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Biochemical evidence for the role of the bound iron-sulphur centres A and B in NADP reduction by Photosystem I

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The Photosystem I reaction centre contains three iron-sulphur centres. Although extensively characterised at low temperature, the role of these centres in room-temperature electron transport to NADP has never been demonstrated. An iron-sulphur protein was solubilised from spinach Photosystem I particles by butanol extraction. The reduced protein has the characteristic EPR spectrum of a 2[4Fe4S] ferredoxin and is considered to be the solubilised Fe-$S_{A/B}$ centres of Photosystem I. Oxidation-reduction potential titration of the protein showed $E_m \approx -510$ mV. Photosystem I particles depleted of the Fe-$S_{A/B}$ centres were prepared by urea treatment. The depleted preparation had only 15% of the original EPR signals due to the Fe-$S_{A/B}$ centres, showed a rate of $P700^+$ rereduction following flash illumination changed from $t_{1/2} = 12$ ms to $t_{1/2} = 1$ ms, and catalysed $NADP^+$ photoreduction at only 5% of the initial rate. Reconstitution with the solubilised protein led to recovery of the EPR spectrum (80%) and low-temperature electron transfer from P700 to Fe-S_A. The rereduction of $P700^+$ returned to $t_{1/2} = 12$ ms and $NADP^+$ reduction was recovered to 90% of the initial rate. Oxidative destruction of the iron-sulphur centres in the solubilised protein prevented reconstitution. The results show that the Fe-$S_{A/B}$ a centres can be removed from Photosystem I and reconstituted, and that these iron-sulphur centres are essential components of the overall electron transfer to $NADP^+$.

Introduction

Photosynthetic electron transport in oxygenic photosynthetic organisms catalyses the oxidation of water and the reduction of $NADP^+$. $NADP^+$ reduction is catalysed by the Photosystem I reaction centre. Photosystem I is a membrane bound multipigment-protein complex containing the reaction-centre chlorophyll P700, a number of low-potential redox centres thought to be involved in electron transfer to a soluble ferredoxin, and binding sites for the ferredoxin and the electron donor plastocyanin [1]. Electrons are transferred from the soluble ferredoxin to $NADP^+$ by a flavoprotein ferredoxin-$NADP^+$ oxidoreductase, which is a peripheral protein associated with the thylakoid membrane surface.

The low-potential membrane bound redox centres thought to form the electron acceptor complex have been characterised by EPR and absorption spectroscopy. Models of electron transfer in the reaction centre are based on low-temperature (< 77 K) EPR spectroscopy or measurements of the kinetics of reduction of $P700^+$ following flash illumination. The acceptor complex includes two components: A0, probably a chlorophyll, and A1, possibly a quinone, which are thought to act as intermediary electron carriers to three iron-sulphur centres, Fe-S_x, Fe-S_A and Fe-S_B. Fe-S_x is a 4Fe-4S centre thought to be bound between the two major polypeptides of the reaction centre. Fe-S_A and Fe-S_B are 4Fe-4S centres bound to a 10 kDa polypeptide which forms part of the reaction-centre complex [2,3]. Although reduction of all these components as a result of electron transfer at low temperature is extensively documented, the role of the iron-sulphur centres in electron transfer to $NADP^+$ at room temperature has not been directly demonstrated.

The Fe-$S_{A/B}$ protein can be removed from Photosystem I by a number of procedures including urea [4] and ethylene glycol [5] treatment. It can be isolated in

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Abbreviation: P700, the reaction centre chlorophyll donor of Photosystem I.

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active form either by acetone [6] or butanol [7] extraction of Photosystem I preparations. Golbeck and his co-workers in a series of elegant experiments [9–11] have shown that the kinetic and EPR characteristics of the Photosystem I complex can be reconstituted from "apo-Photosystem I" and the Fe-$S_{A/B}$ protein. These experiments open the way for a biochemical investigation of the role of the Fe-$S_{A/B}$ protein in $NADP^+$ reduction. We have now confirmed the work of Golbeck and his co-workers showing reconstitution of the iron-sulphur centres and have investigated the ability of a reconstituted system to reduce $NADP^+$. The results show that the Fe-$S_{A/B}$ protein is required for $NADP^+$ reduction.

