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Reconstitution of the iron-sulfur clusters in the isolated F_A/F_B protein: EPR spectral characterization of same-species and cross-species Photosystem I complexes

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The ESR spectra of the F_A/F_B iron-sulfur clusters in spinach and *Synechococcus* sp. PCC 6301 Photosystem I complexes differ slightly, but characteristically. In the fully reduced state, the spinach F_A/F_B resonances appear at $g = 2.051$, 1.941, 1.923, 1.887, while the *Synechococcus* sp. PCC 6301 F_A/F_B resonances appear at $g = 2.047$, 1.937, 1.917 and 1.879; both are independent of the details of Photosystem I isolation or the method of reduction. When the spinach or *Synechococcus* sp. PCC 6301 F_A/F_B holoprotein is removed from the thylakoid membrane by solvent extraction, the ESR resonances broaden and the iron-sulfur clusters become labile, degrading rapidly to the level of zero-valence sulfur. We show that the clusters can be reinserted in vitro by incubating the isolated F_A/F_B apoprotein for 12 h with $FeCl_3$ and Na_2S in the presence of β-mercaptoethanol under strictly anaerobic conditions. Upon chemical reduction with $Na_2S_2O_4$ at pH 10, the rebuilt spinach F_A/F_B protein shows the broadened resonances of F_A and F_B, but when rebound to a *Synechococcus* sp. PCC 6301 Photosystem I core protein, the hybrid spinach-*Synechococcus* Photosystem I complex shows sharpened resonances with g-values of 2.052, 1.941, 1.922 and 1.886. These g-values are similar to those of the native spinach Photosystem I complex. In contrast, when a spinach or *Synechococcus* sp. PCC 6301 F_A/F_B holoprotein is rebound to a Photosystem I core protein from the same species, the F_A/F_B resonances appear identical to their respective control Photosystem I complexes. These results indicate that the altered ESR spectrum reported earlier on reconstitution of a solvent-extracted spinach F_A/F_B holoprotein with a *Synechococcus* sp. PCC 6301 Photosystem I core protein (Golbeck et al. (1988) FEBS Lett. 240, 9–14) is a consequence of the cross-species reconstitution and does not result from damage to the F_A/F_B holoprotein incurred during isolation. This in vitro reconstitution protocol circumvents the need to isolate the labile F_A/F_B holoprotein, and makes possible reinsertion of the iron-sulfur clusters following modification of the F_A/F_B apoprotein or the *psaC* gene.

Introduction

We reported earlier [1,2] that the peripheral low-molecular-mass polypeptides, including the 8.9 kDa, F_A/F_B protein, could be removed from a spinach or a *Synechococcus* sp. PCC 6301 Photosystem I complex by treatment with chaotropic agents followed by sucrose gradient ultracentrifugation. The resulting preparation, termed the Photosystem I core protein, demonstrated charge separation between P700 and F_X at both room and cryogenic temperatures. Shortly thereafter, we reported that the F_A/F_B polypeptide, isolated from spinach thylakoid membrane fragments by acetone/methanol extraction [3,4], could be rebound to the *Synechococcus* sp. PCC 6301 Photosystem I core protein [5]. Although complete restoration of electron flow from P700 to F_A and F_B was achieved, the iron-sulfur clusters in the interspecies complex showed a slightly different set of g-values than those of the control *Synechococcus* sp. PCC 6301 Photosystem I complex. We found that the liberated spinach F_A/F_B protein was extremely sensitive to oxidative denaturation, resulting in the loss of the iron-sulfur clusters soon after removal from the thylakoid membrane. However, the inclusion of di-

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Abbreviations: Chl, chlorophyll; ESR, electron spin resonance; CP 1, chlorophyll protein 1; PAGE, polyacrylamide gel electrophoresis; DCPIP, dichlorophenolindophenol; SDS, sodium dodecyl sulfate; β-ME, β-mercaptoethanol.

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thionite during the isolation procedure stabilized the iron-sulfur clusters against oxidative degradation and allowed limited studies of the isolated F_A/F_B protein. In contrast, the *Synechococcus* sp. PCC 6301 F_A/F_B apoprotein could be readily recovered after chaotrope-induced removal from the Photosystem I complex, but the iron-sulfur clusters were already degraded to the zero-valence sulfur state [2]. We showed that the iron-sulfur clusters in the *Synechococcus* sp. PCC 6301 F_A/F_B apoprotein could be reinserted using $FeCl_3$, Na_2S and β-mercaptoethanol concomitant with rebinding of the holoprotein to the *Synechococcus* sp. PCC 6301 Photosystem I core protein [6]. Significantly, the F_A/F_B iron-sulfur clusters in the same-species reconstituted complex showed *g*-values identical to those of the native *Synechococcus* sp. PCC 6301 Photosystem I complex. Since we are aware of no systematic study of differences in the ESR spectra of the F_A/F_B clusters among Photosystem I complexes from various species, we questioned whether the altered *g*-values in the cross-species reconstituted complex were due to subtle, unrealized differences between species or whether the altered *g*-values were due to damage incurred to the F_A/F_B holoprotein during exposure to organic solvent.

We therefore chose to reexamine the issue of the integrity of the reconstituted Photosystem I complex using a different experimental approach. First, we performed careful measurements of the EPR spectrum of reduced F_A/F_B clusters in Photosystem I complexes of two higher plants (spinach and tobacco) and four cyanobacteria (*Synechococcus* sp. PCC 6301, *Synechococcus* sp. PCC 7002, *Synechocystis* sp. PCC 6803, and a thermophilic species of *Synechococcus* sp.). This permitted us to detect subtle differences in the *g*-values of the F_A/F_B clusters between species. Second, we studied the sensitivity of the isolated F_A/F_B protein to oxidative denaturation and adopted a method for reinserting the iron-sulfur clusters into the F_A/F_B apoprotein in vitro using $FeCl_3$ and Na_2S and β-mercaptoethanol. This allowed us to use a protein of known history in the reconstitution protocol, thereby bypassing the organic extraction step to acquire F_A/F_B holoprotein. Lastly, we performed same-species and cross-species reconstitution of the Photosystem I complexes using spinach and *Synechococcus* sp. PCC 6301 F_A/F_B proteins and their respective Photosystem I core proteins. This permitted us to determine whether the altered EPR spectrum reported earlier on reconstituting a spinach F_A/F_B holoprotein with a *Synechococcus* sp. PCC 6301 Photosystem I core protein was a consequence of the cross-species reconstitution or whether it resulted from a damaged F_A/F_B holoprotein. Careful examination of the amino acid sequences of known F_A/F_B proteins (or the amino acid sequences deduced from *psaC* genes) among various eukaryotic and prokaryotic organisms, along with precise measurement of the ESR spectra of the F_A and F_B clusters, suggest that the differences seen among various species are not inherent to the F_A/F_B proteins but are due to specific, and more complex, interactions between this protein and the Photosystem I core protein.

