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# EVIDENCE FOR THE ROLE OF A BOUND FERREDOXIN AS THE PRIMARY ELECTRON ACCEPTOR OF PHOTOSYSTEM I IN SPINACH CHLOROPLASTS

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SUMMARY

The presence of a bound electron transport component in spinach chloroplasts with an EPR spectrum characteristic of a ferredoxin has been confirmed. The ferredoxin is photoreduced at 77 °K or at room temperature, it is not reduced in the dark by $Na_2S_2O_4$. The distribution of the ferredoxin in subchloroplast particles has been investigated. The ferredoxin is enriched in Photosystem I particles and it is proposed that it functions as primary electron acceptor for Photosystem I.

The EPR spectra indicate the presence of two components which are photoreduced sequentially. It is proposed that they may represent two active centres of a single protein.

## INTRODUCTION

Malkin and Bearden[1] have reported that the lamellae from spinach chloroplasts contain a bound compound which has an EPR signal similar to that of ferredoxin. They further reported that this ferredoxin was reduced when the chloroplast lamellae were illuminated at 77 °K, indicating that the ferredoxin was reduced in a solid state reaction and is therefore very close to the chlorophyll involved in the primary photochemical reaction. They proposed that this ferredoxin was the primary electron acceptor in photosynthetic electron transport. We have now confirmed and extended these important experiments. We have shown that an EPR signal similar to that of a reduced ferredoxin is produced in washed broken spinach chloroplasts by illumination at room temperature or at 77 °K. We have prepared subchloroplast particles by the procedure of Sane *et al.*[2]. Examination of these particles shows that although the ferredoxin is present in both large (Photosystem II) and small (Photosystem I) particles, the ferredoxin is enriched in the small particles. The small particles retain the ability to photoreduce the ferredoxin at 77 °K indicating that it functions as the primary electron acceptor for Photosystem I.

## MATERIALS AND METHODS

Broken washed chloroplasts ($P_1S_1$ and $C_1S_1$) were prepared from greenhouse grown spinach by the method of Whatley and Arnon[3]. Chlorophylls *a* and *b* were measured spectrophotometrically by the method of Arnon[4].

Abbreviation: DCIP, dichlorophenolindophenol.

The two photochemical systems were partially separated by the method of Sane *et al.*[2]. $P_1S_1$ or $C_1S_1$ chloroplasts were finally resuspended in 0.02 M Tris–HCl buffer (pH 7.5) containing 0.15 M KCl. This preparation was passed through an Aminco French pressure cell at 6000 lb/inch$^2$. The French press homogenate was then fractionated by centrifugation at 10 000 × *g* for 30 min, 40 000 × *g* for 30 min and 160 000 × *g* for 60 min (designated in the terminology of Sane *et al.*[2], 1K + 10K, 40K, and 160K fractions). The resulting precipitates were resuspended in 0.02 M Tris-HCl buffer (pH 7.5) containing 0.01 M KCl.

The photosynthetic electron transport activity of the chloroplasts and subchloroplast particles was measured using a Clark type oxygen electrode. The reaction mixture was maintained at 20 °C and illuminated with red light ($8 \cdot 10^4$ ergs/cm$^2$ per s) through a heat filter.

Photosystem I activity was measured as an oxygen uptake with methyl viologen as electron acceptor and dichlorophenolindophenol (DCIP) reduced by sodium isoascorbate as electron donor. The reaction mixture contained: 50 mM Tris-HCl buffer (pH 8.0), 24 mM KCl, 0.6 mM $NaN_3$, 30 $\mu$M methyl viologen, 4 $\mu$M DCIP, 8 mM sodium isoascorbate and 4 mM $NH_4Cl$.

Photosystem II activity was measured as an oxygen evolution with $K_3Fe(CN)_6$ as electron acceptor. The reaction mixture contained: 50 mM Tris–HCl buffer (pH 8.0), 24 mM KCl, 1.6 mM $K_3Fe(CN)_6$ and 4 mM $NH_4Cl$.

Samples for EPR spectroscopy were prepared after standing the chloroplast preparation on ice in the dark for 30 min. $Na_2S_2O_4$ solutions were prepared anaerobically in 1 M Tris–HCl buffer (pH 7.5) and were added in the dark. The samples were frozen in liquid $N_2$ and wrapped in aluminium foil for storage at 77 °K, unless otherwise stated.