Materials and Methods

Chloroplasts were prepared from market spinach [12] and Photosystem I was isolated using either digitonin (high-purity digitonin, Calbiochem) [13] or Triton X-100 [14]. The Fe-$S_{A/B}$ protein was removed from Photosystem I by treatment with 6.8 M urea. 9 M urea in 0.1 M Tris-HCl (pH 8.0) was added dropwise with rapid stirring to a suspension of Photosystem I particles, initial concentration 1.5 mg chlorophyll/ml. The suspension was then diluted with oxygen-free Tris-HCl buffer (pH 8.0) to 250 μg chlorophyll/ml and 6.8 M urea and stirred under argon. The removal of the Fe-$S_{A/B}$ protein was monitored by measuring the rereduction of $P700^+$ by back-reaction from the electron acceptors following laser flash excitation. When the $t_{1/2}$ for rereduction had changed from 12 ms to 1 ms (25–30 min incubation) the treatment was stopped by diluting the reaction mixture 10-times with oxygen-free 20 mM Tris-HCl (pH 8.0). Urea was then removed by washing the preparation over an ultrafiltration membrane (Amicon YM-100) or by centrifugation for 2 h at $150000 \times g$. The preparation was finally concentrated to 1 mg chlorophyll/ml and stored frozen in liquid nitrogen until required. For EPR analysis, depleted Photosystem I particles (12 μg chlorophyll/ml) in 25 mM Tricine-KOH (pH 7.8) with 0.1% β-mercaptoethanol and 0.07% Triton X-100 were incubated with excess Fe-$S_{A/B}$ protein for 10 min in the dark at room temperature under argon. The preparation was then washed over an ultrafiltration membrane (Amicon YM-100) to remove unbound protein and concentrated to 0.5 mg chlorophyll/ml. Reconstitution for optical assays and $NADP^+$ reduction was also carried out in situ adding excess Fe-$S_{A/B}$ protein to the reaction mixture and incubating for 10 min before illumination.

The Fe-$S_{A/B}$ protein was extracted from purified Triton X-100 Photosystem I particles by butanol extraction as described in Ref. 7. The protein was concentrated and freed of butanol by washing on a membrane filter (Amicon YM-5) [10] for use in redox titrations. It was further purified by absorption on DEAE-Fractogel [7] and elution with 0.3 M NaCl in 20 mM Tris-HCl buffer (pH 8.0) for use in reconstitution experiments. This preparation contains contaminating polypeptides of high and low molecular weight. Further purification by passage through a Sephacryl S200 HR (Pharmacia) column in anaerobic 20 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl and 5 mM dithiothreitol resulted in a fraction highly enriched in the Fe-$S_{A/B}$ protein. Analysis by SDS-PAGE showed polypeptides of approx. 10, 15 and 20 kDa in this preparation. The ability of each preparation to reconstitute the 12 ms back-reaction in kinetic optical assays of $P700^+$ rereduction was measured and a 3-fold excess by volume of the preparation used in the experiments described. Spinach ferredoxin, plastocyanin and ferredoxin-$NADP^+$ oxidoreductase were prepared by DEAE-cellulose chromatography of chloroplast extracts [15–17]. Oxidation-reduction potential titrations were carried out as described previously [18] using 30 μM methyl viologen and triquat as mediators. EPR spectra were recorded with a JEOL RE-1X spectrometer fitted with an Oxford Instruments liquid helium cryostat. Kinetic optical measurements of $P700^+$ at 820 nm were made using a laboratory-built spectrophotometer [19]. Samples were excited at 1 Hz at 337 nm with an 800 ps flash from a nitrogen laser (Photochemical Research Associates, LN1000) as actinic light source. $NADP^+$ reduction was measured as the change in A_{340} following 30 s periods of illumination of the reaction mixture in a cuvette by saturating white light.

Results

Properties of the isolated Fe-$S_{A/B}$ protein

The isolated Fe-$S_{A/B}$ protein has the characteristic EPR spectrum of a soluble 2(4Fe4S) iron-sulphur protein (Fig. 1). Oh-Oka et al. [8] have reported the redox potential of the centres to be -470 and -560 mV, the two centres being distinguished by different temperature-dependence of their EPR signals. We have carried out titrations on the preparation obtained by butanol extraction. We found that the redox centres were unstable under the conditions we normally use for titration, but that they could be stabilised by the presence of 25% glycerol in the medium. In these titrations a single wave was observed on the titration with $E_m \approx 510$ mV. The spectra recorded in the titration were typical of a 2[4Fe4S] ferredoxin. There was no major effect of temperature on the relative signal sizes at different potentials and we did not obtain evidence for different potentials for the two centres. Plotting the titration curve for different parts of the spectrum, or at 10 K or 40 K, did not materially affect the midpoint potential. The preparation essentially showed the properties of the higher-potential component described by Oh-Oka

