Biochimica et Biophysica Acta, 679 (1982) 146–155
Elsevier Biomedical Press

BBA 41020

EFFECT OF TEMPERATURE ON THE PHOTOREDUCTION OF CENTRES A AND B IN PHOTOSYSTEM I, AND THE KINETICS OF RECOMBINATION

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(Received 9th September, 1981)

Key words: Iron-sulfur protein; Photosystem I; Temperature effect; ESR; (Spinach chloroplast)

When spinach Photosystem I particles, frozen in the dark with ascorbate, are illuminated at low temperatures, one electron is transferred from *P*-700 to either iron-sulphur centre A or B. It was found that the proportion of centre A or B reduced depended on the temperature of illumination. At 25 K, reduction of centre A, as detected by ESR spectroscopy, was strongly preferred. At higher temperatures, at about 150 K, there was an increased proportion of reduced centre B. Reduction of B was more strongly preferred in particles frozen in 50% glycerol. The kinetics of dark reoxidation of A^- and B^- at various temperatures were followed by observing the radical signal of $P\text{-}700^+$, and also by periodically cooling to 25 K to measure the ESR spectra of the iron-sulphur centres. The recombination of A^- and $P\text{-}700^+$ occurred at lower temperatures than that at of B^-; at 150–200 K, centre B was the more stable electron trap. Dark reoxidation of both centres was more rapid in samples that were illuminated at 25 K than in samples illuminated at 150–215 K. In no case was net electron transfer between centres A and B observed. Differences in *g* values of the ESR spectra in particles illuminated at 25 and 200 K indicate that the iron-sulphur centres are in altered conformational states. It is concluded firstly that, in the frozen state, the rates of dark electron transfer decrease in the sequence $A^- \rightarrow P\text{-}700^+ > B^- \rightarrow P\text{-}700^+ > B^- \rightarrow A$; secondly, that when centres A or B are photoreduced, a temperature-dependent conformational change takes place which slows down the rate of recombination with $P\text{-}700^+$.

Introduction

It was observed by Malkin and Bearden [1] that illumination of chloroplast PS I, at temperatures around 20 K, resulted in the photo-oxidation of *P*-700 and reduction of an ESR-detectable iron-sulphur protein. Later studies using chemical reduction and illumination at room temperature [2,3] showed that the spectrum of the iron-sulphur protein was too complex to be accounted for by just one centre. To explain the redox titration data on the centres it was proposed [4] that there were two iron-sulphur centres A and B. A is the centre which is first reduced by illumination at 20 K, and has a characteristic ESR spectrum with approximate *g* values 2.05, 1.94 and 1.86. It may be noted that it has subsequently been observed that there are probably at least two intermediate electron acceptors between the primary donor *P*-700 and centres A and B [5–8]. These are termed A_1 and A_2 (or X) and if they are photoreduced even at very low temperatures they can be rapidly reoxidized by $P\text{-}700^+$. The iron-sulphur centres A and B therefore act as relatively stable traps for electrons from these primary acceptors. The donor to A and B is thought to be X, which may also be an iron-sulphur protein [9].

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Abbreviations: PS, photosystem; Chl, chlorophyll.

In spinach PS I particles the midpoint redox potential of centre A is more positive (approx. −550 mV) than that of centre B (approx. −590 mV) [3,4]. As expected from these values, centre A is the first to be reduced by chemical reduction at room temperature. However, in the cyanobacterium *Phormidium laminosum* [10], it was found that partial chemical reduction produced a different ESR signal with approximate g values 2.07, 1.94 and 1.89. This spectrum was identified as that of reduced centre B, which in these particles had a more positive potential than centre A. However, on illumination at low temperatures, centre A was still preferentially reduced, indicating that in the frozen state the electron-transfer pathway from *P*-700 to A is more facile than from *P*-700 to B. Similar effects were observed in spinach PS I in 50% glycerol, which had the effect of making the midpoint potential of centres A and B less negative [11].

Nugent et al. [12] found that in chloroplasts from a strain of barley, centre B had a less negative potential than A. They also noted that after illumination at 15 K and storage in the dark at 77 K for a day or at 125 K for a few minutes, the spectrum of reduced A decayed to a greater extent than that of B.

Ke and co-workers [13,14] and Visser et al. [15] have used optical and ESR spectroscopy to study the kinetics of recombination of electrons from the acceptors of PS I with photo-oxidized *P*-700, over the temperature range 13–225 K. The decay was found to occur in at least two phases, and the extent of each phase of recombination decreased with decreasing temperature until at 13 K no recombination was observed. Ke et al. [14] also followed the temperature-dependent decay of the signal due to centre A reduced by illumination at 13 K, and showed that this was in parallel with the decay of $P\ 700^{+}$.

We have now extended these measurements by studying the effect of illumination at different temperatures on the photoreduction of centres A and B and their dark recombination. We used chloroplasts and purified PS I particles from spinach (*Spinacia oleracea*) for this work, as these have been most widely used in studies of photosynthetic primary reactions.