Materials and Methods

Isolation of the Photosystem I core protein and the F_A/F_B polypeptide

The Photosystem I complex was isolated from spinach (*Spinacea oleracea*, market-purchased), tobacco (*Nicotiana tabacum*, supplied by G. Cheniae and D. Blubaugh, University of Kentucky), *Synechococcus* sp. PCC 6301 (*Anacystis nidulans* TX-20, grown in-house, culture from UTCC, Austin, TX), *Synechococcus* sp. PCC 7002 (grown in-house, culture from D. Bryant, Penn State University), *Synechocystis* sp. PCC 6803 (supplied by L. McIntosh, Michigan State University), and thermophilic *Synechococcus* sp. (*Synechococcus vulcanus*, supplied by J. Brand, University of Texas) membrane fragments with Triton X-100 followed by sucrose density ultracentrifugation [2]. The Photosystem I core protein was isolated from spinach and *Synechococcus* sp. PCC 6301 Photosystem I complexes with 6.8 M urea followed by ultrafiltration over a YM-100 membrane and sucrose-density ultracentrifugation in 0.04% Triton X-100 [1,2]. The spinach and *Synechococcus* sp. PCC 6301 F_A/F_B holoproteins were isolated using methanol/acetone [3,4] and butanol [7,8] extraction of lyophylized thylakoid fragments. The extracted material was centrifuged to remove small membrane fragments and the clear supernatant was concentrated over a YM-5 ultrafiltration membrane with ultrapure nitrogen. Even though dithionite was present throughout the isolation procedure [5], the F_A/F_B clusters were found to degrade rapidly if any additional purification steps, including ion-exchange chromatography, were included. Therefore, to prevent iron-sulfur denaturation, the crude protein fraction from spinach and *Synechococcus* sp. PCC 6301 was used directly after solvent extraction of the thylakoid membranes. The elapsed time from addition of the solvent to freezing of the preparation was 1 h. The spinach F_A/F_B apoprotein was isolated by treating a Photosystem I complex similar to that described for *Synechococcus* sp. PCC 6301 [5]. The complex was incubated with 6.8 M urea for 30 min to dissociate the low-molecular mass polypeptides, followed by dialysis against 25 mM Tris (pH 8.3) and ultrafiltration over a YM-100 membrane. The filtrate, which contains the F_A/F_B apoprotein, was concentrated over a YM-5 membrane. One consequence of this abbreviated isolation procedure is that in addition to the F_A/F_B holoprotein or apoprotein, the preparation contains several of the peripheral low molecular mass polypeptides that are associated with the Photosystem I complex. At least two

of these polypeptides (probably the *psaD* and *psaE* gene products) also appear to rebind to the Photosystem I core protein upon reconstitution with the F_A/F_B holoprotein (see Ref. 6). A detailed report on the polypeptide composition of the native and reconstituted *Synechococcus* sp. PCC 6301 complexes will appear shortly.

Oxidative denaturation of the F_B and F_A iron-sulfur clusters

The F_A/F_B apoprotein was prepared by allowing air-oxidation of the iron-sulfur clusters in the freshly isolated protein. Preliminary studies, using ESR spectroscopy of the chemically reduced protein as the assay, indicated that the F_A/F_B clusters in the isolated protein degrade with a half-time of about 10 min at 37 °C. The apoprotein could also be prepared through controlled oxidation of the iron-sulfur clusters by treating the freshly-isolated F_A/F_B protein for 2 h with 3 M urea and 5 mM $K_3Fe(CN)_6$ in 50 mM Tris, pH 8.3 (see Refs. 10, 11). The urea and $K_3Fe(CN)_6$ were removed by dialysis for 24 h in 50 mM Tris (pH 8.3) and 5 mM Tiron, followed by dialysis for an additional 24 h in 50 mM Tris (pH 8.3) to remove the iron-Tiron chelate and the excess Tiron. The fraction containing the F_A/F_B apoprotein was concentrated over a YM-5 membrane.

Reconstitution of the F_B and F_A iron-sulfur clusters

Reconstitution of the F_A/F_B iron-sulfur clusters was performed according to the following protocol: (i) a solution of 50 mM Tris (pH 8.3) was purged with oxygen-free nitrogen in a closed reaction vessel; (ii) after 2 hours, β-mercaptoethanol was added through a septum to a final concentration of 1%; (iii) the F_A/F_B apoprotein was added to a concentration of 5 μg/ml (approx. 1 nmol/ml iron-sulfur cluster) and the solution was purged with oxygen-free nitrogen; (iv) after 10 min, an aliquot of 30 mM $FeCl_3$ was slowly added to a final concentration of 0.3 mM; (v) after 5 min, an aliquot of 30 mM Na_2S was slowly added to a final concentration of 0.3 mM. This solution was allowed to incubate in the dark and at room temperature for 12 h. The solution was transferred under a flow of oxygen-free nitrogen to an ultrafiltration cell equipped with a YM-5 membrane and concentrated with ultrapure nitrogen at 4 °C. β-mercaptoethanol (0.01%) was present throughout the entire procedure.

Rebinding of the F_A/F_B holoprotein to the Photosystem I core protein

The native-F_A/F_B and reconstituted-F_A/F_B proteins were rebound at an approx. 100-fold excess molar ratio to the Photosystem I core protein (10 μg Chl/ml) in 50 mM Tris (pH 8.3) and 0.1% β-mercaptoethanol. After 1 h incubation at room temperature, the solution was washed twice over a YM-100 membrane with 50 mM Tris (pH 8.3) and 0.04% Triton X-100 to remove unbound F_A/F_B protein. The reconstituted Photosystem I complex was further purified by sucrose-density ultracentrifugation in 0.04% Triton X-100.

Reconstitution of the F_A/F_B apoprotein in the presence of the Photosystem I core protein

The reconstituted-F_A/F_B polypeptide was rebound to the Photosystem I core protein by incubating the F_A/F_B apoprotein with $FeCl_3$, Na_2S and β-mercaptoethanol as described above, but in the presence of Photosystem I core protein at 10 μg Chl/ml. After 12 h, the reaction vessel was uncapped and the solution was transferred under a flow of oxygen-free nitrogen to an ultrafiltration cell equipped with a YM-100 membrane and washed twice with 50 mM Tris (pH 8.3) containing 0.04% Triton X-100. The washed solution was diluted to 500 μg Chl/ml followed by dialysis for 12 h in 50 mM Tris (pH 8.3) containing 5 mM Tiron. The residual Tiron and Tiron-iron chelate were removed by dialysis for 12 h in 50 mM Tris (pH 8.3) containing 0.04% Triton X-100. The reconstituted Photosystem I complex was concentrated to 1000 μg Chl/ml.

