Characterization of the Multiple Forms of Cytochrome b_{559} in Photosystem II†

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ABSTRACT: Cytochrome b_{559} is an essential component of the photosystem II (PSII) protein complex. Its function, which has long been an unsolved puzzle, is likely to be related to the unique ability of PSII to oxidize water. We have used EPR spectroscopy and spectrophotometric redox titrations to probe the structure of cytochrome b_{559} in PSII samples that have been treated to remove specific components of the complex. The results of these experiments indicate that the low-temperature photooxidation of cytochrome b_{559} does not require the presence of the 17-, 23-, or 33-kDa extrinsic polypeptides or the Mn complex (the active site in water oxidation). We observe a shift in the g value of the EPR signal of cytochrome b_{559} upon warming a low-temperature photooxidized sample, which presumably reflects a change in conformation to accommodate the oxidized state. At least three redox forms of cytochrome b_{559} are observed. Untreated PSII membranes contain one high-potential (375 mV) and one intermediate-potential (230 mV) cytochrome b_{559} per PSII. Thylakoid membranes also appear to contain one high-potential and one intermediate-potential cytochrome b_{559} per PSII, although this measurement is more difficult due to interference from other cytochromes. Removal of the 17- and 23-kDa extrinsic polypeptides from PSII membranes shifts the composition to one intermediate-potential (170 mV) and one low-potential (5 mV) cytochrome b_{559}. This large decrease in potential is accompanied by a very small g-value change (0.04 at g_z), indicating that it is the environment and not the ligand field of the heme which changes significantly upon the removal of the 17- and 23-kDa polypeptides. Removal of the 33-kDa polypeptide has no effect on the reduction potential of cytochrome b_{559}; removal of the Mn complex may cause a small decrease in the potential. We propose that the 17- and 23-kDa polypeptides stabilize a very hydrophobic heme environment, which is responsible for the unusually high potential of the high-potential form of cytochrome b_{559}. The lower potential forms of cytochrome b_{559} that result when the extrinsic polypeptides are removed probably reflect a conformation in which the heme is more accessible to the solvent. Our results demonstrate the importance of performing redox titrations to identify the forms of cytochrome b_{559} that are present in a PSII sample and indicate that the intermediate- and low-potential forms are not heterogeneous, denatured forms of cytochrome b_{559}.

Our understanding of the molecular basis of light-driven electron transport in photosynthesis has been significantly advanced by the crystal structure of the bacterial photosynthetic reaction center (Deisenhofer et al., 1984). Because of the similarity between some of the components of the photosynthetic electron-transport chain in bacteria and in photosystem II (PSII)[1] (Okamura et al., 1982) and the homology between the primary sequences of the two proteins that bind these components in bacteria and two proteins in PSII known as D1 and D2, it has been proposed that the structure of PSII is analogous to that of the bacterial reaction center (Michel & Deisenhofer, 1988, and references cited therein). The isolation of a PSII core complex consisting of D1, D2, and cytochrome b_{559} and containing several of the electron-transfer components common to bacteria and PSII provides experimental support for this proposal (Nanba & Satoh, 1987). This partial structural model for PSII must now be extended to include the components unique to PSII and to account for the ability of PSII to oxidize water. One important difference between the structures of PSII and the bacterial reaction center is the presence of cytochrome b_{559}, a component that has long been known to be intrinsic to the PSII complex [Knaff & Arnon, 1969; reviewed by Cramer and Whitmarsh (1977)], but whose function is still debated. As a component of the most highly purified PSII core complex, cytochrome b_{559} is likely to have a structural role in PSII (Thompson et al., 1986) and may have a function related to the ability of PSII to oxidize water, such as a role in the assembly of the Mn compex (Cramer et al., 1986), which is the active site in water oxidation, or a role in the protection of PSII from the extremely powerful oxidant that it must generate to oxidize water (Thompson & Brudvig, 1988). These recent results and proposals emphasize the importance of understanding the structure and function of cytochrome b_{559} to fully understand electron transport and water oxidation in PSII.

