample is frozen, low temperature illumination results in the photoreduction of centre B:

$$\text{P-700 FeS}_B \text{FeS}_A^- \rightarrow \text{P-700}^+ \text{FeS}_B^- \text{FeS}_A^-$$

In spinach PS I samples that contain glycerol [5], and in barley [6], *Phormidium laminosum* [7] and *Dunalliella parva* [8] PS I samples, centre B can be photoreduced even when centre A is oxidised, i.e., the electron is transferred to centre A in some reaction centres and to centre B in others. The fact that the redox state P-700$^+$ FeS$_B^-$

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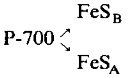
Abbreviations: Fe-S, iron-sulphur centre; PS I, Photosystem I; PS II, Photosystem II; P-700, reaction centre chlorophyll of Photosystem I; EPR, electron paramagnetic resonance.

FeS_A is stable at low temperature, without transfer of the electron from centre B to centre A suggests that these centres function in parallel rather than in series:

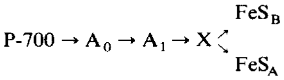


$$\text{P-700} \begin{smallmatrix} \nearrow \text{FeS}_B \\ \searrow \text{FeS}_A \end{smallmatrix}$$

The transient photooxidation of P-700 that can be observed following a laser flash when centres A and B are chemically reduced led to the discovery of another electron acceptor, termed centre X, assumed to function before centres A and B [9,10]. The EPR spectrum of centre X is a broad signal with *g*-values of 2.08, 1.87 and 1.77; it can be observed in PS-1 samples frozen under strongly reducing conditions, or it can be transiently observed following low-temperature illumination of PS-I samples which have centres A and B reduced:

$$\text{P-700XFeS}_B^- \text{FeS}_A^- \xrightarrow{h\nu} \text{P-700}^+ \text{X}^- \text{FeS}_B^- \text{FeS}_A^-$$

Centre X is therefore thought to function in the photochemical electron-transfer sequence between the intermediary acceptors and the iron-sulphur centres A and B:



$$\text{P-700} \rightarrow \text{A}_0 \rightarrow \text{A}_1 \rightarrow \text{X} \begin{smallmatrix} \nearrow \text{FeS}_B \\ \searrow \text{FeS}_A \end{smallmatrix}$$

Although centre X has an unusual EPR spectrum in terms of its *g*-values and temperature-dependence, both its optical [11] and its Mössbauer [12] spectra are characteristic of a 4Fe4S centre. Spin quantitation of X has been attempted [13] but is extremely difficult because of the problems in obtaining an accurate spectrum of X. We found that about 1.5 P-700 were oxidised for each X reduced during illumination at 8 K, but that there was only 0.75 Centre A per X when X was frozen in the fully reduced state. We concluded that accurate measurement was not possible because of the experimental difficulties, but that the centres were present in approximately equivalent amounts. However, it was noted in Ref. 13 that the amplitude of the X^- EPR signal observed on low temperature illumination (when only one electron can be transferred to the electron acceptors) was consistently smaller than the amplitude of the X^- signal in samples reduced at room tempreature (when more than one electron can be transferred to the electron acceptors). It was originally suggested that the smaller X^- signal amplitude observed during low-temperature illumination was due to the light intensity being non-saturating. However, it may be that a consistent error in the simulation of the spectrum of X led us to underestimate the amount of X by 30%, and that there are in fact two X per centre A and that 8 K illumination would therefore result in reduction of half the X present, i.e. 1 per P-700 oxidised. While the Fe determinations of Golbeck et al. [14] suggest that there are not more than three 4Fe4S centres per P-700, Mössbauer spectroscopic studies [12] have suggested that there may be a fourth low-potential iron-sulphur centre present. Chemical reduction of centres A and B resulted in reduction of 50% of the Fe-S centres present while attempts to reduce X by illumination of the very dense sample only reduced a further 20% of the Fe-S centres. If the two centres A and B represent only half the centres present there may be two lower potential centres.