Fig. 1. The EPR spectrum of the isolated Fe-$S_{A/B}$ protein. The protein in 20 mM Tris-HCl (pH 8.0) containing 0.3 M NaCl was reduced with 0.7% $Na_2S_2O_4$ for 5 min before freezing. EPR conditions: microwave power, 10 mW; temperature, 15 K; modulation width, 1 mT; instrument gain, 250.

et al. [8] We have previously shown that glycerol affects the properties of the Fe-$S_{A/B}$ centres in situ [20], so it may be that while stabilising the centres it changes the environment sufficiently to remove the differences reported by Oh-Oka et al. [8] The results confirm that the low potential of the Fe-$S_{A/B}$ centres in the reaction centre is a property of the Fe-$S_{A/B}$ protein itself, but show that the characteristic EPR spectrum of the two centres in vivo is the result of binding to Photosystem I.

The role of the Fe-$S_{A/B}$ protein in NADP$^+$ reduction by Photosystem I

The role of the Fe-$S_{A/B}$ protein in electron transport was investigated using digitonin Photosystem I particles. Similar results were obtained with Triton X-100 particles but with large variations between preparations. Inactivation and reactivation of the Photosystem I preparations was initially monitored by measuring the rereduction of P700$^+$ by back-reaction from the electron acceptors following laser flash illumination (Fig. 2). Before urea treatment the preparations showed rereduction of P700$^+$ with a half-time of about 12 ms, reflecting the back-reaction from Fe-$S_{A/B}$, Fig. 2-1. During the treatment, the rate of rereduction increased to about 1 ms, Fig. 2-2, reflecting the back reaction from Fe-S_A. Readdition of the isolated Fe-$S_{A/B}$ protein resulted in a recovery of the original kinetics, Fig. 2-3. In Photosystem I preparations, the low-temperature photooxidation of P700 and concomitant reduction of Fe-S_A can be observed by EPR spectrometry (Fig. 3 right); after urea treatment, Fe-S_A is decreased to about 15% of the original, Fig. 3-2. After reconstitution with the Fe-$S_{A/B}$ protein, P700 oxidation is again coupled to Fe-S_A reduction and the signal size of Fe-S_A is restored to about 80% of that in the starting material (Fig. 3-3). If samples are illuminated at room temperature and frozen under illumination, both centres A and B are reduced. Fig. 3 (left) shows that both centres A and B are recovered on reconstitution. After reconstitution the spectra show slight distortion, which may suggest that the reconstituted structure is not completely identical to the original.

NADP$^+$ reduction by washed spinach thylakoids requires addition of the soluble 2Fe2S ferredoxin; all

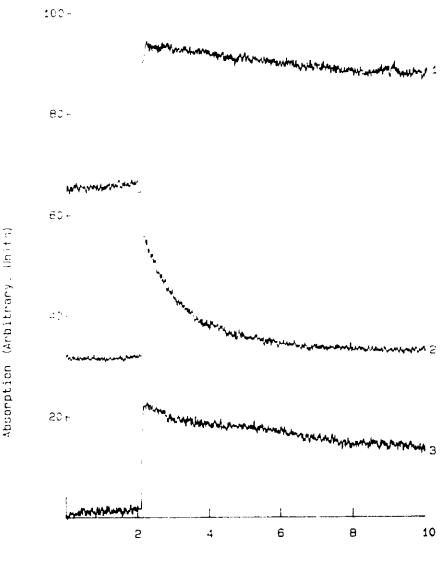

Fig. 2. Kinetics of rereduction of P700$^+$ following oxidation by laser flash illumination. (1) Digitonin Photosystem I particles. (2) Digitonin Photosystem I particles following urea treatment to remove Fe-$S_{A/B}$ as described in the text. (3) Urea-treated digitonin particles reconstituted with the Fe-$S_{A/B}$ preparation. The reaction mixture contained: Photosystem I particles (25 μg chlorophyll/ml) in 20 mM Tricine-KOH (pH 8.0), 6.7 mM sodium ascorbate and 68 μM dichlorophenol indophenol. 30 repetitions were averaged at 1 Hz flash repetition rate.