Experimental Procedure

Spinach chloroplasts were prepared as described in Ref. 16. PS I particles, extracted from spinach chloroplasts with Triton X-100 and further purified as described in Ref. 17, were provided by Dr. M.C.W. Evans. The chlorophyll : *P*-700 ratio was 45–50.

ESR spectra were recorded on a Varian E4 spectrometer. The sample temperature was controlled by an Oxford Instruments ESR9 liquid helium flow cryostat, modified with a cover so that the whole sample tube was in the helium gas space. All the temperature changes were made with the sample inside the cavity. The temperature was changed by a combination of restricting the helium flow and heating the gas. The time taken for adjustment of sample temperature was less than 1 min. Temperatures were measured by means of the gold/iron vs. chromel thermocouple below the sample. These were checked by a carbon thermometer inside a sample tube containing mineral oil. Discrepancies of up to 5 degrees were observed between the two readings, and during measurements the themocouple readings were corrected by a calibration curve.

Samples were illuminated with a Barr and Stroud 330 W light source and fibre optic light guide through a Schott glass heat filter, type KGl (H.V. Skan, Ltd., Solihull B90 4AL, U.K.). Normally, the light source was placed as close to the front of the cavity as possible and used at full power. By using a carbon thermometer in frozen samples, it was found that illumination increased the sample temperature by less than 1 degree over the temperature range of these studies.

Results

Effect of temperature at which samples are illuminated: light-induced reduction of centre B

The effect of temperature on the photochemical reduction of centres A and B was investigated. Samples were treated with ascorbate before freezing in the dark so that *P*-700 was reduced and centres A and B oxidized. Therefore, at low temperature only one electron would be expected to transfer per reaction centre, either to A or B. This was found to be the case up to 225 K in the absence of glycerol. It should be emphasized that in the experiments described, the

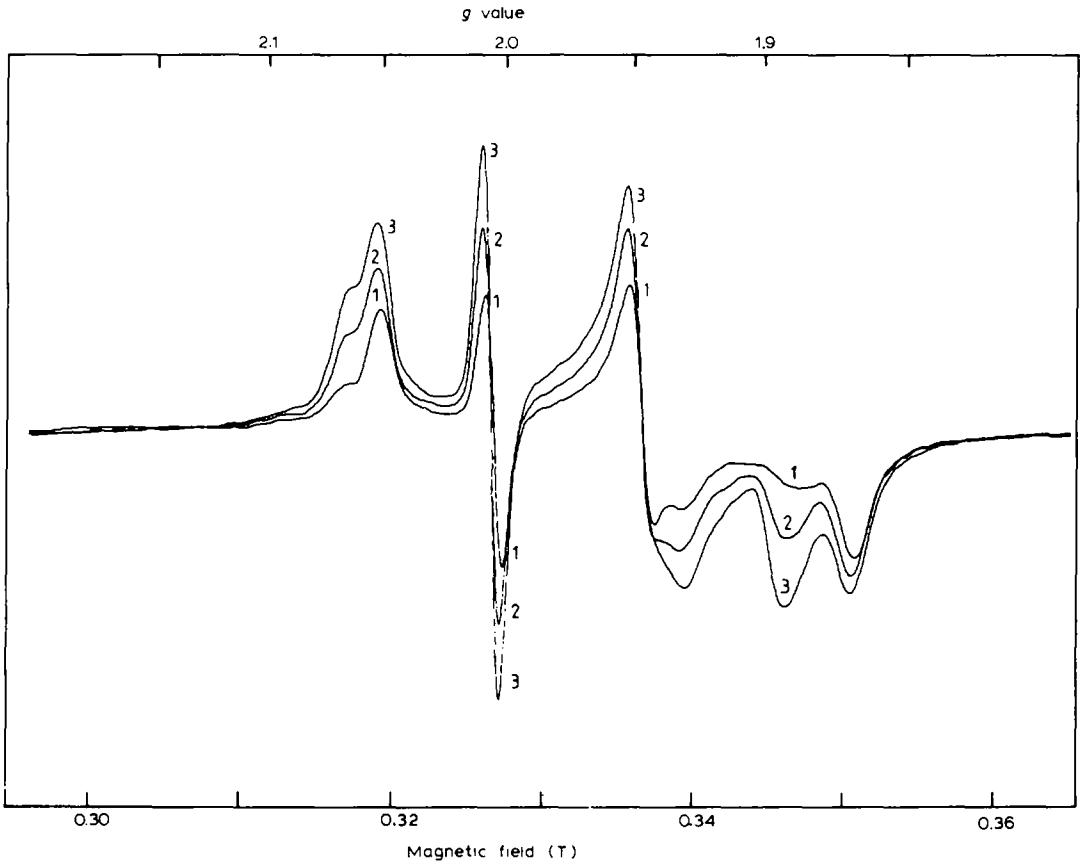


Fig. 1. ESR spectra of spinach PS I particles, 2.8 mg Chl/ml with 10 mM sodium ascorbate, illuminated with saturating light at the following temperatures: 1, 25 K; 2, 200 K; 3, 215 K. Illumination was then continued as the samples were cooled to 25 K for measurement of the spectra. Instrument settings were: microwave power, 20 mW, frequency 9.18 GHz, modulation amplitude 1 mT.

signals observed were due to isolated reduced centres A and B. The additional features due to interaction between A and B, such as the g 1.92 signal [2,3,10], were not observed.