Spectroscopic methods

Flash-induced absorption transients were determined at 698 nm using a single beam spectrophotometer consisting of a 250 W quartz-tungsten source, a monochromator before and after the sample cuvette, and a silicon photodiode (United Detector Technology PIN-10D). A shutter opened the measuring beam 1 ms prior to the flash, and a sample-and-hold circuit nulled the measuring beam immediately after the shutter opening. The signal was changed into a voltage with a 1 K resistor, amplified with an EG&G Model 113 preamplifier (300-kHz bandwidth) and digitized with a Nicolet 4094A Digital Oscilloscope. The data were transferred to a Macintosh IIci computer over an RS-232 line using software written in FORTH (Creative Solutions, Rockville, MD). The sample was repetitively flashed with a Phase-R DL1200 flashlamp-pumped dye laser (50 mJ pulse energy for 400 ns FWHM at 660 nm with sulforhodamine B as the organic dye). The optical measurements were performed at 20 °C in a 1 cm pathlength cuvette containing 5 μg Chl/ml, 0.033 mM DCPIP or PMS, and 1.7 mM ascorbate in 50 mM Tris (pH 8.3). ESR studies were performed on a Varian E-109 spectrometer equipped with an Air Products LTD liquid helium transfer cryostat. The spectrometer was interfaced to a Macintosh Plus computer via a Keithley digital voltmeter (Model 195A) and an IEEE-488 bus controller (I/O Tech Mac 488A) for signal averaging and baseline subtraction. The frequency was measured with a Hewlett-Packard Model 5340A microwave counter, and the magnetic field strength was measured with a Varian Model 906790 Gaussmeter. The precision of

the measurement, using a calibrated time base and diphenylpicrylhydrazyl as a field marker, allowed g-values to be determined with a relative error of no more than ± 0.001 at $g = 2.000$. Sample temperatures were monitored with a thermistor situated directly below the sample tube. Light-minus-dark difference spectra were obtained by illuminating the sample with a 150 W xenon lamp.

Analytical methods

Acid-labile sulfide and P700 were determined as described in Ref. 11. Chlorophyll was determined in 80% acetone [12].

Results

ESR spectra of isolated spinach and cyanobacterial Photosystem I complexes

The ESR spectra of the F_A/F_B clusters in isolated spinach and *Synechococcus* sp. PCC 6301 Photosystem I complexes are shown in Fig. 1. When the sample is illuminated during freezing, both iron-sulfur clusters become photochemically reduced, leading to resonances at $g = 2.051$, 1.941, 1.923 and 1.887 in spinach and $g = 2.047$, 1.937, 1.917 and 1.879 in *Synechococcus* sp. PCC 6301 (Fig. 1a). Within the tolerances of the measurement (see Materials and Methods), identical g-values were determined for spinach Photosystem I complexes measured on five separate occasions and for *Synechococcus* sp. PCC 6301 complexes measured on four separate occasions. When the complexes were frozen in darkness and illuminated, only one electron could be transferred from P700, leading to the reduction of 85% of F_A and 15% of F_B. Consistent with the above analysis, the $g = 1.86$ resonance of F_A^- in spinach showed reproducible differences from *Synechococcus* sp. PCC 6301 (the former is located downfield of the latter). These differences are not related to the nature of the Photosystem I preparation, nor to the method of reduction of the iron-sulfur clusters. Similar g-values are found for the F_A/F_B clusters in digitonin, octyl glucoside, and Triton Photosystem I complexes (Fig. 1a,b), as well as in intact spinach thylakoid membranes (not shown). Likewise, identical g-values are found in Photosystem I complexes reduced in darkness with dithionite at pH 10.5 and reduced photochemically by freezing during illumination at pH 8.3 (not shown).

The ESR spectra of the F_A/F_B iron-sulfur clusters in isolated tobacco, *Synechococystis* sp. PCC 6803, *Synechococcus* sp. PCC 7002, and thermophilic *Synechococcus* sp. Photosystem I complexes are shown in Fig. 2. The tobacco F_A/F_B iron-sulfur clusters (Fig. 2a) show resonances at $g = 2.051$, 1.941, 1.924 and 1.887, and appear identical to those of spinach. *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 (Fig. 2) show similar g-values of 2.050, 1.938, 1.918 and 1.878

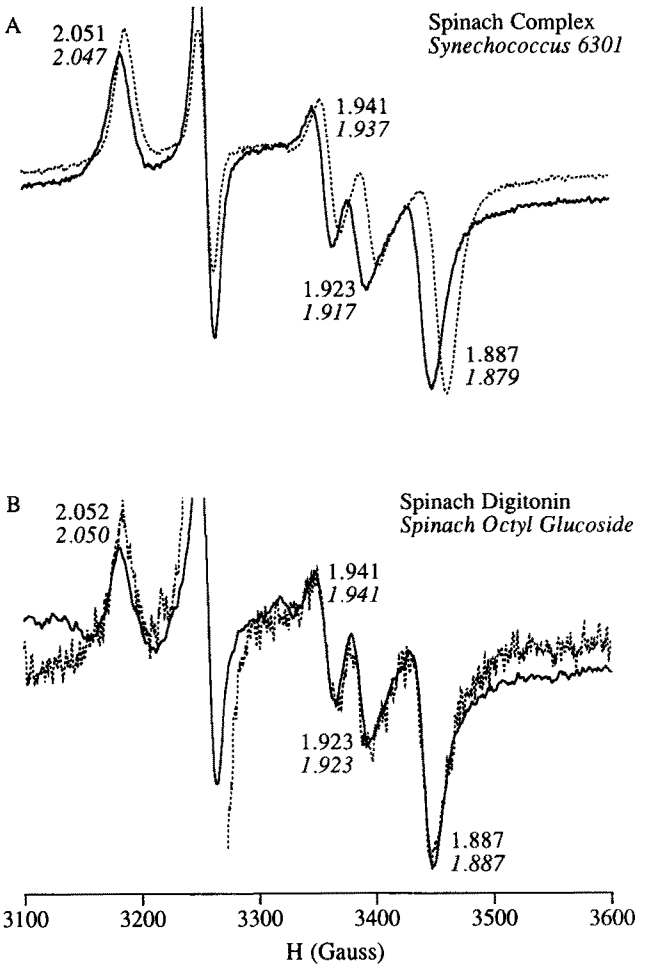

Fig. 1. ESR spectra of (A) spinach (solid line) and *Synechococcus* sp. PCC 6301 (dotted line) Photosystem I complexes isolated with Triton X-100 and (B) spinach Photosystem I particles isolated with digitonin (solid line) and octyl glucoside (dotted line). The samples were illuminated during freezing and the resonances were resolved by subtracting the light-off (before light on) from the light-on spectrum. All samples were suspended in 50 mM Tris buffer (pH 8.3) containing 1 mM sodium ascorbate and 0.03 mM DCPIP at 300 μg Chl/ml. Spectrometer conditions: temperature, 19 K; microwave power, 20 mW; microwave frequency, 9.128 GHz; receiver gain, $5.0 \cdot 10^3$; modulation amplitude, 10 G at 100 kHz.