We have previously demonstrated that it is possible to reduce the intermediary acceptors of PS I samples by illumination at approx. 230 K [2]. Under these conditions, dithionite can rereduce the P-700^+ formed on illumination and so result in more than one electron being transferred to the acceptor complex. We have now used this technique to photochemically reduce centre X and have measured the X and P-700 signal sizes in the dark and also under continuous illumination during the reduction of X. The results suggest that there may be two X components in the PS I reaction centre or that the behaviour of PS I centres is heterogeneous under these conditions.

Materials and Methods

Chloroplasts were isolated from market spinach as described in Ref. 15. PS I was isolated from the chloroplasts using the non-ionic detergent Triton X-100 as described in Ref. 16. The resulting PS I preparations had a P-700 to chlorophyll ratio of approx. 1 : 40 and lacked the components of the

PS II reaction centre and the b_6-f complex as determined by EPR and optical spectrometry.

EPR samples with the iron-sulphur centres A and B reduced were prepared by anaerobic addition of sodium dithionite (final concentration, 0.1% w/v) to PS I particles (1 mg chlorophyll/ml) in 0.1 M Tris-HCl (pH 9.0). The samples were frozen in complete darkness after 10–15 min. dark equilibration. No redox mediators were added.

210 or 230 K illumination was carried out in an unsilvered dewar containing ethanol using a 1000 W projector. The bath was cooled with solid CO_2. The temperature was measured with an alcohol thermometer and maintained within 1 K of the required temperature by addition of solid CO_2 during illumination. The time-course of illumination was followed for 50 min, when the reduction of X was found to be consistently 80% or more of the signal size in a sample frozen under illumination. The same projector was used to illuminate samples inside the EPR cryostat. EPR spectra were recorded with a Jeol FelX spectrometer at X-band with 100 kHz field modulation. The sample temperature was maintained with an Oxford Instruments liquid helium cryostat. For kinetic measurements the spectrometer was interfaced to a Datalab DL920 transient recorder and DL400B signal averager.

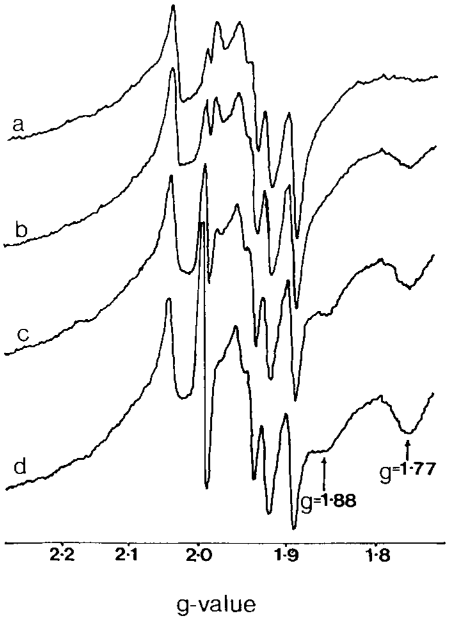


Fig. 1. EPR spectra recorded at intervals during the reduction of iron-sulphur centre X by 210 K illumination of PS I particles with iron-sulphur centres A and B reduced before freezing. EPR conditions: microwave power, 10 mW, modulation amplitude, 10 G, temperature, 7.5 K, frequency, 9.1 GHz. Spectra were recorded in the dark after (a) 0, (b) 4, (c) 12 and (d) 40 min of illumination at 210 K.

Results

We have earlier reported the use of 230 K illumination to gradually reduce the intermediary aceptors of PS I. Here we have used a lower temperature, 210 K, to reduce centre X. At 230 K, X is reduced very rapidly and reaches its maximum signal size in less than 5 min. The rate of this reaction is slowed down approx. 10-fold by decreasing the temperature to 210 K. The lower temperature required to gradually reduce X is presumably a consequence of the much slower back-reaction time from X^- than from the reduced intermediary acceptors. Fig. 1 shows EPR spectra recorded during the course of 210 K illumination of a dithionite-containing PS I sample frozen with the iron-sulphur centres A and B reduced. During the course of 210 K illumination, the $g = 1.77$ and $g = 1.87$ peaks of centre X (arrowed) gradually appear. The broad $g = 2.08$ peak of X is obscured by the large $g = 2.04$ signal of centres A and B. Fig. 1 also shows that a $g = 2.00$ radical signal, due to the intermediary acceptors A_0 and A_1, appears during 210 K illumination.