One of the most interesting properties of cytochrome b_{559} is its unusually high and variable reduction potential. The reduction potential ranges from about 370 to about 0 mV; the shift from high to low potentials often correlates with damaging treatments (Cramer & Whitmarsh, 1977). Similar decreases in reduction potential have been observed upon isolation of other b cytochromes, but the reduction potentials of the native cytochromes are usually much lower, in the range $E_m = 0 \pm 100$ mV (Cramer et al., 1979). Cytochrome b_{559} is thought to exist in two or three forms, known as "high

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[1] Abbreviations: Chl, chlorophyll; DCBQ, 2,5-dichloro-p-benzoquinone; E_m, reduction potential; E_{mn}, reduction potential at pH n; EPR, electron paramagnetic resonance; HP, high potential; IP, intermediate potential; kDa, kilodaltons; LP, low potential; MES, 2-(N-morpholino)-ethanesulfonic acid; PSII, photosystem II; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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potential" (370 mV) and "low potential" (60–80 mV; Cramer & Whitmarsh, 1977) and in a few studies "intermediate potential" (240 mV; Horton & Croze, 1977). The high- and low-potential forms are thought to be distinguished by different g values of the EPR signal of the oxidized heme.

The protein ligands of the heme have been shown, by a combination of EPR, resonance Raman, and absorption spectroscopies, to be two histidines (Babcock et al., 1985). However, the two proteins [9 kDa (α) and 4 kDa (β)] known to bind the heme each contain only one His (Herrmann et al., 1984), leading to the proposal that the heme cross-links two proteins. This cross-linked structure of the cytochrome has been suggested to be the cause of the instability of the high-potential form (Babcock et al., 1985). Because there are two copies of cytochrome b_{559} per PSII complex, the heme must be bound by either $\alpha\alpha$ and $\beta\beta$ or $2\alpha\beta$ complexes. The latter is more likely due to the homogeneous behavior of the cytochrome on cation-exchange columns during purification (Cramer et al., 1986).

There is limited information on the position of the heme relative to other components of the PSII complex. The 9- and 4-kDa cytochrome b_{559} proteins are predicted to each span the membrane one time, with the His ligand about five amino acids from the thylakoid membrane surface (Herrmann et al., 1984). Recent proteolysis and antibody binding studies have shown that the orientation of the 9-kDa protein is such that the His ligand is close to the outer membrane surface, suggesting that both heme irons are about 7.5 Å from the outer membrane surface (Tae et al., 1988). Finally, the D1/D2/cytochrome b_{559} preparation (Nanba & Satoh, 1987) and the photooxidation of cytochrome b_{559} at 77 K (Knaff & Arnon, 1969) point to a close association between cytochrome b_{559} and the D1/D2 core proteins.

Cytochrome b_{559} is thought not to be a component of the main electron-transfer chain in PSII because the kinetics of its oxidation and reduction are slow, and the extent of these reactions under physiological conditions is quite small (Cramer & Whitmarsh, 1977). The high-potential form of cytochrome b_{559} has often been correlated with water oxidation activity, but exceptions to this correlation have now been found (Briantais et al., 1985; Ghanotakis et al., 1986). Because cytochrome b_{559} can be reduced as well as oxidized by PSII, it has often been proposed to function in a cyclic electron-transfer pathway, perhaps as a protective mechanism (Heber et al., 1979). Thompson and Brudvig (1988) have recently shown that cytochrome b_{559} rereduces oxidized chlorophyll and have suggested that this reduction is part of a cyclic electron-transfer pathway which serves to protect PSII from photoinhibition.

To understand the molecular basis of the unusual redox properties of cytochrome b_{559} and to evaluate the various proposals for its function, we have investigated its structure, as reflected by its EPR and redox properties, in PSII samples. These studies provide a fresh view of cytochrome b_{559} because most past studies have been performed on thylakoid membrane preparations, in which interference from other cytochromes is a limitation. It is also now possible to do a number of well-defined biochemical manipulations of the PSII complex to remove specific extrinsic polypeptides and the Mn complex. By examining the effects of these treatments on both the EPR and redox properties of cytochrome b_{559}, these studies provide new insight into the structure of cytochrome b_{559} in PSII.