When a sample was illuminated at 25 K, the ESR spectrum (Fig. 1, curve 1) shows that it was principally centre A that was reduced, as expected. This gives rise to the features at g 2.05, 1.94 and 1.86. The narrow radical at g 2.0026 is due to $P\text{-}700^+$. When illuminated at higher temperatures and immediately cooled down, with the light still on, it was found that between 200 and 215 K, there was an increased intensity of features at g 2.065, 1.93 and 1.89 due to centre B. The intensity of reduced centre A was not diminished. Thus, at the higher temperature more reaction centres could undergo photochemical reaction and there was an increased probability of electron transfer to centre B. Possible explanations for this are that the rate of electron transfer between the donor X and B is temperature dependent and or that conformational change produces a more favourable pathway for electron transfer at higher temperatures. In addition, the relative rates of recombination of A^- and B^- with $P\text{-}700^+$ at the higher temperatures must be taken into account.

Fig. 2 shows a similar experiment with spinach chloroplasts. Samples frozen in the dark (spectrum a) in the presence of ascorbate show a chlorophyll radical at g 2 and the reduced 'Rieske' iron-sulphur protein [18] at g 1.90. Illumination at 25 K (spectrum b) produced a spectrum mostly due to centre A^-, although the small feature at g 1.93 suggests a

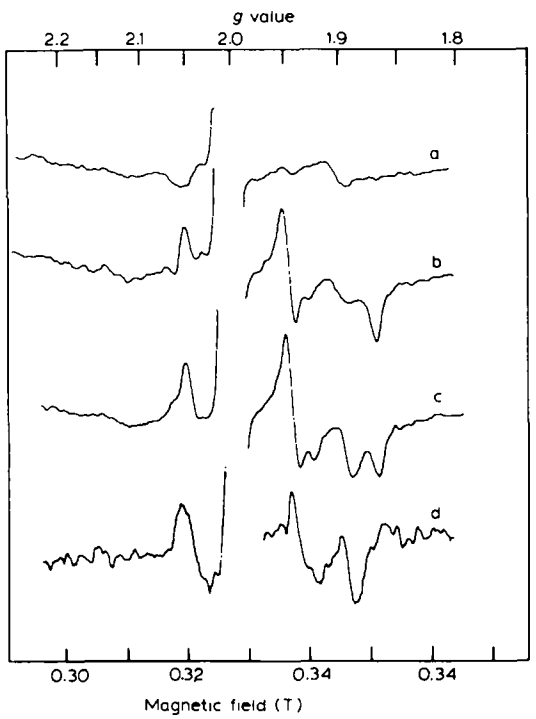


Fig. 2. ESR spectra of spinach chloroplasts, 4 mg Chl/ml with 10 mM ascorbate, (a) frozen in the dark, (b) illuminated at 25 K, (c) illuminated at 215 K. d is the computed difference between b and c. Conditions of illumination and spectral measurement were as for Fig. 1.

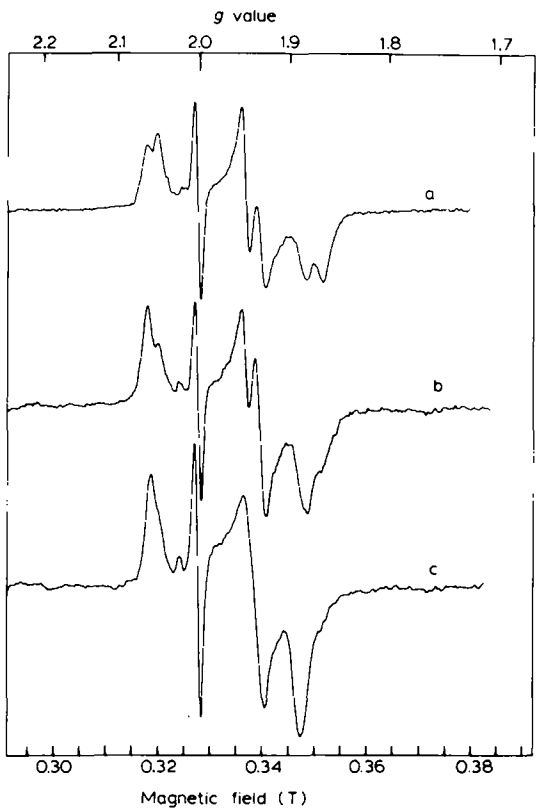


Fig. 3. ESR spectra of a sample of PS I particles in 50% (v/v) glycerol, containing 1.5 mg Chl/ml and 10 mM ascorbate, illuminated at the temperatures (a) 25 K, (b) 130 K, (c) 200 K. Other conditions as for Fig. 1.

small amount of photoreduced B^-. Illumination at 215 K (spectrum c) induced an enhanced signal due to B^- as seen by the difference spectrum (d). Therefore, the preferential reduction of centre B in PS I particles at higher temperatures is not an artifact of Triton treatment.