and 2.048, 1.937, 1.917 and 1.878, respectively. The F_A/F_B resonances in the thermophilic species of *Synechococcus* sp. (Fig. 2b) occur at $g = 2.051$, 1.937, 1.918 and 1.879 and are similar to those of the mesophilic species (Fig. 1a). In general, g-values of the F_A/F_B clusters are similar in the four different cyanobacterial Photosystem I complexes and are shifted consistently to higher field compared with those in the spinach and tobacco Photosystem I complexes.

Oxidative denaturation and reconstitution of the F_A/F_B iron-sulfur clusters

The iron-sulfur clusters in the isolated spinach F_A/F_B protein are sensitive to oxidative denaturation after the protein is liberated from the thylakoid membrane. As

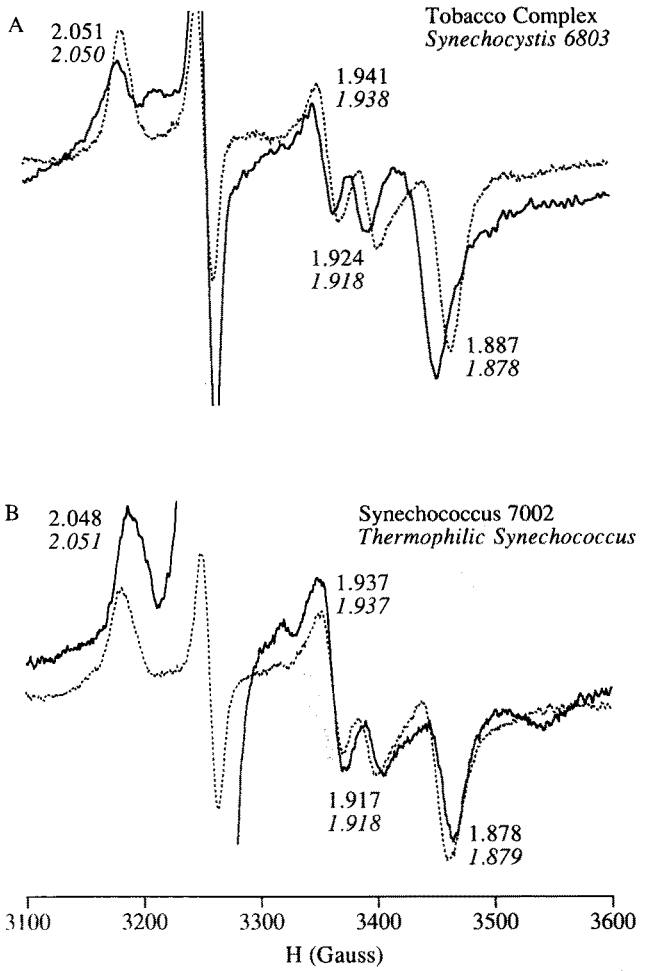

Fig. 2. ESR spectra of (A) tobacco (solid line) and *Synechocystis* sp. PCC 6803 dotted line) and (B) *Synechococcus* sp. PCC 7002 (solid line) and the thermophilic *Synechococcus* sp. (dotted line) Photosystem I complexes isolated with Triton X-100. The samples were illuminated during freezing and the resonances were resolved by subtracting the light-off (before light on) from the light-on spectrum. All samples were suspended in 50 mM Tris buffer (pH 8.3) containing 1 mM sodium ascorbate and 0.03 mM DCPIP at 300 μg Chl/ml. Spectrometer conditions: temperature, 19 K; microwave power, 20 mW; microwave frequency, 9.128 GHz; receiver gain, $5.0 \cdot 10^3$; modulation amplitude, 10 G at 100 kHz.

shown in Fig. 3a, the ESR resonances of F_A and F_B degrade with an approximate half-time of about 10 min after exposure to 37 °C, even under highly reducing conditions. There does not appear to be differential sensitivity of either the F_A or F_B cluster to this mode of denaturation. The iron-sulfur clusters can also be forced into oxidative denaturation at 0 °C by exposure to 3 M urea and 5 mM $K_3Fe(CN)_6$ for 10 min (Fig. 3b); this method allows for controlled denaturation under known conditions [13]. In both instances, the acid-labile sulfide is oxidized to the level of zero-valence sulfur and the non-heme iron is lost from the protein (data not shown).

We found that the oxidatively-denatured iron-sulfur clusters can be reconstituted by incubating the isolated spinach F_A/F_B apoprotein with $FeCl_3$ and Na_2S in the presence of β-mercaptoethanol (see Ref. 14). The ESR spectrum of the reconstituted F_A/F_B protein is shown in Fig. 3b. The chemically-reduced spectrum of the native F_A/F_B protein shows principal resonances at $g = 2.05$, 1.96, 1.94 and 1.89 (Fig. 3a). After oxidative denaturation with urea and $K_3Fe(CN)_6$, little or no chemically-reduced F_A or F_B is observed (Fig. 3b). The lack of iron-sulfur clusters is also reflected in the loss of the chemical oxidized-minus-reduced difference spectrum of the denatured protein (data not shown). However, after reconstitution with $FeCl_3$ and Na_2S in the presence of β-mercaptoethanol, the characteristic ESR spectrum of F_A and F_B is regained (Fig. 3b). The efficiency of reconstitution of the F_A/F_B clusters is greater than 90% after 12 h of incubation with $FeCl_3$ and Na_2S and β-mercaptoethanol. In the absence of