In Fig. 2 the amplitude of the $g = 1.77$ X^- signal and the $g = 2.0$ radical signal are plotted as a function of the duration of 210 K illumination. It can be seen that the increase in the amplitude of the $g = 1.77$ signal due to centre X is biphasic; the signal size increases rapidly during the first 12 min of 210 K illumination and then continues to increase, but at a slower rate. The amplitude of the $g = 2.0$ radical signal of the intermediary acceptors, on the other hand, increases only slowly during the first 30–40 min of 210 K illumination, but increases rapidly after that time. Therefore, while it is not possible to completely separate the reduction of X from the reduction of the intermediary acceptors, only about 16% of the maximum $g = 2.0$ signal appears during the first 12 min of 210 K illumination; during the same period the

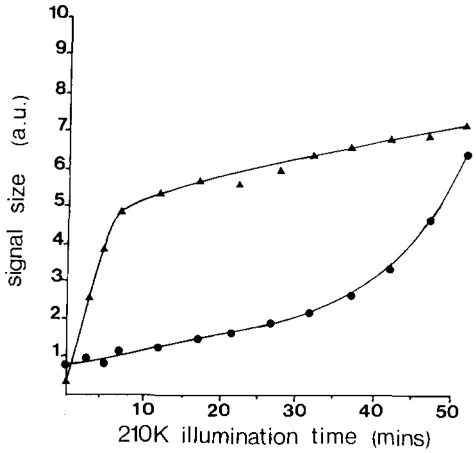


Fig. 2. Changes in the amplitude of the $g = 1.77$ signal of iron-sulphur centre X and the $g = 2.00$ signal of A_0A_1 during the course of 210 K illumination of a sample of PS I particles frozen with iron-sulphur centres A and B reduced before freezing. EPR conditions as in Fig. 1. Spectra were recorded in the dark. ▲——▲, Fe-S_X^- recorded in the dark; ●——●, $A_0^-A_1^-$ recorded in the dark.

$g = 1.77$ signal reaches 70% of the signal size in a sample frozen under illumination.

Fig. 3 shows the result of an experiment where, in addition to the amplitude of the $g = 1.77$ X^- signal, the amplitude of the $g = 2.0$ radical signal was measured at 7.5 K, first in the dark and then under continuous illumination. The reversible light-minus-dark $g = 2.0$ signal is due to the oxidised primary donor, P-700$^+$, generated transiently on illumination by reversible transfer of an electron to centre X:

$$\text{P-700X} \xrightarrow{h\nu} \text{P-700}^+\,X^-$$

Since no reversible P-700$^+$ signal is observed in samples that have X completely reduced, the amplitude of the light-minus-dark $g = 2.0$ signal would be expected to be related to the extent of reduction of X^- in a ratio of $(1 - X^-)$, reflecting the proportion of X that is oxidised. However, as Fig. 3 shows, the amplitude of the light-minus-dark $g = 2.0$ signal decreases linearly with the period of 210 K illumination and does not follow the X^- signal size. During the first 10 min of 210 K illumination, the X signal size increses rapidly from 0 to 60% of the signal size in a sample frozen under illumination; during the same period the light-minus-dark $g = 2.0$ radical signal size decreases by only 13%. This result is not compatible with the view that the $g = 1.77$ signal is due to a single component that receives electrons from P-700. It can, however, be explained by the presence of two centres, both of which contribute to the $g = 1.77$ signal. Reduction of one of these centres would be expected to increase the amplitude of the $g = 1.77$ signal to 50% of its maximum value without significantly decreasing the light-minus-dark $g = 2.0$ signal, since the electron from P-700 could be transferred to the second $g = 1.77$ centre.