Fig. 3 shows the effect of temperature of illumination on PS I particles in 45% glycerol, the type of medium in which most of the optical measurements of $P\text{-}700^+$ decay have been measured (e.g., Refs. 13–15). As reported by Evans and Heathcote [11] the effect of glycerol is to increase the extent of photoreduction of B relative to A. In samples illuminated at 25 K, centres A and B were reduced with approximately equal probability (Fig. 3a). Illumination at higher temperature increased the proportion of B reduced until at 200 K (Fig. 3c) the spectrum is an almost pure spectrum of reduced centre B. In this case, there was a clear decrease in the amount of centre A that remained reduced, as the samples were illuminated at higher temperatures. It is likely that this is due to a faster rate of recombination of reduced A with $P\text{-}700^+$.

These experiments also demonstrate as previously noted [10] that since it is possible to obtain stable samples with either A or B reduced, then there is no electron transfer between the two centres at 25 K.

Kinetics of recombination of centres B^- and A^- with $P\text{-}700^+$

After illumination at various temperatures to cause charge separation and measuring the EPR signals at 25 K the rate of recombination was followed by raising the temperature of the sample, in the dark, for a fixed amount of time. During this time the rate of decay of the $P\text{-}700^+$ radical could be followed [13]. To measure the spectra of the iron-sulphur

centres the temperature was quickly lowered to 25 K. The rate of recombination could be followed by repeatedly raising and lowering the temperature of the same sample.

The results obtained are most easily explained by considering first the recombination process in glycerol-treated particles in which B^- was principally the reduced species. After illumination at 200 K, the dark decay of the signal was approximately exponential with a half-time about 3.5 min. The decay of the B^- signal was in parallel with the slower phase of the decay of the $P\text{-}700^+$ radical (Fig. 4).

When the dark recombination was carried out at 150 K, the rate of recombination was clearly dependent on the temperature of illumination. This is illustrated by the spectra of Fig. 5. The sample illuminated at 25 K (spectrum a) showed a considerable amount of recombination after 1 min in the dark at 150 K (b). The sample illuminated at 200 K (spectrum c) showed little recombination after 1.5 min at 150 K (d). The rate of recombination of centre B in the dark at 150 K after illumination at various temperature is shown in Fig. 6a. The quantity of reduced B was measured from the size of the g 2.07 feature. Two phases of recombination were seen: a fast phase (less than 1 min) and a slow phase. The proportion of the slow phase of recombination was greater when the illumination took place at higher temperatures.

In PS I particles in the absence of glycerol, the rate of recombination of photoreduced centre A could be measured (Fig. 7). This can be seen most easily in the features at g 2.05 and 1.86. If the sample was illuminated at 24 K (spectrum a) the signal due to A^- rapidly decayed at 150 K (b). In samples illuminated at 215 K (spectrum c), the reduced centre A was more stable to recombination (d). The rate of recombination of A was generally more rapid than that of B. The recombination curves, like those for B^- signal in glycerol-treated PS I particles, were biphasic and the proportion of the slower phase was