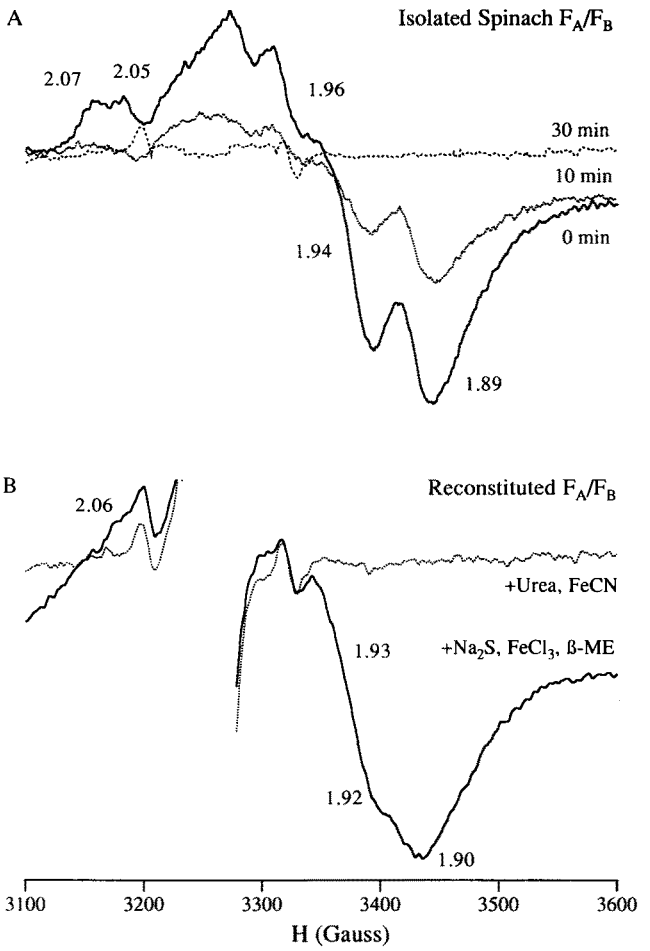

Fig. 3. ESR spectrum of the (A) chemically-reduced F_A/F_B protein after 0, 20 and 60 min of heating to 37 °C and (B) after reconstitution with $FeCl_3$, Na_2S and β-mercaptoethanol. The samples in (A) were warmed directly in the ESR sample tube in the presence of excess sodium dithionite and 0.033 mM methyl viologen in 0.1 M glycine (pH 10). The spectra were resolved by subtracting the oxidized spectrum from the chemically reduced spectrum and amplifying the difference 5-fold in software. Spectrometer conditions: temperature, 19 K; microwave power, 20 mW; microwave frequency, 9.128 GHz; receiver gain, $5.0 \cdot 10^3$; modulation amplitude, 10 G at 100 kHz.

added sodium sulfide, yield decreases to 40%; the sulfide for reconstitution is most likely derived from the protein-bound zero-valence sulfur released by nucleophilic displacement with β-mercaptoethanol. No reconstitution is observed in the absence of either β-mercaptoethanol or added iron. However, if the apoprotein is not dialyzed against Tiron, some residual iron is present, and a 10% yield of F_A and F_B reconstitution is observed in the presence of β-mercaptoethanol alone. The only difference with the freshly-isolated F_A/F_B holoprotein is that there appears to be a broadening or merging of the high- and mid-field resonances after in vitro reinsertion of the iron-sulfur clusters. Although the reconstituted F_A/F_B spectrum appears more axial in appearance than the control F_A/F_B spectrum, we believe that the shape of the spectrum is due to a freezing-in of a large variety of conformational states rather than to a change in the symmetry of the spin system.

Rebinding of the reconstituted-F_A/F_B polypeptide to the Photosystem I core protein

Similar to our earlier work, we found that when the freshly-isolated spinach F_A/F_B holoprotein is rebound to the *Synechococcus* sp. PCC 6301 Photosystem I core protein, there is a transition in the room-temperature, flash-induced absorption change from a 1.2 ms optical transient due to the $P700^+ F_X^-$ backreaction to a 30-ms optical transient due to the $P700^+ [F_A/F_B]^-$ backreaction (see Fig. 5, Ref. 5). When an attempt is made to rebind the spinach F_A/F_B apoprotein to the *Synechococcus* sp. PCC 6301 Photosystem I core protein, there is no change in the 1.2 ms backreaction between $P700^+$ and F_X^-. However, when the rebuilt spinach F_A/F_B protein is rebound to the *Synechococcus* sp. PCC 6301 Photosystem I core protein, the 1.2 ms optical transient due to the $P700^+ F_X^-$ backreaction is replaced (within a mixing time of less than 1 min) by the 30 ms transient characteristic of the $P700^+ [F_A/F_B]^-$ backreaction (see Fig. 6, Ref. 15). The iron-sulfur clusters in the spinach F_A/F_B apoprotein can also be reconstituted in the presence of the *Synechococcus* sp. PCC 6301 Photosystem I core protein. Under these conditions, electron flow from P700 to F_A/F_B is also reestablished, except that 12 h of incubation is required due to the relatively slow kinetics of reinsertion of the iron-sulfur clusters into the F_A/F_B apoprotein.

The ESR spectrum of the reconstituted spinach-*Synechococcus* sp. PCC 6301 Photosystem I complex is compared to the native spinach Photosystem I complex in Fig. 4. This cross-species complex was prepared with F_A/F_B apoprotein isolated by treating a spinach Photosystem I complex with chaotropes followed by rebuilding the iron-sulfur clusters and rebinding to the *Synechococcus* sp. PCC 6301 Photosystem I core protein. When the reconstituted spinach-*Synechococcus* sp. PCC 6301 Photosystem I complex is illuminated during freezing, the F_A/F_B resonances sharpen and appear similar as those in the control complex. The spectrum is characterized by interaction between F_A and F_B which gives rise to *g* values of 2.052, 1.941, 1.922 and 1.886. This spectrum is nearly identical to that of the F_A/F_B clusters in the native spinach Photosystem I complex (shown also in Fig. 4a) but is unlike the native *Synechococcus* sp. PCC 6301 Photosystem I complex (*g*-values of 2.047, 1.937, 1.917 and 1.879; see Fig. 1a). The *g*-values, moreover, do not depend on whether the hybrid spinach-*Synechococcus* sp. PCC 6301 Photosystem I complex is photochemically reduced at pH 8.3 or chemically reduced in the dark at pH 10.5 (data not shown). The spectrum is also identical to the reconstituted Photosystem I complex prepared using a solvent-extracted spinach F_A/F_B holoprotein (note that the *g*-values of the reconstituted cross-species Photosystem I complex shown here are more accurate than those shown in Ref. 5 due to precise calibration of the ESR spectrometer). Indeed, when either the solvent-isolated spinach F_A/F_B holoprotein or the chaotrope-isolated (and iron-sulfur reconstituted) F_A/F_B protein is rebound to the *Synechococcus* sp. PCC 6301 Photosystem I core protein, frozen in darkness, and illuminated at 16 K, F_A (*g*-values of 2.05, 1.94 and 1.86) is approx. 85% photoreduced, and F_B (*g*-values of 2.07, 1.92 and 1.89) is approx. 15% photoreduced.