EPR spectra were obtained using a Varian E4 EPR spectrometer operating at 10 mW and a frequency of 9.170 GHz. First derivative EPR spectra were obtained using a 100-kHz modulation of magnetic field. Samples were cooled to 18 °K by a stream of helium gas passing through a quartz assembly placed inside the EPR cavity. The temperature was monitored by a carbon resistance thermometer placed upstream of the sample. Measurement of the temperature dependence of the $g=1.94$ signal showed that the maximum size of signal was obtained at 18 °K.

## RESULTS

Fig. 1 shows the EPR spectrum at 18 °K obtained with illuminated broken ($P_1S_1$ or $C_1S_1$) spinach chloroplast preparations, in the presence and absence of $S_2O_4^{2-}$. The signal was seen either if the chloroplasts were prepared in the dark and illuminated at 77 °K essentially as described by Malkin and Bearden[1] or if the samples were prepared under normal room illumination and frozen in the light. Samples prepared carefully in the dark did not show any signal even in the presence of $Na_2S_2O_4$. We found that it was necessary to keep the preparations in the dark for 30 min at 0 °C before the $g=1.94$ signal completely disappeared. The EPR spectra show a signal with components at $g=1.95$, $g=1.93$, $g=1.90$ and $g=1.87$ and also at $g=2.05$, although the latter may be obscured by the $g=2.04$ signal due to copper, as reported by Malkin and Bearden[1].

The $g=2.05$ component can be seen more easily in subchloroplast particles

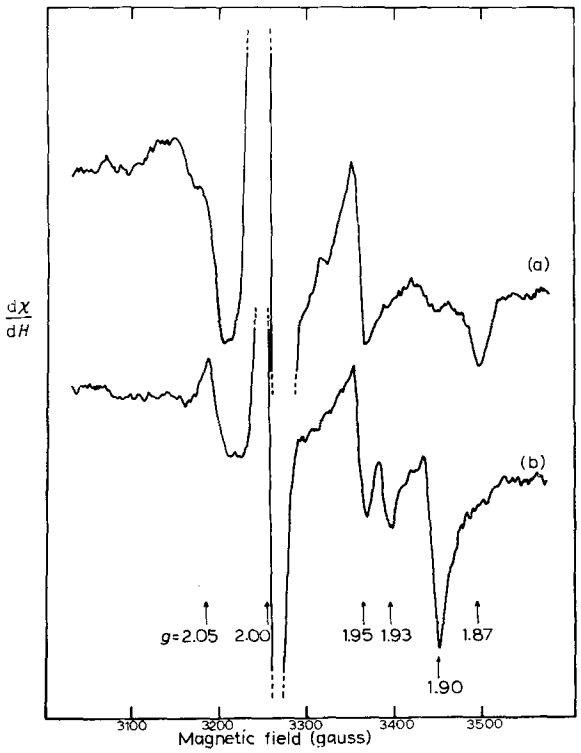


Fig. 1. Low-temperature EPR signals of spinach chloroplasts (a) prepared in the dark and illuminated at 77 °K for 3 min and (b) prepared under room illumination in the presence of 0.02 M $Na_2S_2O_4$ (pH 7.5). $P_1S_1$ chloroplasts were equivalent to 1.7 mg chlorophyll per ml. EPR spectra were then recorded at 18 °K with the following instrument settings: frequency, 9.170 GHz; power, 10 mW; modulation amplitude, 6.3 gauss; scan rate, 1000 gauss/min; gain, 2000.

(see Fig. 3). Some of the $g$ values reported by Malkin and Bearden[1] are different from those reported here, however, a qualitative comparison of the spectra show that the same compound or compounds are involved. Malkin and Bearden[1] found that the components at $g=1.93$ and $g=1.90$ were only seen when $S_2O_4^{2-}$ was added, while the $g=1.87$ component was not seen in the presence of $S_2O_4^{2-}$. We have found, however, that all the components can be seen in spectra obtained in the presence or absence of $S_2O_4^{2-}$, but that the intensity of each component is different under different conditions. The spectra also show a large signal in the $g=2.00$ region which is ascribed to the chlorophyll free radical[5]. In the dark, particularly in the presence of $S_2O_4^{2-}$ the size of this signal is much reduced (see Figs 3 and 4).