In a further experiment, centre X was gradually reduced by 210 K illumination and at successive intervals during the course of illumination, the sample was removed from the 210 K ethanol/solid CO_2 bath, cooled to 7.5 K and four signal sizes measured: the $g = 2.0$ radical signal size and the $g = 1.77$ X^- signal, each recorded first in the dark and then under saturating illumination. the signal sizes were measured at 7.5 K, the temperature optimum for the X^- signal, so that any heating effect during illumination should decrease rather than increase the amplitude of the $g = 1.77$ signal. There was no irreversible increase in the amplitude of either the $g = 2.0$ radical signal or the $g = 1.77$ X^- signal after illumination of the sample at 7.5 K. The amplitude of the total $g = 1.77$ X^- signal

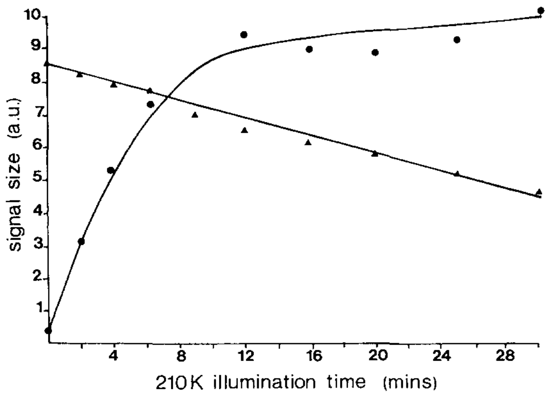


Fig. 3. Changes in the amplitude of the $g = 1.77$ of iron-sulphur centre X and of the reversible light-induced $g = 2.00$ signal of P-700 during the course of 210 K illumination of a sample of PS I particles prepared with iron-sulphur centres A and B reduced before freezing. Spectra were recorded at 7.5 K as in Fig. 1. ●——● Fe-S_X^-, recorded in the dark; ▲——▲ P-700$^+$, light-dark.

recorded under illumination increases rapidly during the first 10 min of 210 K illumination and then continues to increase, but at a slower rate. This again is not the expected result if there is only one X component. If the amplitude of the $g = 1.77$ signal recorded under continuous saturating illumination represents the total X content, i.e., both the stably reduced X in the redox state P-700 X^- and also the reversibly reduced X in the redox state P-700^+ X^-, no increase in the total amplitude of the signal (recorded under illumination) would be expected during 210 K illumination if there were one X per reaction centre. If, however, there were two X's, an approximate doubling in the amplitude of the signal would be expected, and this is indeed observed. Fig. 4 shows the amplitude of the reversible, light-minus-dark $g = 2.0$ and $g = 1.77$ signals. The two signal sizes decrease linearly and in parallel, indicating that at 7.5 K all the electrons that leave P-700 on photooxidation are transferred to the $g = 1.77$ component(s).

If there are two X centres it is possible that the back reaction kinetics of P-700^+ X^- might differ for the two centres. Fig. 5a shows the kinetics of the decay of the $g = 1.77$ signal following a laser flash. the half-time for the decay of the signal was approx. 250 ms. This decay time did not change appreciably as the $g = 1.77$ signal appeared during 210 K illumination (Fig. 5b), although the maximum amplitude of the transient signal did decrease. There is therefore no evidence for two kinetically distinct centres.

To ensure that a magnetic interaction between the intermediary acceptors of PS I and centre X was not affecting their signal sizes, the $g = 2.0$

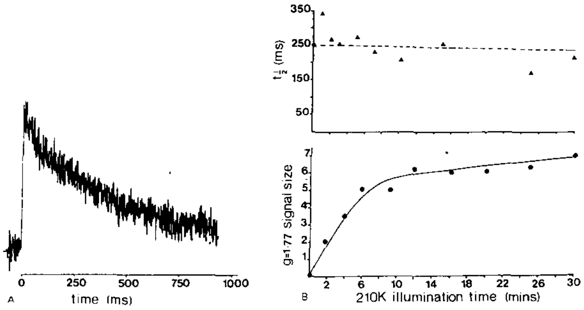


Fig. 5. (A) Kinetics of the decay of the $g = 1.77$ signal of iron-sulphur centre X induced by laser flash (0.6 μs) illumination of PS I particles frozen with iron-sulphur centres A and B reduced. EPR conditions as in Fig. 1. 256 repetitions were averaged with a repetition rate of 0.5 Hz. (B) The decay time (t) of the flash induced $g = 1.77$ signal of iron-sulphur centre X in PS I particles frozen with iron-sulphur centres A and B reduced, as a function of time of illumination of the sample at 210 K and reduction of iron-sulphur centre X.