To determine whether the *g*-values of the cross-species ESR spectrum are due to inherent features of the hybrid spinach-*Synechococcus* sp. PCC 6301 reconstitution or to an altered F_A/F_B protein, we performed

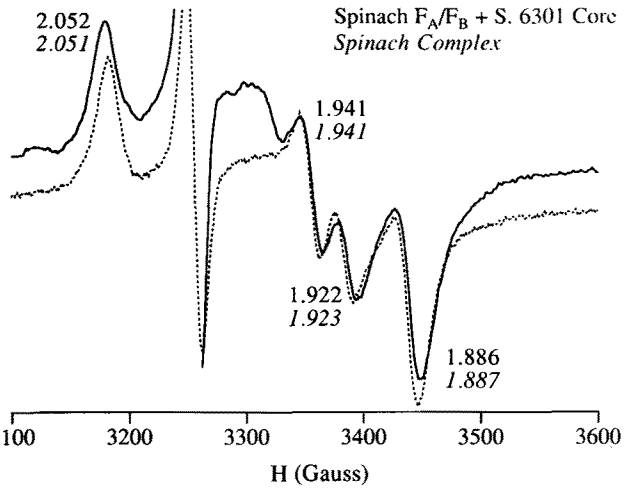

Fig. 4. ESR spectra of the photochemically-reduced, reconstituted spinach F_A/F_B protein with the *Synechococcus* sp. PCC 6301 core protein (solid line) and the control spinach Photosystem I complex (dotted line). The samples were suspended in 50 mM Tris buffer (pH 8.3) containing 1 mM sodium ascorbate and 0.03 mM DCPIP at 300 μg Chl/ml. The spectra were resolved by subtracting the light-off (before light-on) from the light-on spectrum. Spectrometer conditions: temperature, 19 K; microwave power, 20 mW; microwave frequency, 9.128 GHz; receiver gain, $5.0 \cdot 10^3$; modulation amplitude, 10 G at 100 kHz.

same-species reconstitution studies. Because no differences were found in the integrity of the F_A/F_B protein obtained by solvent extraction or chaotrope treatment (followed by rebuilding the iron-sulfur clusters), the former, simpler method was used to obtain F_A/F_B holoprotein for the following studies. The results of reconstituting a *Synechococcus* sp. PCC 6301 F_A/F_B protein with a *Synechococcus* sp. PCC 6301 Photosystem I core protein are shown in Fig. 5a. The yield of the *Synechococcus* sp. PCC 6301 F_A/F_B holoprotein was consistently low; however, we were able to isolate sufficient protein to show that the ESR spectrum of the reconstituted Photosystem I complex was identical to that of the native *Synechococcus* sp. PCC 6301 Photosystem I complex. The results of reconstituting a spinach F_A/F_B protein with a spinach Photosystem I core protein are shown in Fig. 5b. The difficulty encountered here was that the yield of the spinach Photosystem I core protein was low; the spinach complex appears to be more stable than the *Synechococcus* sp. PCC 6301 complex, and hence there is considerable denaturation of F_X in the process of ensuring that all of the F_A/F_B polypeptide is removed. A similar destruction of F_X was noted by Hoshina et al. [16] when hot ethylene glycol was used to remove the F_A/F_B protein from a spinach Photosystem I complex. As shown in Fig. 5b, the *g*-values after rebinding the spinach F_A/F_B protein to the spinach Photosystem I core are identical to those in the native spinach Photosystem I complex (the low yield of both *Synechococcus* sp. PCC 6301 F_A/F_B holoprotein and spinach Photosystem I core protein precluded the reciprocal cross-species reconstitution study). We conclude that the slightly altered ESR spectrum of the hybrid spinach-*Synechococcus* sp. PCC 6301 Photosystem I complex is due to inherent protein-protein interactions between the F_A/F_B polypeptide and the Photosystem I core protein and not to damage incurred to the F_A/F_B protein during isolation.

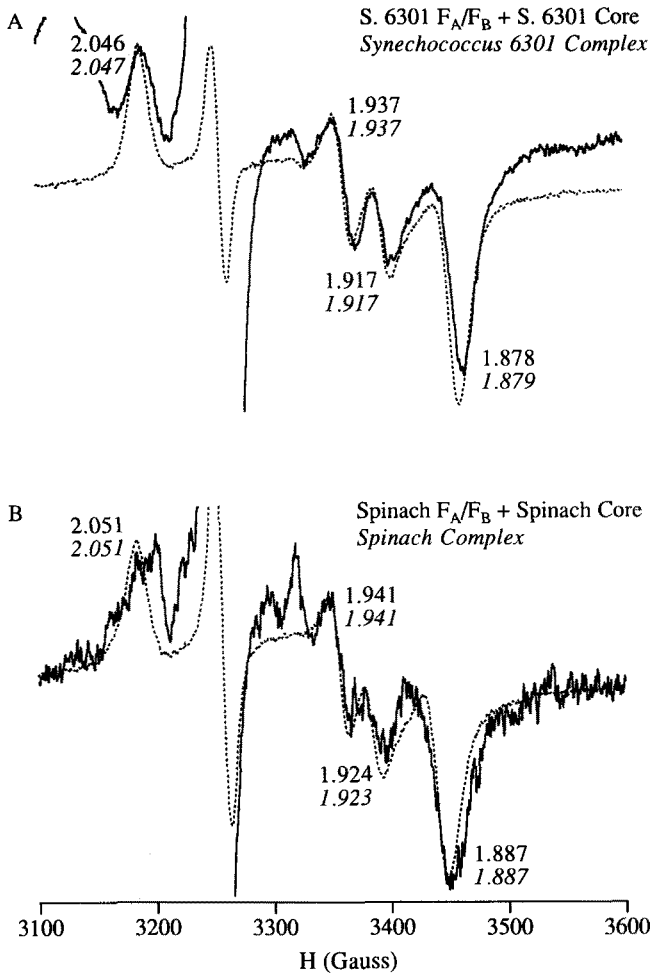

Fig. 5. ESR spectra of (A) the photochemically-reduced reconstituted *Synechococcus* PCC 6301 F_AF_B protein with the *Synechococcus* PCC 6301 Photosystem I core protein (solid line) compared with the control *Synechococcus* 6301 complex (dotted line); and (B) the reconstituted spinach F_A/F_B protein with the spinach Photosystem I core protein (solid line) compared with the control spinach Photosystem I complex (dotted line). The samples were suspended in 50 mM Tris buffer (pH 8.3) containing 1 mM sodium ascorbate and 0.03 mM DCPIP at 300 μg Chl/ml. The spectra were resolved by subtracting the light-off (before light on) from the light-on spectrum. Spectrometer conditions: temperature, 19 K; microwave power, 20 mW; microwave frequency, 9.128 GHz; receiver gain, $5.0 \cdot 10^3$; modulation amplitude, 10 G at 100 kHz.