Fig. 2 compares the EPR spectra of chloroplasts and subchloroplast particles prepared by the procedure of Sane *et al.*[2]. The EPR samples were prepared and illuminated at room temperature in the presence of $S_2O_4^{2-}$ to obtain the maximum signal size. The spectra in this figure were taken from a single experiment and the chlorophyll concentrations of the different fractions were similar to permit a direct comparison to be made. Table I shows the photochemical activities, chlorophyll content and the relative size of the EPR signals (in arbitrary units) in the different fractions. The spectra were qualitatively similar from all three preparations. The signal size in

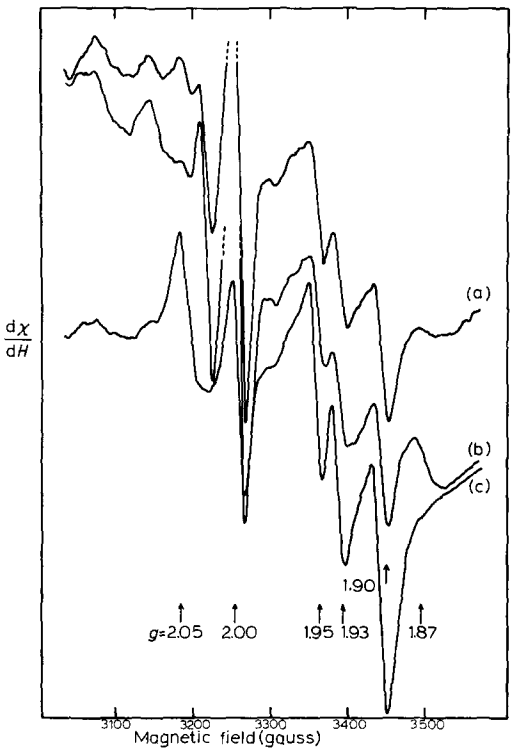


Fig. 2. Low-temperature EPR signals in (a) spinach chloroplasts, (b) 1K + 10K particles and (c) 160K particles. Chloroplasts ($C_1S_1$) and particles (1.9 mg chlorophyll per ml) were illuminated ($4 \cdot 10^4$ ergs/cm$^2$ per s) for 20 min at 20 °C in the presence of 0.02 M $Na_2S_2O_4$ (pH 7.5). EPR spectra were then recorded as in Fig. 1, except with gain, 1250.

TABLE I

DISTRIBUTION OF CHLOROPHYLLS, PHOTOCHEMICAL ACTIVITIES AND EPR SIGNAL SIZE IN CHLOROPLASTS AND SUBCHLOROPLAST PARTICLES

Chlorophyll *a* and *b* concentrations are expressed as mg chlorophyll per ml. Photosystem I and II activities are expressed as μequiv $e_2^-$ per mg chlorophyll per h. EPR signal size is expressed in arbitrary units.

| *Fraction* | *Chloro-phyll a* | *Chloro-phyll b* | *Ratio chloro-phyll a/b* | *Photo-system II activity* | *Photo-system I activity* | *EPR signal size* |
|---|---|---|---|---|---|---|
| Original chloroplasts | 1.38 | 0.48 | 2.90 | 80 | 125 | 15 |
| 1K + 10K | 1.35 | 0.50 | 2.70 | 23 | 65 | 12 |
| 160K | 1.51 | 0.34 | 4.45 | 0 | 156 | 25 |

preparations of 160K particles was, however, approximately twice as large as that from the original chloroplasts. As can be seen from Table I this increase in signal size may be correlated with the increased chlorophyll *a/b* ratio and the fact that only Photosystem I activity is observed in the 160K particles. The signal from the 1K + 10K particles was slightly smaller than that from the chloroplasts, correlating with a