Experimental Procedures

PSII membranes were isolated from spinach leaves by using a modified version (Beck et al., 1985) of the isolation procedure of Berthold et al. (1981). The PSII membranes exhibited O_2-evolution activities of 450–600 μmol of O_2/(mg of Chl·h). PSII membranes were treated with 2 M NaCl as previously described (Thompson et al., 1986) to remove the 17- and 23-kDa polypeptides. The resulting samples retained 20–30% O_2-evolution activity, which increased to 70–80% in the presence of 5 mM $CaCl_2$, as is typical of samples depleted of these polypeptides (Ghanotakis et al., 1984a). The samples used for the redox titrations were treated twice with 2 M NaCl to maximize the removal of the 17- and 23-kDa polypeptides. Removal of the 17-, 23-, and 33-kDa polypeptides was accomplished with a 1 M $CaCl_2$ treatment, in which 200 mM NaCl was included in all buffers to ensure retention of the intact Mn complex (Miller et al., 1987). Samples lacking all three polypeptides and all four Mn were prepared by first treating with 1 M $CaCl_2$ and then with either 0.8 M Tris, pH 8.2 (Yocum et al., 1981), or 5 mM NH_2OH (Tamura & Cheniae, 1987). These samples retained less than 10% O_2-evolution activity. The samples were pretreated with 1 M $CaCl_2$ because we observe that neither the Tris nor the NH_2OH treatment removes all three polypeptides completely. SDS–polyacrylamide gel electrophoresis of PSII membranes treated according to the above procedures typically indicate that less than 10% of the 17-kDa polypeptide and less than 20% of the 23- and 33-kDa polypeptides remain; Mn determinations (Yocum et al., 1981) show that less than 10% of the Mn remain in typical Tris- and NH_2OH-treated samples.

The EPR studies were performed on untreated PSII samples or PSII samples treated as above and then treated with ascorbate to reduce cytochrome b_{559}. The ascorbate treatments consisted of two washes (centrifugation at 35000g for 10 min followed by resuspension of the pellet to a concentration of about 0.5 mg of Chl/mL) in an ascorbate-containing buffer. Both 2 M NaCl and 1 M $CaCl_2$ treated PSII were treated with 5 mM ascorbate, and Tris-treated PSII was treated with 15 mM ascorbate, in a buffer consisting of 25 mM MES–NaOH, pH 6.5, 200 mM NaCl, 15 mM $CaCl_2$, and 30% ethylene glycol. The ascorbate was removed with one wash for experiments involving warming a photooxidized sample to 0 °C (annealing) to prevent rereduction of the photooxidized cytochrome b_{559} by the excess ascorbate. Chemical oxidation of cytochrome b_{559} was accomplished with 2 mM K_2IrCl_6 (dissolved in the buffer solution and used immediately) or 1 mM DCBQ (from a 25 mM stock solution in ethanol). All isolation procedures, treatments, and EPR experiments were performed in dim green light.

EPR spectra of PSII samples at concentrations of 4–6 mg of Chl/mL were collected before and after 10-min illumination at 77 K and after 2-min annealing at 0 °C. The following spectrometer conditions were used: microwave frequency, 9.1 GHz; microwave power, 0.2 mW; magnetic field modulation frequency, 100 kHz; magnetic field modulation amplitude, 20 G; sample temperature, 10 K. Integration of the g_z turning point of the EPR signal was used to determine the amount of photooxidized cytochrome b_{559}. A sample oxidized with 2 mM K_2IrCl_6 was used as an EPR intensity standard corresponding to two ferricytochrome b_{559} per PSII (de Paula et al., 1985). Myoglobin azide (Bolard & Garnier, 1972) was used as a g value standard (g_z = 2.82, g_y = 2.20; Aasa & Vänngård, 1975). We estimate an uncertainty of about ±0.01 in the g values; the major limitation is the broad line width of the cytochrome b_{559} resonance.

Spectrophotometric redox titrations were performed by using a dual-wavelength Aminco DW2000 spectrophotometer, monitoring the absorbance at 560 nm with a reference

wavelength of 540 nm, similar to the procedure of Horton and Croze (1977). The potential was measured with a Corning combination Pt, Ag/AgCl electrode, calibrated against equimolar solutions of ferri- and ferrocyanide (Reilly, 1973). PSII samples were diluted to 0.13 mg of Chl/mL in a buffer consisting of 50 mM MES–NaOH, pH 6.0, 15 mM $CaCl_2$, 5 mM NaCl, and 30% ethylene glycol. The $CaCl_2$ was left out of the buffer for the titration of NH_2OH-treated PSII; this was found to have no effect on the titration behavior. The following mediators were added to a final concentration of 40 μM each from a 4 mM combined stock solution in ethanol: hydroquinone (E_{m7} = 280 mV); 1,2-naphthoquinone (E_{m7} = 143 mV); 1,4-naphthoquinone (E_{m7} = 36 mV). The ferricyanide used as a titrant will also act as a mediator ($E_m \approx 415$ mV at this ionic strength; Reilly, 1973), since it is known to readily oxidize cytochrome b_{559}. The sample was maintained anaerobic throughout the titration under a stream of argon. Ferricyanide and dithionite were used as titrants; samples containing HP cytochrome b_{559} were initially fully oxidized by the addition of 1–2 mM ferricyanide. The sample was gently mixed by inversion of the titration cell before each potential and absorbance measurement. By using a three-necked titration vessel, we were able to invert the cell without bringing the sample into the neck with the needle bearing the argon gas. The 560-nm peak observed at potentials below −100 mV (or below −25 mV in the case of untreated PSII) was taken to correspond to 100% reduced cytochrome b_{559}. Each complete titration was performed in about 5 h. PSII membranes are significantly more stable than thylakoid membranes and are, thus, less likely to be damaged during long titrations.