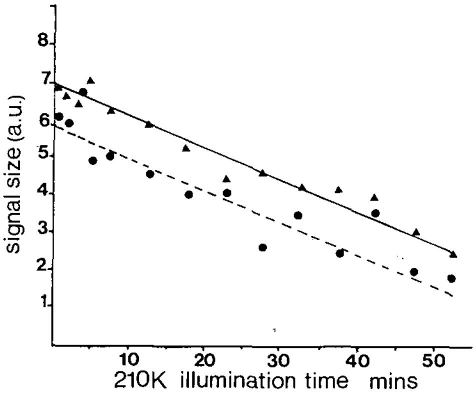


Fig. 4. Variation in the amplitude of the reversible $g = 1.77$ signal of iron-sulphur centre X and $g = 2.00$ signal of P-700^+ induced by illumination at 7.5 K of PS I particles prepared with iron-sulphur centres A and B reduced before freezing, during the course of illumination at 210 K. ●- - - - -●, Fe-S X^- light-dark; ▲———▲, P-700 $^+$ light-dark.

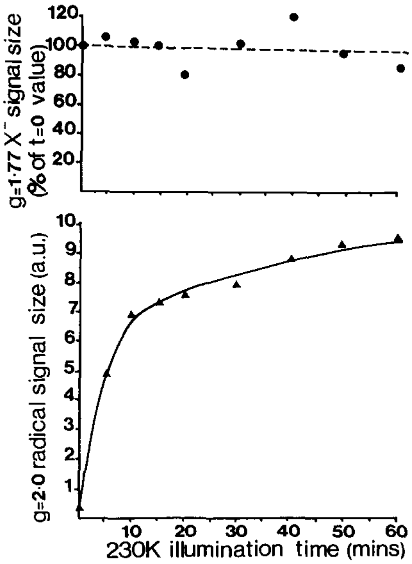


Fig. 6. The signal size of the $g = 1.77$ signal of iron-sulphur centre X during the reduction of A_0A_1 by 230 K illumination of PS I particles frozen with iron-sulphur centres A, B and X reduced. EPR conditions as in Fig. 1.

intermediary acceptor components were reduced by 230 K illumination of an A, B and X-reduced sample. At intervals during 230 K illumination, the sample was cooled to 7.5 K and the $g = 2.0$ and 1.77 signal sizes measured in the dark. Although such an interaction was reported in Ref. 17, as Fig. 6 shows, no significant change in the amplitude of the $g = 1.77$ signal was observed during reduction of the intermediary acceptors. This result suggests that a magnetic interaction is not contributing to the increase in the $g = 1.77$ signal size observed in Fig. 4. The discrepancy between the results obtained here and in Ref. 17 apparently reflects the different sample preparation conditions, with A_0A_1 reduced at around 200 K or at room temperature. It is not clear why this should occur unless the reduction of frozen samples prevents conformational changes in the protein.

Discussion

The results presented here show that the reduction of X at low temperature is biphasic, suggesting that two electron-accepting centres contribute to the spectra at $g = 1.77$. Alternative explanations could be proposed, for example that the population of PS I is heterogeneous or that X is in equilibrium with an unidentified component. We consider that it is unlikely that there is a heterogeneous population, as there is no evidence for this in the reduction of Fe-S_A or of A_1A_0. It is impossible to exclude the possibility of an undetected intermediate in equilibrim with X. However, we think this is unlikely in view of the Mössbauer and spin quantitation experiments which suggest there may be two X centres, although the presence of two X centres, Fe-S_A and Fe-S_B would require 16Fe per P-700, in conflict with published values of 12. We consider that our results support the proposal that there are two X centres for three reasons.