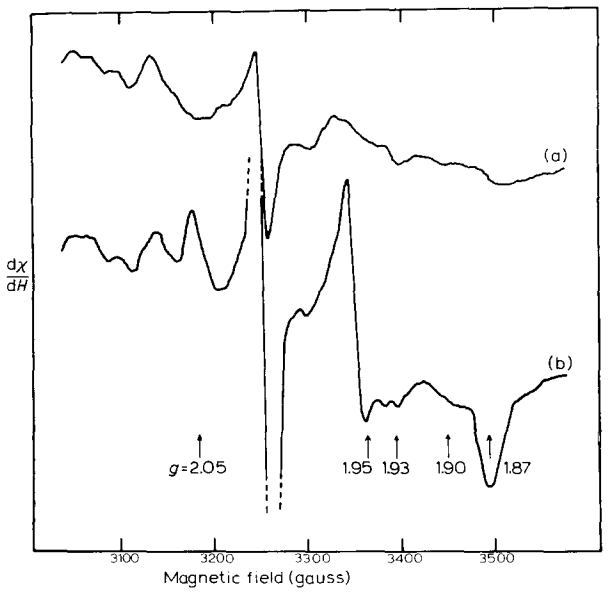


Fig. 3. Low-temperature EPR signals in 160K particles (4.7 mg chlorophyll per ml). (a) EPR sample frozen in dark; (b) illuminated at 77 °K ($4 \cdot 10^4$ ergs/cm$^2$ per s) for 3 min. EPR spectra were then recorded as in Fig. 1, except with gain, 1000.

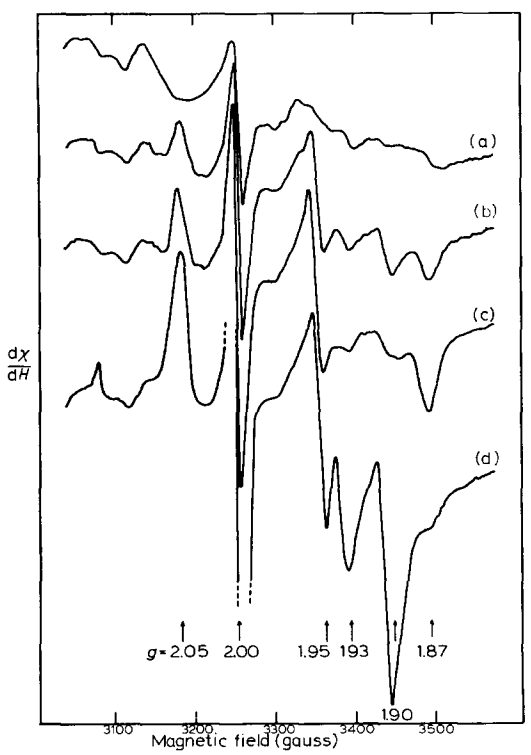


Fig. 4. Low-temperature EPR signals in 160K particles (4.7 mg chlorophyll per ml) in the presence of 0.02 M $Na_2S_2O_4$ (pH 7.5). (a) EPR sample frozen in dark; (b) EPR sample frozen in room light but stored at 77 °K in dark; (c) EPR sample frozen in dark and illuminated at 77 °K ($4 \cdot 10^4$ ergs/cm$^2$ per s) for 3 min; (d) EPR sample illuminated at 20 °C ($4 \cdot 10^4$ ergs/cm$^2$ per s) for 3 min and frozen in room light. EPR spectra were then recorded as in Fig. 1, except with gain, 1000.

slight decrease in the chlorophyll *a/b* ratio. The decrease in signal size is, however, small and may not be significant.

Figs 3 and 4 show the conditions required for the reduction of the bound ferredoxin in 160K particles. If the particles are kept in total darkness and frozen in liquid nitrogen no signal is observed in the $g=1.94$ region either in the presence or absence of $Na_2S_2O_4$ (Figs 3a and 4a). Illumination of the particles at 77 °K with relatively low light intensities ($4 \cdot 10^4$ ergs/cm$^2$ per s) for 3 min results in the reduction of the ferredoxin and the signals shown in Figs 3b and 4c are observed. There does not appear to be any significant difference in the extent of reduction in the presence or absence of $S_2O_4^{2-}$. Fig. 4b shows that if a sample is prepared under room illumination and frozen and stored at 77 °K in the dark, a signal similar to that seen as a result of illumination at 77 °K is obtained. If a sample, prepared in the presence of $S_2O_4^{2-}$ is illuminated ($4 \cdot 10^4$ ergs/cm$^2$ per s for 3 min) at room temperature and then frozen and stored at 77 °K a much larger signal is obtained (Fig. 4d).

## DISCUSSION

The results described in this paper confirm that spinach chloroplasts contain a bound ferredoxin which may be photoreduced at 77 °K.

We have shown that the ferredoxin is associated with the Photosystem I (160K) particles obtained by French press treatment of chloroplasts and that these particles have the ability to photoreduce the ferredoxin at 77 °K. We would therefore propose that this ferredoxin is the primary electron acceptor of Photosystem I.