The titration data were analyzed by using the SIMPLEX and quasi-Newton curve-fitting routines of the SYSTAT program on a Macintosh SE computer. Each curve was fit to the sum of one to three Nernst curves for one-electron-reduction processes by fitting two to five parameters until further changes in the parameters were well within the error of the data (about ±2 mV and ±3% reduced cytochrome b_{559}). Because of the overlap between the titration curves of the different forms, we estimate a final uncertainty of ±10 mV in the reduction potential and ±5% in the fraction of each form of cytochrome b_{559}.

RESULTS

Electron Paramagnetic Resonance Spectroscopy. EPR has been used to investigate the effects of treatments that remove the extrinsic polypeptides and the Mn of PSII on two properties of cytochrome b_{559}. One important assay of the structure of cytochrome b_{559} is low-temperature photooxidation, since this property is likely to be related to its function. Untreated PSII membranes, in which cytochrome b_{559} is mostly reduced in the dark, have been shown to photooxidize one cytochrome b_{559} per PSII when illuminated at 77 K (de Paula et al., 1985; photochemistry is limited to one stable charge separation per reaction center at this temperature). Treatments that remove the extrinsic 17- and 23-kDa polypeptides are known to lower the reduction potential of cytochrome b_{559} such that it is oxidized in the dark (Larsson et al., 1984). de Paula et al. (1987) have shown that when samples lacking the 17- and 23-kDa polypeptides are prereduced with ascorbate, 77 K illumination again results in the photooxidation of cytochrome b_{559}. In an effort to determine which component(s) of PSII may be required for the low-temperature photooxidation of cytochrome b_{559}, we have treated PSII membranes with successively harsher but well-defined treatments known to remove the extrinsic polypeptides and Mn; 2 M NaCl was used to remove the 17- and 23-kDa polypeptides, 1 M $CaCl_2$ was used to remove the 17-, 23-, and 33-kDa polypeptides, and 1 M $CaCl_2$, followed by 0.8 M Tris (pH 8.2), was used to remove all three polypeptides and four Mn. Ascorbate treatment of these samples typically reduced all but about 20% (NaCl-treated PSII) to 40% ($CaCl_2$- and Tris-treated PSII) of the cytochrome b_{559}. Figure 1 shows that after prereduction of these samples with ascorbate, 77 K illumination oxidizes about one cytochrome b_{559} per PSII, as in untreated PSII membranes. Previous studies indicated that after removal of the 17- and 23-kDa polypeptides, further removal of calcium (a cofactor essential for O_2-evolution activity in these samples) prevented the low-temperature photooxidation of cytochrome b_{559} (de Paula et al., 1987). However, more recent results have been inconclusive, probably due to the well-known difficulty of completely removing the calcium. This indicates that it is necessary to quantitate the calcium in the sample to resolve this question. Therefore, although cytochrome b_{559} is certainly affected by the removal of the 17- and 23-kDa polypeptides, as shown by its lower reduction potential, neither the extrinsic polypeptides nor the Mn is required to preserve the structure of the PSII complex necessary for low-temperature photooxidation of cytochrome b_{559}.