Discussion

We have shown that the F_B and F_A iron-sulfur clusters in the isolated 8.9 kDa protein from Photosystem I can be reconstituted from the oxidatively-denatured apoprotein with $FeCl_3$ and Na_2S in the presence of β-mercaptoethanol. Evidence for the in vitro reconstitution of the F_A/F_B iron-sulfur clusters includes: (i) regeneration of the chemically reduced ESR spectrum of F_A^- and F_B^- in the isolated protein, (ii) restoration of the 30-ms optical transient between $P700^+$ and $[F_A/F_B]^-$ after rebinding to the Photosystem I core protein, and (iii) regeneration of the characteristic light-induced ESR spectrum of interacting F_A^- and F_B^- after rebinding to the Photosystem I core protein and illumination before and during freezing. The ESR spectrum after freezing in the dark and illumination at 16 K sharpens and appears similar to a native spinach or *Synechococcus* sp. PCC 6301 Photosystem I complex, with 85% photoreduction of F_A and 15% photoreduction of F_B. This differs from the 50% ratio of photoreduction of F_A and F_B reported earlier [5]; the difference is that the latter sample was reconstituted in the ESR sample tube and frozen in situ after 10 min of incubation, whereas the sample in this study was manipulated for a longer period of time, including exhaustive ultrafiltration and dialysis before freezing. Indeed, differences in the ratios of photoreduced F_A and F_B have been found between species [17,18], and the presence of 60% glycerol

leads to a reversal of the roles of the low temperature photoreduction of F_A and F_B in the same species [19]. Perhaps there is a time-dependent reorientation of the F_A/F_B protein on the Photosystem I core protein that leads to differential docking of the polypeptide, and hence preferential photoreduction of one iron-sulfur cluster over another. The results of this study show that after incubating a sufficient period of time, the cross-species reconstitution of a spinach F_A/F_B protein with a *Synechococcus* sp. PCC 6301 Photosystem I core protein leads to photochemical properties similar to either the control spinach or *Synechococcus* sp. PCC 6301 complex.

One feature with the reconstitution protocol shown here is that an accurate estimation of the yield of reconstitution of the F_A/F_B protein is difficult to assess because the native or reconstituted holoprotein is labile in the absence of the Photosystem I core protein. In this study, we have ensured quantitative rebuilding of the F_A/F_B iron-sulfur clusters and rebinding to the Photosystem I core protein because we have added the F_A/F_B protein it back to the Photosystem I core protein in large excess. Previous studies from our laboratory have shown that only a slight excess of the F_A/F_B holoprotein is necessary to guarantee complete reconstitution of the Photosystem I complex [5]. Any given preparation can therefore suffer a significant degradation of the F_A/F_B clusters before the amount of F_A/F_B holoprotein becomes substoichiometric and thus incapable of reconstituting an intact Photosystem I complex. The reconstitution of the F_A/F_B clusters in the presence of the Photosystem I core protein circumvents the problem of cluster lability: the affinity of the reconstituted F_A/F_B holoprotein to the Photosystem I core protein is high and the F_A/F_B clusters are stable when the holoprotein is rebound to the Photosystem I core. One additional consequence is that since the F_A/F_B apoprotein is stable, the reconstitution protocol allows its isolation and purification followed by rebuilding the iron-sulfur clusters to produce a competent F_A/F_B holoprotein. This, in turn, can be followed by rebinding to the Photosystem I core protein to produce a functional Photosystem I complex.

Turning our attention finally to the differences in the ESR spectra, it is of interest to know whether the *g*-values are inherent to the native Photosystem I complexes or whether they result entirely as a consequence of the amino acid sequences of the F_A/F_B proteins. This question can be addressed, in part, by same-species and cross-species reconstitution studies of the Photosystem I complex. Our studies show that the *g*-values of the F_A/F_B clusters in the spinach Photosystem I complex differ slightly but characteristically from those in *Synechococcus* sp. PCC 6301. Moreover, when the spinach F_A/F_B protein is reconstituted with the *Synechococcus* sp. PCC 6301 Photosystem I core protein, the resulting ESR spectrum is more like the native spinach Photosystem I complex than the *Synechococcus* sp. PCC 6301 complex. Although rebinding to the Photosystem I core protein is necessary for the F_A/F_B resonances to narrow and sharpen, this one example might suggest that the ESR spectral differences originate within the F_A/F_B protein itself and not from the specific interactions between this protein and the Photosystem I core.

This conclusion, however, is weakened considerably by consideration of the amino acid sequences of various F_A/F_B proteins. As shown in Table I, the F_A/F_B proteins are very highly conserved whether encoded in the

TABLE I

Comparison of the amino acid sequences of the psaC gene products of several species

Only amino acids which differ from those of *Synechococcus* sp. PCC 7002 are shown. Slashes indicate the begining or ends of determined sequences; stars indicate the C-terminus; question marks indicate undetermined amino acids. Names of organisms and references: *Synechococcus* sp. PCC 7002 [20]; *Nostoc* sp. PCC 8009, [20]; *Synechocystis* sp. PCC 6803, [21]; *Synechococcus* sp. PCC 6301, Bryant, unpublished data; *Synechococcus vulcanus*, [22]; tobacco, [24]; liverwort, [25]; spinach, [26]; pea, [27]; maize, [28]; wheat, [27]; barley, [29].

	10	20	30	40	50	60	70	80
7002	MSHSVKIYDTCIGCTQCVRACPLDVLEMVPWDGCKAGQIASSPRTEDCVGCKRCETACPTDFLSIRVYLGAETTRSMGLAY*							
8009	T	T		A				*
6803		T	I	K	A	S	V	WH	*
6301	/	/						
S. vulcanus	/A T	T	/					
C. paradoxa	A T	T		R N	A	S		G *
Tobacco		T	I	K	A	S	V	WH	*
Liverwort	A A	T	I	K	A	SR	V	N	S *
Spinach		T	I	K	A	S	V	WH	G *
Pea		T	I G	K	A	S	V	WH	*
Maize	H	T	I	K	A	S	V	P	A S *
Wheat		T	I	K	A	S		P	A S *
Barley	/	T	I	K	A	S	V	P	A S *