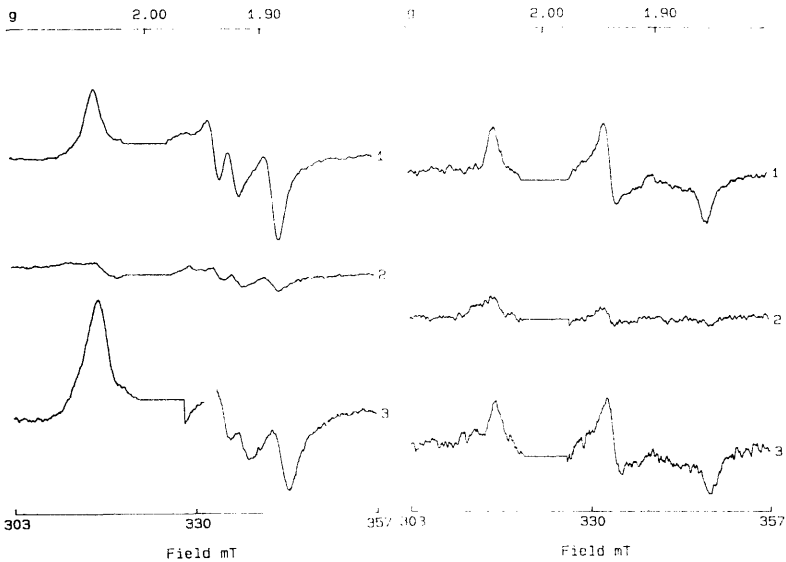

Fig. 3. EPR spectra of digitonin Photosystem I particles. (Right) Light-minus-dark difference spectra of samples reduced with 30 mM sodium ascorbate, frozen in the dark and illuminated at 15 K in the EPR cavity. (Left) Spectra of samples reduced with sodium ascorbate, illuminated at room temperature and frozen under illumination. (1) Control samples of digitonin Photosystem I particles before treatment. (2) Urea-treated particles with the Fe-$S_{A/B}$ protein removed. (3) Urea-treated particles after reconstitution with the Fe-$S_{A/B}$ protein preparation. The samples contained 500 μg/chlorophyll/ml. Urea treatment and reconstitution are described in the text. EPR conditions: Microwave power, 10 mW; temperature, 15 K; modulation width, 1.25 mT. Instrument gain, 500. The $g = 2.00$ region of the spectra has been deleted for clarity.

other protein components are bound to the thylakoids. After isolation of the Photosystem I by digitonin treatment, plastocyanin and $NADP^+$-ferredoxin oxidoreductase are also required. Urea treatment of the digitonin particles results in almost complete loss of $NADP^+$ reduction activity. Activity is reconstituted by addition of the Fe-$S_{A/B}$ protein preparation (Table I).

In the results shown almost all of the $NADP^+$ reduction activity of the digitonin preparation is lost, the residual activity of about 5% is rather lower than the residual Fe-S_A observed by EPR. The ability to reconstitute the $NADP^+$ reduction by replacing the Fe-$S_{A/B}$ protein depends on the retention of Fe-S_X, this centre is also damaged by the urea treatment and a balance

TABLE I

$NADP^+$ reduction by digitonin Photosystem I particles

The reaction mixture contained 25 mM Tricine-KOH (pH 7.8), 6.7 mM sodium ascorbate, 68 μM dichlorophenol indophenol, 0.7% Triton X-100, 0.1% β-mercaptoethanol, digitonin Photosystem I particles (10 μg chlorophyll/ml), 1.5 μM plastocyanin, 5 μM ferredoxin, 0.5 mM $NADP^+$, ferredoxin-$NADP^+$ oxidoreductase and Fe-$S_{A/B}$ protein in excess. $NADP^+$ reduction was followed by measuring the change in A_{340} at 30 s intervals following illumination.