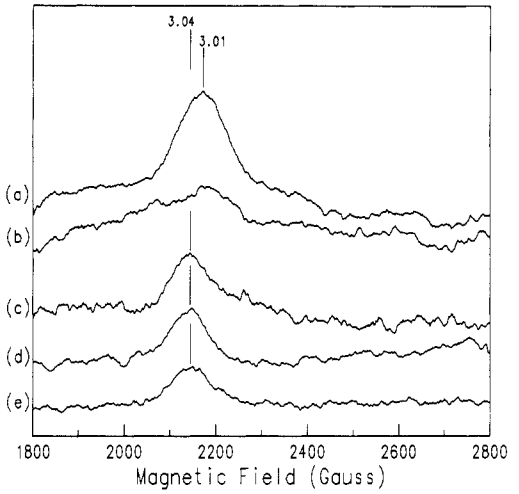

FIGURE 1: EPR spectra of the g_z turning point region of the oxidized cytochrome b_{559} signal demonstrate photooxidation of cytochrome b_{559} after removal of the extrinsic polypeptides and Mn. (a) Dark scan of 2 M NaCl treated PSII (g_z = 3.01). (b) Dark scan of 2 M NaCl treated PSII after treatment with ascorbate. (c–e) Illuminated (10 min at 77 K) minus dark scans of the following ascorbate-treated PSII samples (note that all have g_z = 3.04): (c) 2 M NaCl treated; (d) 1 M $CaCl_2$ treated; (e) 1 M $CaCl_2$ treated followed by treatment with 0.8 M Tris, pH 8.2.

Another probe of the structure of cytochrome b_{559} is the EPR signal of the oxidized heme. The following g values have been reported by Bergström and Vänngård (1982) for the signals of the high potential (untreated thylakoid membranes) and low potential (Tris-treated thylakoid membranes) forms of cytochrome b_{559}: g_z = 3.08 and g_y = 2.16 (untreated); g_z = 2.94 and g_y = 2.25 (Tris-treated). We observe comparable g values in untreated PSII samples photooxidized at low temperature. However, we find that there is a small but reproducible shift to lower g_z = 3.05 (and higher g_y = 2.18) upon warming such a sample to 0 °C. Chemical oxidation with K_2IrCl_6 or DCBQ also results in this lower g_z-value EPR signal. A similar g-value shift occurs in polypeptide-depleted PSII samples: 77 K photooxidation yields an EPR signal with g_z = 3.04 and g_y = 2.17. Warming such a sample to 0 °C shifts the EPR signal to g_z = 3.01 and g_y = 2.19. These results are shown in Figure 2: for both untreated and NaCl-treated PSII samples, 77 K illumination produces an oxidized cyto-

Table I: EPR Signals of Cytochrome b_{559} in PSII

	untreated PSII		2 M NaCl treated PSII	
	photooxidized	annealed[a]	photooxidized	annealed[a]
g_z	3.08	3.05	3.04	3.01
g_y	2.15	2.18	2.17	2.19

[a] EPR signals produced by chemical oxidation of untreated PSII or present in NaCl-treated PSII in the dark (cytochrome b_{559} is oxidized in the dark in this case because the ambient potential is higher than its decreased E_m) exhibit the same g values as the corresponding annealed signals.

chrome b_{559} species with a higher g_z value (and lower g_y value) than those observed after such a sample is annealed at 0 °C. We also observe that the chemically oxidized cytochrome b_{559} in untreated PSII and the dark-oxidized cytochrome b_{559} in NaCl-treated PSII exhibit the same g values as the corresponding photooxidized and then annealed signals (data not shown). Table I summarizes the observed g values.

The various treated PSII samples exhibit the same g_z value upon low-temperature photooxidation, as shown in Figure 1. The dark-oxidized EPR signals also exhibit the same (lower) g_z value (Figure 1a and data not shown). This indicates a similarity in the structure of cytochrome b_{559} in the different types of treated samples.

Spectrophotometric Redox Titrations. The reduction potential of cytochrome b_{559} is another important indicator of the structure and environment of the heme. In an effort to understand the unusual aspects of the reduction potential of this cytochrome—it is much higher than that of many b cytochromes and it is extremely labile—we have examined the changes in titration behavior that occur upon successive removal of the extrinsic polypeptides and Mn from PSII membranes.

Redox titrations were performed by monitoring the absorbance at 560 nm to determine the fraction of reduced cytochrome b_{559} at each potential. Figure 3 shows an example of the difference spectra obtained during the course of a titration. A comparison of the titration behavior of three types of PSII samples is shown in Figure 4. It is apparent that untreated PSII membranes (Figure 4a) exhibit a significantly higher overall reduction potential than that of the treated PSII samples (Figure 4b,c). Each titration consists of an initial reductive titration followed by a more rapid oxidative titration to determine whether the titration behavior is reversible. The data shown in Figure 4 indicate that little or no damage (which is expected to decrease the potential) occurs during the course of the titrations.