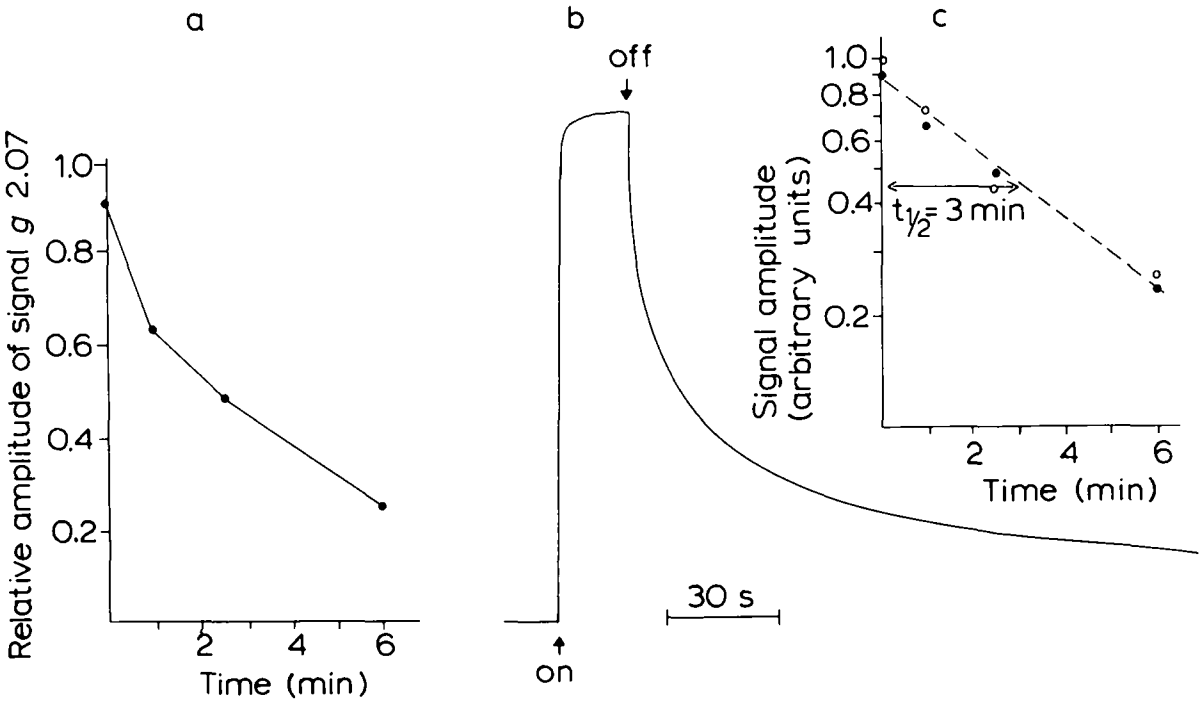


Fig. 4. Kinetics of recombination of B^- with $P\text{-}700^+$ in PS I particles with 50% (v/v) glycerol, prepared as for Fig. 3. After illumination at 200 K, samples were maintained at this temperature, then after varying lengths of time the temperature was decreased to 25 K to measure the signal at g 2.07 due to B^-. (a) Decrease of B^- signal with time; (b) decrease of g 2.002 signal, measured at 200 K; (c) the slower phases of curves a and b, plotted semilogarithmically; •, reduced centre B; ○, oxidized P-700.

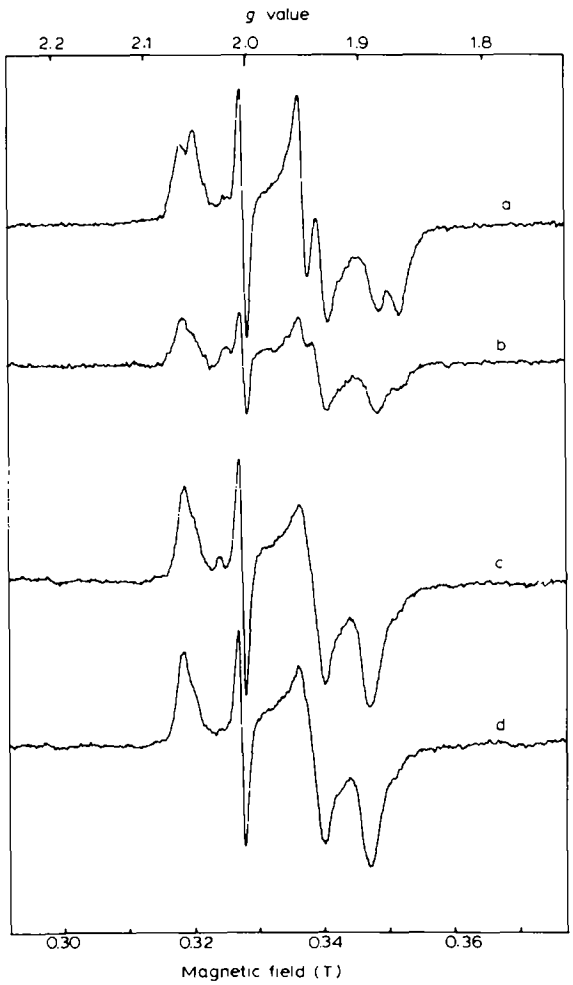


Fig. 5. ESR spectra of PS I particles in 50% glycerol, prepared as for Fig. 3. (a) After illumination at 25 K; (b) the same after 1 min at 150 K; (c) after illumination at 200 K; (d) the same after 1.5 min at 150 K. Spectra were recorded at 25 K under the same conditions as Fig. 1.