(1) The lack of correlation between the amplitude of the total $g = 1.77$ X^- signal and the amplitude of the reversible, light-minus-dark $g = 2.0$ P-700^+ signal. The appearance of 60 % of the maximum $g = 1.77$ signal during the first 10 min of 210 K illumination with only a 16% decrease in the amplitude of the reversible $g = 2.0$ signal due to P-700^+ indicates that P-700 photooxidation is virtually unaffected by reduction of one-half of the X content of the sample. This suggests that the maximum $g = 1.77$ signal represents two components. If both these components can accept electrons from P-700, it would then be possible to reduce one of these components and so induce half the maximum $g = 1.77$ signal size without significantly decreasing the p-700^+ signal size:

no $g = 1.77$ X^- signal:

$$\text{P-700 } X_1X_2 \xrightarrow{h\nu} \text{P-700}^+ \, X_1X_2^-$$

half max. $g = 1.77$ X^- signal:

$$\text{P-700 } X_1^- X_2 \xrightarrow{h\nu} \text{P-700}^+ \, X_1^- X_2^-$$

max. $g = 1.77$ X^- signal:

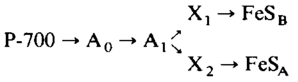
$$\text{P-700 } X_1^- X_2^- \xrightarrow{h\nu} \text{no P-700}^+ \text{ signal observed on illumination.}$$

(2) The fact that the amplitude of the reversible $g = 1.77$ X^- signal induced by saturating low-temperature illumination is always less than half the amplitude of the $g = 1.77$ signal observed either in samples in which X was reduced at room temperature and then frozen, or in samples fully reduced by 210 K illumination. If the $g = 1.77$ signal were due to a single component, saturating low temperature illumination would be expected to induce the maximum, or nearly the maximum signal size. If however there were two $g = 1.77$ centres, the amplitude of the $g = 1.77$ signal that appears on low temperature illumination would always be smaller than the maximum $g = 1.77$ signal, since at low temperature only one electron is available from P-700 photooxidation for transfer to the electron acceptors. In samples reduced at room temperature or by 210 K illumination more than one electron can be transferred to the acceptors and therefore the signal size would be approximately double that induced by low-temperature illumination.

During the course of 210 K illumination, the amplitude of the $g = 1.77$ signal recorded under illumination at 7.5 K increases to approximately twice the original signal size. If there were only one $g = 1.77$ centre, no increase in the signal size would be expected.

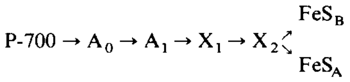
(3) When a sample with Fe-S_A and Fe-S_B reduced is illuminated at 210 K about half of the X is reduced with essentially no reduction of A_0A_1; as the second fraction of X is reduced A_0A_1 becomes reduced. This result would be compatible with a model in which two X centres become reduced in each reaction centre, A_0A_1 becoming reduced only when both Fe-S_X centres are reduced. If the results reflected heterogeneity in the centres, reduction of A_0A_1 would be expected to start as soon as any X was reduced.

Each of these findings suggests that there are two $g = 1.77$ components. Because the $g = 2.08$, 1.87 and 1.77 peaks attributed to centre X are very broad and all except the $g = 1.77$ peak are obscured by the strong and sharp signals of the iron-sulphur centres A and B, it is difficult to obtain accurate g-values in order to ascertain whether the two components have different EPR spectra. The finding that centres A and B appear to function in parallel [7], suggests that the two $g = 1.77$ or X components may also function in parallel:



$$\begin{tikzcd} & & & X_1 \arrow[r] & \mathrm{FeS_B} \\ \text{P-700} \arrow[r] & A_0 \arrow[r] & A_1 \arrow[ur] \arrow[dr] & & \\ & & & X_2 \arrow[r] & \mathrm{FeS_A} \end{tikzcd}$$

Alternatively the two components may function in series with a branchpoint in the electron-transfer sequence occurring after the second X component:



$$\begin{tikzcd} & & & & & \mathrm{FeS_B} \\ \text{P-700} \arrow[r] & A_0 \arrow[r] & A_1 \arrow[r] & X_1 \arrow[r] & X_2 \arrow[ur] \arrow[dr] & \\ & & & & & \mathrm{FeS_A} \end{tikzcd}$$

Kinetic measurements of the forward reaction would be the only way to distinguish between these two possibilities.

Acknowledgements

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