The titrations of the various types of PSII samples typically exhibit three waves, which we attribute to three forms of cytochrome b_{559}, which will be called high potential (HP), intermediate potential (IP), and low potential (LP). The number of components needed to fit the data in Figure 4 can

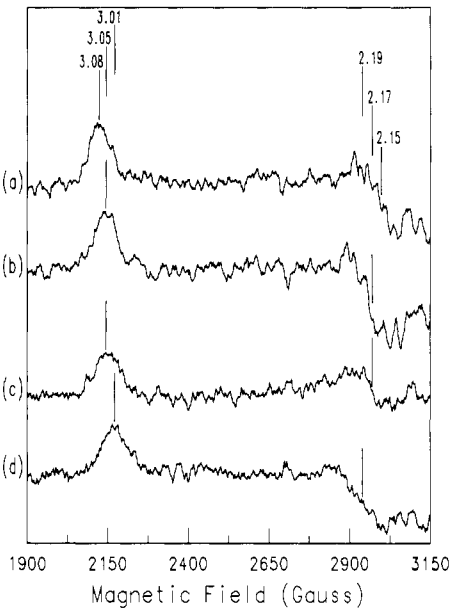

FIGURE 2: EPR spectra of photooxidized and annealed cytochrome b_{559}. (a) 77 K photooxidized minus dark spectrum of untreated PSII, $g_z = 3.08$, $g_y = 2.15$, (b) 77 K photooxidized and 2 min 0 °C annealed minus dark spectrum of untreated PSII, $g_z = 3.05$, $g_y = 2.18$. (c) 77 K photooxidized minus dark spectrum of NaCl-treated PSII, $g_z = 3.04$, $g_y = 2.17$. (d) 77 K photooxidized and annealed minus dark spectrum of NaCl-treated PSII, $g_z = 3.01$, $g_y = 2.19$.

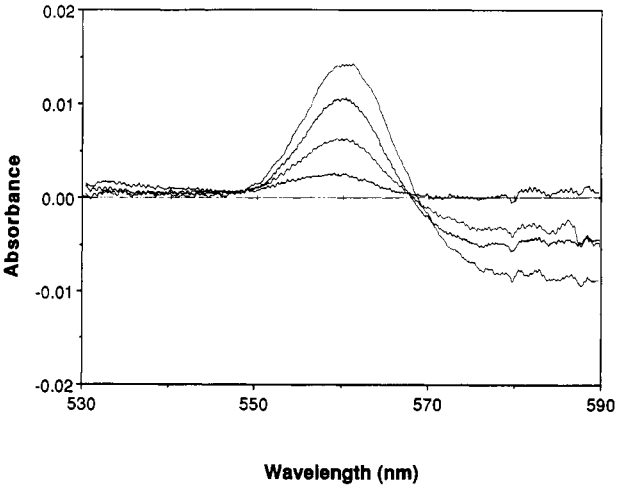

FIGURE 3: Dual-wavelength optical absorption difference spectra obtained periodically during a titration of NaCl-treated PSII. The sample was initially fully oxidized with 1 mM ferricyanide to obtain the background spectrum for all reduced minus oxidized difference spectra: (top to bottom) −94 minus 450 mV; 84 minus 450 mV; 161 minus 450 mV; 231 minus 450 mV.

be estimated visually from the spread of the curves. Since a single-component curve for a one-electron process spans 120

Table II: Reduction Potentials[a] of Cytochrome b_{559} in PSII

	thylakoids	untreated PSII	NaCl-treated (minus 17- and 23-kDa polypeptides)	$CaCl_2$- and NH_2OH-treated (minus 17-, 23-, and 33-kDa polypeptides and 4 Mn)
HP	383 (38%)	373 (45%)	370 (19%)	*b*
IP	*b*	226 (31%)	164 (43%)	178 (55%)
LP	*b*	56 (24%)	−3 (38%)	9 (45%)
		Data Fit to Consensus Values for Three Forms of Cytochrome b_{559}		
HP		375 (44%)	375 (18%)	
IP		228 (31%)	171 (42%)	171 (58%)
LP		57 (25%)	3 (40%)	3 (42%)

[a] Reduction potentials are given in millivolts vs the standard hydrogen electrode; errors are approximately ±10 mV (±5%); therefore, elsewhere in this paper we report reduction potentials to the nearest 5 mV. [b] Titration not performed over this potential range.