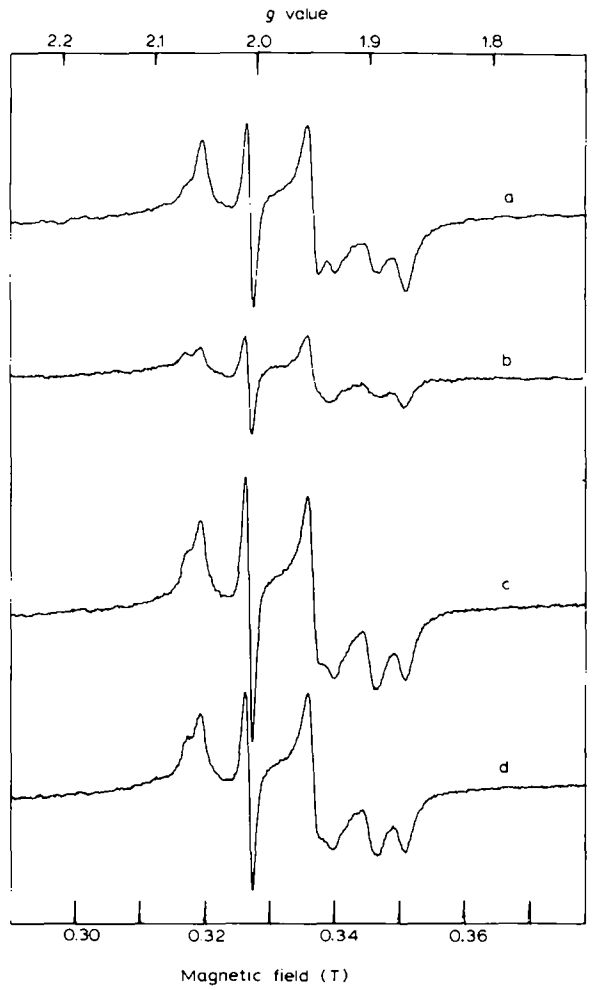


Fig. 7. ESR spectra of PS I particles, prepared without glycerol as for Fig. 1; (a) after illumination at 24 K; (b) the same after 1 min at 205 K; (c) after illumination at 215 K; (d) the same after 1 min at 205 K.

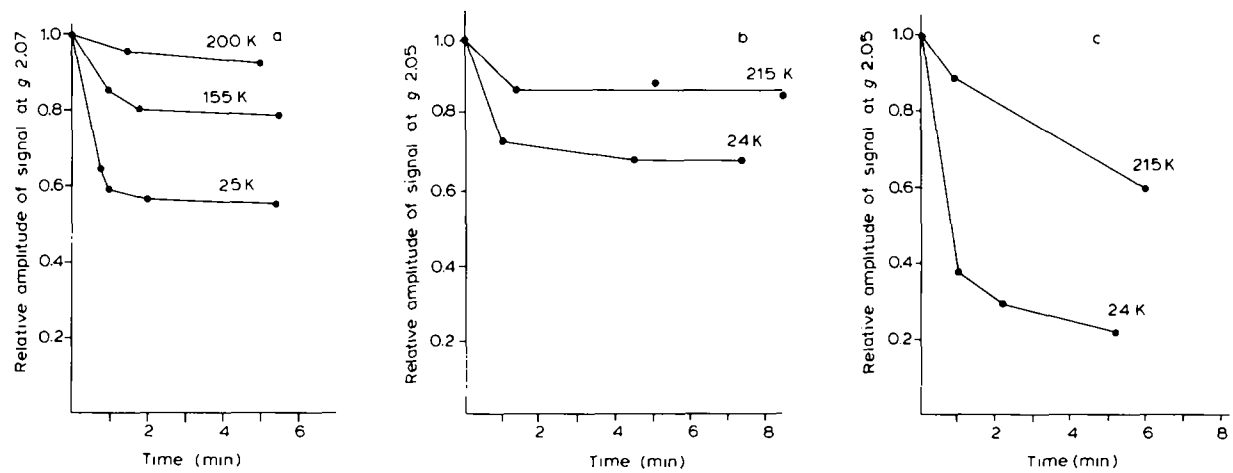


Fig. 6. (a) Kinetics of dark reoxidation of centre B^- at 150 K. After illumination at the temperatures indicated, samples were maintained in the dark at 150 K for varying periods of time, then taken to 25 K for measurements as for Fig. 4. (b) Kinetics of dark reoxidation of centres A^- at 150 K, after illumination at the temperatures indicated. The extent of A reduction was estimated from the amplitude at g 2.05, corrected for the lineshape of the B^- signal. (c) Kinetics of dark reoxidation of A^- at 200 K, after illumination at the temperatures indicated.