	Rate of $NADP^+$ reduction (μmol/mg chlorophyll per h)
(1) Native Photosystem I	718
(2) Urea-treated Photosystem I (Fe-$S_{A/B}$ removed)	28
(3) Urea-treated Photosystem reconstituted with Fe-$S_{A/B}$	673
(3) Urea-treated Photosystem I reconstituted with Fe-$S_{A/B}$:	
minus ferredoxin	0
minus ferredoxin-NADP oxido-reductase	0
(4) Urea-treated Photosystem I with oxidised Fe-$S_{A/B}$ protein	30

has to be chosen between removing as much of the Fe-$S_{A/B}$ as possible and obtaining high levels of reconstitution. Following reconstitution, the $NADP^+$ reduction activity is restored to about 90% of the initial activity, a recovery similar to that observed for the Fe-S_A by EPR. Following urea treatment, plastocyanin is no longer required for $NADP^+$ reduction, kinetic experiments show that plastocyanin can no longer catalyse fast electron donation to $P700^+$. However, it seems that the damaged donor side is then sufficiently accessible to the artificial donors, ascorbate and dichlorophenol indophenol, to permit good rates of $NADP^+$ reduction. Although the maximum initial rate of $NADP^+$ reduction is not completely recovered, it may be that this reflects limitation by electron donation rather than failure to fully reconstitute the electron acceptors. Destruction of the iron-sulphur centres of the Fe-$S_{A/B}$ protein by exposing it to oxygen prevents reconstitution of $NADP^+$ reduction, confirming that the iron-sulphur centres are essential. The residual activity of the urea-treated preparation is not inhibited by the oxidised protein.

The Fe-$S_{A/B}$ preparation is not free of other polypeptides. The results presented were obtained with the relatively crude preparation from the DEAE column. Qualitatively the same results are obtained using the more purified fraction from the Sephacryl column. However, the extent of reconstitution is lower, 50% rather than 90%. We cannot therefore exclude the possibility that other proteins may be required to form the binding sites on the reaction centre. The present experiments show that the Fe-$S_{A/B}$ protein is required for $NADP^+$ reduction by Photosystem I. Further work will be required to determine the role of other low-molecular-weight polypeptides in forming binding sites for soluble electron carriers, and controlling the rate of electron flow through the reaction centre.

References

1 Evans, M.C.W. and Bredenkamp, G. (1990) Physiol. Plant. 79, 415–420.
2 Oh-oka, H., Takahashi, Y., Wada, K., Matsubara, H., Ohyama, K. and Ozeki, H. (1987) FEBS Lett. 218, 52–54.
3 Hoj, P.B., Svendsen, I.B., Scheller, H.V. and Moller, B.L. (1987) J. Biol. Chem. 262, 12676–12684.
4 Golbeck, J.H. (1987) Biochim. Biophys. Acta 895, 167–204.
5 Hoshina, S., Sakurai, R., Kunishima, N., Wada, K. and Itoh, S. (1990) Biochim. Biophys. Acta 1015, 61–68.
6 Wynn, R.M. and Malkin, R. (1988) FEBS Lett. 229, 293–297.
7 Oh-oka, H., Takahashi, Y., Matsubara, H. and Itoh, S. (1988) FEBS Lett. 234, 291–294.
8 Oh-oka, H., Itoh, S., Saeki, K., Takahashi, Y. and Matsubara, H. (1991) Plant Cell Physiol. 32, 11–17.
9 Golbeck, J.H., Mehari, T., Parrett, K. and Ikegami, I. (1988) FEBS Lett. 240, 9–14.
10 Mehari, T., Parrett, K.G., Warren, P.V. and Golbeck, J.H. (1991) Biochim. Biophys. Acta 1056, 139–148.
11 Parrett, K.G., Mehari, T. and Golbeck, J.H. (1990) Biochim. Biophys. Acta 1015, 341–352.
12 Ford, R.C. and Evans, M.C.W. (1983) FEBS Lett. 160, 159–164.
13 Boardman, N.H., (1971) Methods Enzymol. 23, 268–276.
14 Williams-Smith, D.L., Heathcote, P., Sihra, C.K. and Evans, M.C.W. (1978) Biochem. J. 170, 565–572.
15 Katoh, S. (1973) Methods Enzymol. 23, 408–413.
16 Buchanan, B.B. and Arnon, D.I. (1973) Methods Enzymol. 23, 413–440.
17 Shin, M. (1973) Methods Enzymol. 23, 440–447.
18 Evans, M.C.W., Reeves, S.G. and Cammack, R. (1974) FEBS Lett. 49, 111–114.
19 Hubbard, J.A.M., Corrie, A.R., Nugent, J.H.A. and Evans, M.C.W. (1989) Biochim. Biophys. Acta 977, 91–96.
20 Evans, M.C.W. and Heathcote, P. (1980) Biochim. Biophys. Acta 590, 89–96.