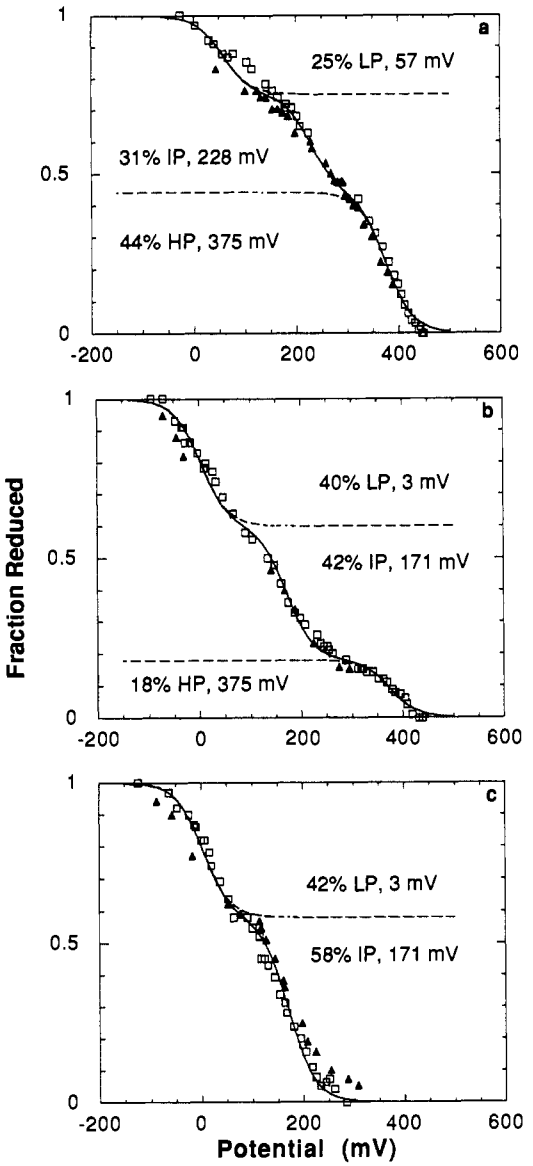

FIGURE 4: Redox titrations of three types of PSII samples. In all cases a reductive titration (□) was followed by an oxidative titration (▲), and the curves are plots of the best fit using the consensus potentials given in Table II. (a) Untreated PSII; (b) 2 M NaCl treated PSII, which is depleted of the 17- and 23-kDa extrinsic polypeptides; (c) 1 M $CaCl_2$ followed by 5 mM NH_2OH treated PSII, which is depleted of the 17-, 23-, and 33-kDa extrinsic polypeptides and four Mn.

mV (for 10–90% reduction), the span of curves 4a,b (≈400 mV) suggests three components, and the span of curve 4c (≈300 mV) suggests two components. The titration data for each type of sample were fit to a linear combination of up to three Nernst equations for single-electron reductions; the parameters obtained in these fits are given in Table II. Three components were necessary to fit curves 4a,b, because the residuals of the two-component fit were larger than the error in the data. In several cases, when the reduction potential of a form was quite similar in different types of samples, the potentials were averaged to determine the consensus values for the reduction potentials of the different forms of cytochrome b_{559}. The results of final fits of the titration data, using these consensus values for the potentials of the different forms, are also given in Table II and were used to generate the curves shown in Figure 4. The HP form exhibits a well-defined potential of 375 mV. The reduction potentials of the two lower potential forms decrease about 50 mV upon removal of the

Table III: Distribution of Redox Forms of Cytochrome b_{559} in PSII

	content[a] (%)		predicted[b] (%)			observed (%)		
sample	intact	−17, −23	HP	IP	LP	HP	IP	LP
untreated	70	30	35	50	15	44	31	25
NaCl-treated	30	70	15	50	35	18	42	40

[a] Determined on the basis of the hydroquinone sensitivity of O_2-evolution activity. [b] On the basis of one HP and one IP in intact centers and one IP and one LP in centers lacking the 17- and 23-kDa polypeptides.

polypeptides: the IP form decreases from E_m = 230 to 170 mV and the LP form from 55 to 5 mV.[2] Neither of the two lower potential forms exhibits a heterogeneous reduction potential, as might be expected if these forms are the result of nonspecific damage.

Untreated PSII samples contain 44% HP, 31% IP, and 25% LP cytochrome b_{559}. The low fraction of the HP form could be due to damage or could reflect the in vivo composition of PSII. A similar fraction of HP cytochrome b_{559} (38%) is observed in a titration of thylakoid membranes (see Table II), although it is more difficult to establish the fraction of HP cytochrome b_{559} in thylakoids due to interference from other cytochromes. This result suggests that the PSII isolation procedure is not the cause of the low fraction of the HP form. It is also unlikely that damage occurring during the titration has decreased the fraction of the HP form, in view of the excellent reversibility of the titration (see Figure 4a). Further, the results of a rapid 1 mM ferrocyanide reduction are also consistent with the results of the rigorous titration. This indicates that the titration conditions (mediators, anaerobicity) are not the cause of the presence of the IP form. We conclude that the 40% HP cytochrome b_{559} which we observe in both thylakoids and PSII samples reflects the actual in vivo composition of the PSII complex, consistent with the fact that many titration studies of untreated thylakoid membranes have reported a significant fraction of low-potential cytochrome b_{559} (Cramer & Whitmarsh, 1977). Although this could be due to an intrinsic heterogeneity among different PSII centers, we propose that each intact PSII complex contains one HP and one IP cytochrome b_{559}.