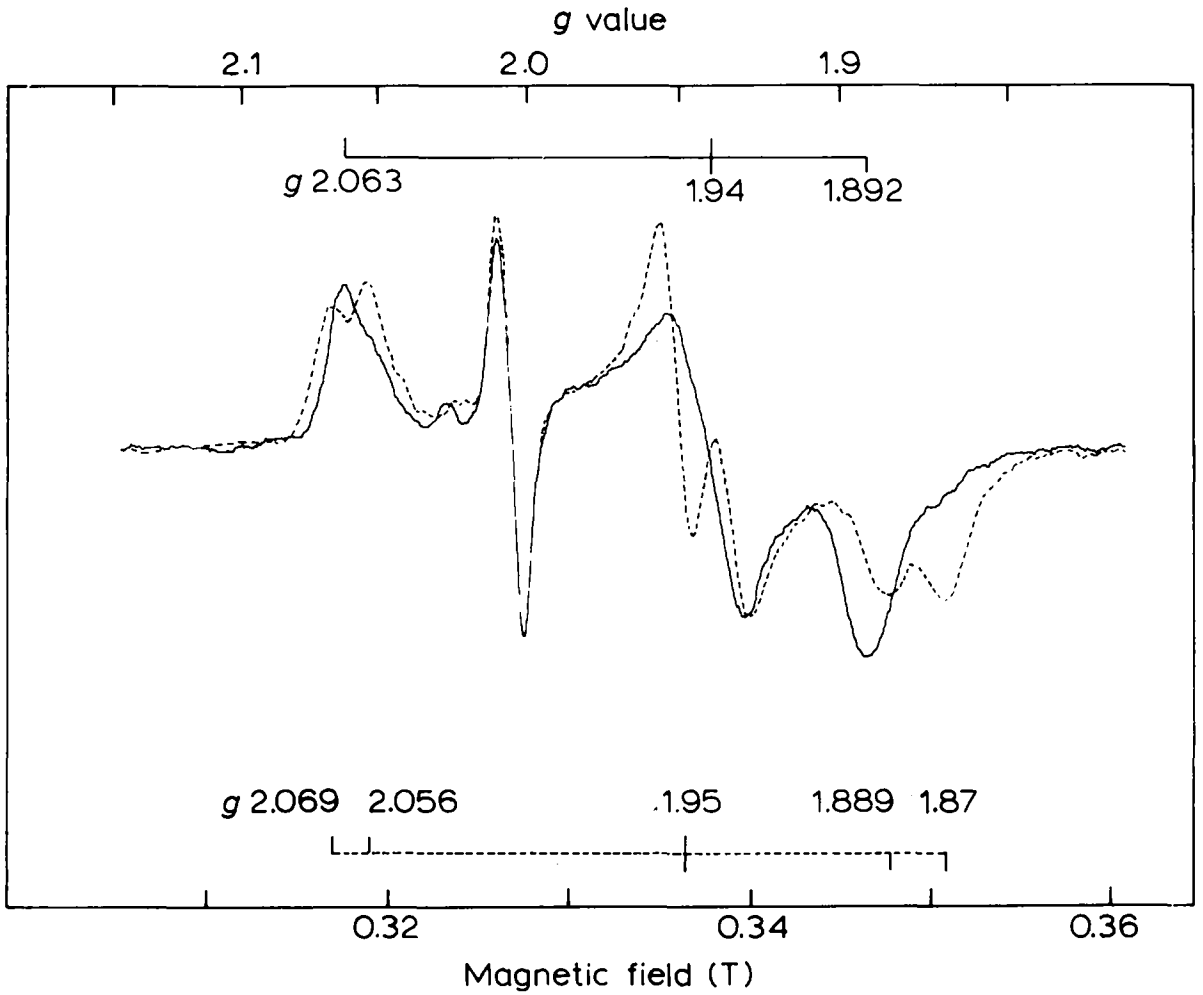


Fig. 8. Comparison of ESR spectra of PS I particles in 50% glycerol, prepared as for Fig. 3, illuminated at 200 K (———) and 25 K (- - - - -).

greater in samples illuminated at higher temperatures. (Fig. 6b and c).

Evidence for conformational changes of centres A and B

The conclusion from these experiments is that the effect of illumination at 200 K differs from that seen at 25 K and leads to a more stable reduced form of centre B. Fig. 8 shows a detailed comparison of spectra recorded at 25 K or PS I particles, in 50 (v/v) glycerol, illuminated at 25 and 200 K.

The former spectrum contains contributions from reaction centres in which A is reduced. Nevertheless, it can be seen that the g values of centre B^- are different in the two cases: g 2.069, 1.933 and 1.883 in the sample illuminated at 25 K, and g 2.063, 1.94 and 1.892 in the sample illuminated at 200 K. The g values of iron-sulphur proteins are often sensitive to changes in solvent composition and salt concentration (e.g., see Ref. 19) which presumably cause subtle changes in the protein conformation or environment of the iron-sulphur cluster.

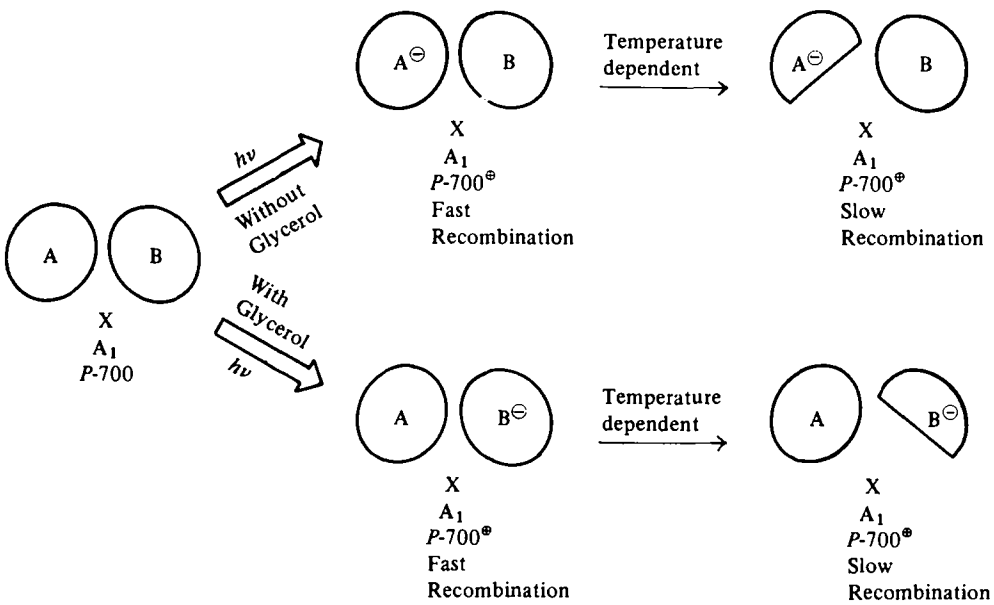
As with centre B, the spectrum of reduced centre A obtained by illumination of PS I particles at 25 K differed from that obtained by illumination at 200 K. It has previously been noted (e.g., Ref. 10) that the