Malkin and Bearden[1] proposed that the ferredoxin was a primary low potential electron acceptor in photosynthesis but did not discuss whether it was the acceptor for Photosystem I, or of Photosystem II as in the scheme of Knaff and Arnon[6]. The Photosystem II (1K + 10K) particles which we have prepared always contain bound ferredoxin, however, in view of the high Photosystem I activity which they exhibit, it seems likely that this is evidence for the failure of the procedure used to completely separate Photosystem I and II rather than for a functional role for the bound ferredoxin in Photosystem II. It has been suggested that other components isolated from chloroplasts might be the primary electron acceptor of Photosystem I, notably in recent years the ferredoxin reducing substance described by Yocum and San Pietro[7] and the $S_{L\text{-}eth}$ fraction prepared by Regitz *et al.*[8]. However, neither of these compounds contains appreciable amounts of iron and the nature of the active centre is unknown.

The ability of chloroplasts and subchloroplast particles to reduce the bound ferredoxin at 77 °K and also at room temperature is very strong evidence for its role as the primary electron acceptor of Photosystem I. It is therefore unlikely that any components isolated from chloroplasts which is not a ferredoxin is the primary electron acceptor. The ferredoxin reducing substance and the $S_{L\text{-}eth}$ fraction may perhaps function later in the electron transport chain or are membrane components required for the operation of the electron transport chain but are not directly involved in it.

Malkin and Bearden[1] were able to observe the components at $g=1.93$ and $g=1.90$ only on addition of $S_2O_4^{2-}$. They state that reduction occurred in the dark, when their preparations aggregated, presumably as a result of pH changes occuring when they added solid unbuffered $Na_2S_2O_4$ to the preparation. We have found that all the

components can be seen as a result of photoreduction at 77 °K but that the intensities vary. In chloroplasts without added reductant the $g=1.95$ and $g=1.87$ components tend to be largest while in the presence of $S_2O_4^{2-}$ the $g=1.93$ and $g=1.90$ signals are larger. In subchloroplast particles all four components can be seen after illumination at 77 °K but the $g=1.93$ and $g=1.90$ components become much larger under some conditions. We have obtained the largest signals after illumination at room temperature in the presence of $S_2O_4^{2-}$ (see Fig. 4d). It is possible that the two pairs of components of the signals, those at $g=1.95$ and $g=1.87$ and those at $g=1.93$ and $g=1.90$ represent two separate electron transport compounds which can be sequentially reduced at 77 °K. The change in the intensities of the different components resembles that seen on reductive titration of 8 iron bacterial ferredoxins where components due to two active centres may be observed to appear sequentially during reduction[9]. It is possible that the two groups of components seen in the chloroplast spectra represent two active sites of a single electron transport protein. Our results do not support the suggestion of Malkin and Bearden[1] that the signals at $g=1.93$ and $g=1.90$ are due to damage to the ferredoxin. Neither do our results suggest that these signals are due to a ferredoxin located elsewhere in the electron transport chain, as might be possible if they were observed only on reduction by $S_2O_4^{2-}$.

The very much larger signals seen after illumination at room temperature in the presence of $S_2O_4^{2-}$ show that only partial reduction is obtained at 77 °K. This is presumably in part because the light does not penetrate the sample, although we obtained large signals using much lower light intensities and shorter illumination times than Malkin and Bearden[1]. It is also possible that only partial reduction is observed because the electron is able to return to the chlorophyll. At room temperature in the presence of $S_2O_4^{2-}$ all components of the intermediary electron transport chain might be expected to be reduced and the photooxidised chlorophyll would be reduced rapidly, preventing the electron returning from the bound ferredoxin, resulting in more complete reduction of the ferredoxin. We have been unable to reduce the ferredoxin with $S_2O_4^{2-}$ in the dark. The failure to reduce the ferredoxin is not unprecedented. Ferredoxins from the photosynthetic bacteria are only slowly reduced by $S_2O_4^{2-}$ (ref. 10). The binding of the ferredoxin in the membrane in close association with chlorophyll might also make it inaccessible to chemical reductants. Malkin and Bearden[1] proposed that the spectra with signals at $g = 2.04$, 1.95 and 1.87 represented a bound plant type ferredoxin, our data do not support this suggestion as they indicate that a ferredoxin with two active centres may be involved. Identification of the nature of the ferredoxin will depend on its isolation in native form.

ACKNOWLEDGEMENTS

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