plastid genomes of eucaryotes or the procaryotic cyanobacteria (the complete amino acid sequence for the *Synechococcus* sp. PCC 6301 F_A/F_B protein has not been determined). The higher plant sequences are approx. 95% identical, while the cyanobacterial sequences (with the sole exception of *Synechocystis* sp. PCC 6803) exhibit greater than 90% identity. Virtually all amino acid substitutions within a class are conservative replacements. Each gene predicts a protein of 81 amino acids (8.8 kDa), although it appears that the N-terminal methionine may be missing from the mature protein. The protein from all sources is predicted to contain nine cysteine residues, eight of which are expected to participate in ligating the two [4Fe-4S] centers F_A and F_B. The sequences can be divided into three groupings which arise from specific amino acid replacements at two positions in the proteins. These are positions 37 and 70–71. At position 37, all higher plants have a lysine residue, while *Cyanophora paradoxa, Synechococcus* sp. PCC 7002, and *Nostoc* sp. PCC 8009 have non-charged amino acids. At positions 70–71, some species have tryptophan-histidine, while other species have non-aromatic amino acids (either glycine-alanine, glycine-asparagine, or glycine-proline). The apparent division between prokaryotes and eukaryotes, however, is broken by *Synechocystis* sp. PCC 6803, which is plant-like in that it contains lysine at position 37 and tryptophan-histidine at positions 70 and 71. In fact, spinach and *Synechocystis* sp. PCC 6803 only differ by one amino acid near the C-terminus of the protein; at position 80, spinach contains glycine, whereas *Synechocystis* sp. PCC 6803 contains alanine. Yet, in spite of this extremely minor change, there are large differences in the *g*-values of the F_A/F_B clusters between these two species. It is difficult to believe that this sole substitution, at the C-terminus of the protein, leads to the changes seen in the *g*-values of both the F_A and F_B clusters. Studies with a tobacco Photosystem I complex bear out this conclusion: the amino acid sequence of the tobacco F_A/F_B protein is identical to that of *Synechocystis* sp. PCC 6803, yet the *g*-values of the tobacco F_A/F_B clusters are identical to those in spinach. This one instance would imply that in addition to narrowing the principal resonances, the Photosystem I core protein (or one or more of the low molecular mass polypeptides) have an important influence on the position of the resonances in the ESR spectrum of the F_A/F_B clusters. It is not understood how and where the F_A/F_B protein binds to the reaction center heterodimer, and species-related differences in the amino acid sequences of the *psaA* and *psaB* proteins in the binding domain probably provide a large contribution to the spectral changes seen in the F_A/F_B protein. Further insight into this issue will probably need to consider a detailed description of the amino acids involved in the F_A/F_B protein binding site on the Photosystem I core protein.

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References

1 Golbeck, J.H., Parrett, K.G., Mehari, T., Jones, K.L. and Brand, J.J. (1988) FEBS Lett. 228, 268–272.
2 Parrett, K.P., Mehari, T., Warren, P. and Golbeck, J.H. (1989) Biochim. Biophys. Acta 973, 324–332.
3 Malkin, R., Aparicio, P.J. and Arnon, D.I. (1974) Proc. Natl. Acad. Sci. 71, 2362–2366.
4 Wynn, R.M. and Malkin, R. (1988) FEBS Lett. 229, 293–297.
5 Golbeck, J.H., Mehari, T., Parrett, K.P. and Ikegami, I. (1988) FEBS Lett. 240, 9–14.
6 Parrett, K.G., Mehari, T. and Golbeck, J.H. (1990) Biochim. Biophys. Acta 1015, 341–352.
7 Oh-oka, H., Takahashi, Y., Kuriyama, K., Saeki, K. and Matasubara, H. (1988) J. Biochem. 103, 962–968.
8 Oh-oka, H., Takahashi, Y., Matasubara, H and Itoh, S. (1988) FEBS Lett. 234, 291–294.
9 Golbeck, J.H., Lien, S. and San Pietro, A. (1977) Arch. Biochem. Biophys. 178, 140–150.
10 Golbeck, J.H. and Kok, B. (1978) Arch. Biochem. Biophys. 188, 233–242.
11 Golbeck, J.H. and San Pietro, A. (1976) Anal. Biochem. 73, 539–542.
12 Arnon, D.I. (1949) Plant Physiol. 24, 1–15.
13 Petering, D., Fee, J.A. and Palmer, G. (1971) J. Biol. Chem. 246, 643–653.
14 Malkin, R. and Rabinowitz, C.J. (1966) Biochem. Biophys. Res. Commun. 23, 822–827.
15 Golbeck, J.H. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. II, pp. 531–538, Kluwer, Dordrecht.
16 Hoshina, S., Sue, S., Kunishima, N., Kamide, K., Wada, K. and Itoh, S. (1989) FEBS Lett. 258, 305–308.
17 Nugent, J.H.A., Møller, B.L. and Evans, M.C.W. (1981) Biochim. Biophys. Acta 634, 249–255.
18 Cammack, R., Ryan, D.D. and Stewart, A.C. (1979) FEBS Lett, 107, 422–426.
19 Evans, M.C.W. and Heathcote, P. (1980) Biochim. Biophys. Acta 590, 89–96.
20 Bryant, D.A., Rhiel, E., Lorimier, R., Zhou, J., Stirewalt, V.L., Gasparich, G.E., Dubbs, J.M. and Snyder, W. (1989) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. II, pp. 1–11 Kluwer, Dordrecht.
21 Anderson, S.L. and McIntosh, L. (1989) Molecular Biology of Cyanobacteria Workshop, Toronto, Canada, Abstract 22.
22 Koike, H., Ikeuchi, M., Hiyama, T. and Inoue, Y. (1989) FEBS Lett. 253, 257–263.
23 Golbeck, J.H. and Bryant D.A. (1990) in Current Topics in Bioenergetics (Lee, C.P. ed.), Vol. 16 (in press).
24 Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongbse, J., Obokata, J.,

Yamaguchi-Shinozaki, K., Kato, A., Tohdoh, N., Shimada, H. and Sugiura, M. (1986) EMBO J. 5, 2043–2049.
25 Ohyama, K., Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T., Sano, S., Umesono, K., Shiki, Y., Takeuchi, M., Chang, Z., Aota, S., Inokuchi, H. and Ozeki, H. (1986) Nature 322, 572–574.
26 Steppuhn, J., Hermans, J., Nechushtai, R., Herrmann, G.S. and Herrmann, R.G. (1989) Curr. Genet. 16, 99–108.
27 Dunn, P.P.J. and Gray, J.C. (1988) Plant Mol. Biol., 11, 311–319.
28 Schantz, R. and Bogorad, L. (1988) Plant Mol. Biol., 11, 239–247.
29 Scheller, H.V., Svendsen, I. and Møller, B.L. (1989) Carlsberg Res. Commun. 54, 11–15.