spectrum of centre A obtained by chemical reduction differs from that obtained by photoreduction at very low temperatures. This effect is not as pronounced as for centre B, but can be seen as slight shifts in the g 2.05 and g 1.86 signals in Fig. 1. This emerged clearly when attempts were made to obtain isolated spectra of A^- and B^- by computer subtraction of spectra such as those of Fig. 1. Because of the shifts in the peak positions, very complex shapes resulted. Once again this is evidence that at temperatures above 150 K, a conformational change in centre A takes place after reduction.

Discussion

From these results it appears that the observation that centre A is the principal electron acceptor for PS I (e.g., Ref. 1) is a consequence of carrying out the photochemical reaction at very low temperatures. At higher temperatures, though still in the frozen state, electrons were transferred from *P*-700 either to A or B. The distribution is not determined directly by the relative redox potential of the centres, but by the kinetically favoured electron-transfer pathways. At physiological temperature there may be a redistribution of electrons between the centres, but in our experiments we have not seen it. The rate of recombination of electrons with $P\text{-}700^+$ was faster than the rate of electron transfer between A and B. The difference in redox potentials between A and B is not therefore seen as being functionally significant, particularly since in some species of plants, B has the more negative potential, whereas in other species it is the other way round [12]. The favoured pathway for electron transfer more probably depends on the presence of an acceptor, soluble ferredoxin, attached to the complex.

The pathway of electron transfer within the PS I reaction centre complex, and the proposed conformational shifts, are summarized in Scheme I. On illumination of frozen chloroplasts or PS I particles at 25 K, the upper pathway is favoured, leading to reduction of A (e.g., Fig. 2b). This reduced form recombines rapidly at 120 K. On illumination at



Scheme I. Electron-transfer pathways and possible conformational changes on the acceptor side of PS I. The circles around centres A and B are to indicate conformational states. It is not known whether they are on the same or different protein molecules.

about 150 K, the reduced A changes to a conformational state which recombines more slowly. The lower pathway, leading to reduction of B, occurs less frequently under these conditions, but since B recombines more slowly, at higher temperatures, there is a significant reduction of B on prolonged illumination (Fig. 2c). The presence of glycerol tends to favour the photoreduction of B, and hence the lower pathway. Scheme I is over-simplified in some ways. For example, it appears from the kinetics that even at 25 K, part of the B that is photoreduced is in the 'reduced' conformation.

The pathway by which electrons are transferred from A^- and B^- back to $P\text{-}700^+$ is not yet clear. The immediate electron donor of electrons is assumed to be the third acceptor, X (or A_2). The photoreduction of X is seen only under conditions where A and B are already reduced, and recombination of X^- with $P\text{-}700^+$ is rapid (less than 1 s) even at 10 K (see, e.g., Ref. 20). The midpoint potential of X is considerably more negative than those of A and B, about −710 mV (Ref. 21 and Chamorovsky, S.K. and Cammack, R., unpublished observations), so electron transfer to $P\text{-}700^+$ via X would involve an unfavourable electron transfer. This might account for the slowness of the recombination compared with the photoreduction.

The nature of the conformational shifts giving rise to the changes in g values of the ESR spectra of A^- and B^-, and the slower rate of recombination, is not known. The reduction of [4 Fe-4 S] clusters in analogue compounds [22] and in high-potential iron-sulphur protein [23] tends to cause a distortion along one axis. Possibly, this leads to conformational shifts in the protein, which are prevented at extremely low temperatures. Such a conformational change might make centre B less accessible for charge recombination with $P\text{-}700^+$.

By observing the ESR signals due to the iron-sulphur centres, some of the complexities of the $P\text{-}700^+$ decay kinetics which have previously been measured [14,15] can now be understood. In the optical experiments, approx. 50% glycerol was present, so the recombining species would be principally B^-. In the absence of glycerol, both A^- and B^- would be involved. The reoxidation of each type of iron-sulphur centre occurs in two phases: a rapid phase; and a slower phase, which is greater at higher temperatures, which we have interpreted as due to iron-sulphur centres that have undergone a conformational change. In addition, there will be a very fast phase of $P\text{-}700^+$ reduction due to recombination of electrons from A_1 or X, since there is a fraction (which we estimate to be 22–50%, depending on temperature) of reaction centres in which neither A or B is reduced because of their frozen conformation.

Acknowledgements

We are grateful to Dr. M.C.W. Evans for advice and material assistance during this project, which was supported by grants from the British Council and the U.K. Science Research Council.

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