The effect of the removal of the 17- and 23-kDa extrinsic polypeptides on cytochrome b_{559} is a decrease in the overall reduction potential through a decrease in the fraction of the HP form (44% to 18%) and a 50-mV decrease in the potentials of both the IP and LP forms. The majority of the PSII sample shifts from a mix of HP and IP to IP and LP. There are no significant further changes in the titration curve upon removal of the 33-kDa polypeptide and four Mn. The similarity between the titration curves of these two treated samples (Figure 4b,c) indicates that the 33-kDa polypeptide and Mn do not have as significant an effect on cytochrome b_{559} as do the 17- and 23-kDa polypeptides, although the Mn may have a small

[2] The following additional data analysis was conducted to test the validity of the observed 50-mV decrease in the reduction potentials of the IP and LP forms upon removal of the 17- and 23-kDa polypeptides. The titration data for untreated PSII include two points obtained during the oxidative titration (the two lowest potential oxidative points) that do not fit the curve and are clearly influencing the position of the IP/LP inflection (see Figure 4a). To verify that this was not the cause of the higher potential of the IP and LP forms, we performed a fit to the data obtained in the reductive titration alone. The resulting parameters were fairly similar (34% at 215 mV and 19% at 51 mV), which indicates that the rapid oxidative titration data were not significantly biasing the fit and that there is indeed a decrease in the potentials of the IP and LP forms upon removal of the polypeptides.

effect (see Discussion). Since these treated samples contain approximately equal amounts of the IP and LP forms (42% IP/40% LP and 58% IP/42% LP), we propose that polypeptide-depleted PSII samples consist of one IP and one LP cytochrome b_{559} per PSII complex.

These interpretations are also supported by the observed loss of O_2-evolution activity during the titration, which should occur in sites lacking the 17- and 23-kDa polypeptides. Hydrophobic reductants, such as hydroquinone, which is present during the titration, are known to reduce and extract Mn in PSII centers depleted of the 17- and 23-kDa polypeptides (Ghanotakis et al., 1984b). Thus, the loss of O_2-evolution activity during the titration can be used to determine the fraction of the sample that is lacking these polypeptides. Table III compares the observed fractions of each form of cytochrome b_{559} with predicted fractions, on the basis of the interpretation that native PSII contains one HP and one IP and PSII lacking the 17- and 23-kDa polypeptides contains one IP and one LP cytochrome b_{559}. We observe loss of 30% of the activity in untreated PSII samples after a reductive titration, as well as after merely incubating the PSII sample under conditions similar to those of the titration, suggesting that 30% of the PSII centers in the untreated sample lack the 17- and 23-kDa polypeptides. If these polypeptide-depleted centers contain one IP and one LP, this predicts 15% LP cytochrome b_{559}, which is roughly consistent with the observed 25% LP. The additional 31% IP observed in this sample cannot also correspond to a polypeptide-depleted fraction. Furthermore, NaCl-treated PSII loses all but 30% of its activity during the titration, suggesting that 30% of the centers retain the 17- and 23-kDa polypeptides. If these intact centers contain one HP and one IP, this predicts 15% HP cytochrome b_{559}, which is again roughly consistent with the observed 18% HP. The 50-mV decrease in the reduction potential of the LP form after the NaCl treatment may be due to a change induced in the sample by the high ionic strength of the treatment. We conclude that the HP form occurs in the presence of the polypeptides and the LP form in their absence.

Discussion

Redox titrations of PSII membranes indicate that there are (at least) three forms of cytochrome b_{559}, HP (375 mV), IP (230/170 mV), and LP (55/5 mV). Untreated PSII appears to contain one HP and one IP cytochrome b_{559}; removal of the 17- and 23-kDa polypeptides changes the composition to one IP and one LP cytochrome b_{559}. The EPR studies are consistent with the titration studies in that the major change in structure, as reflected by both the EPR g values and the reduction potentials, occurs upon removal of the 17- and 23-kDa polypeptides, with no further significant changes in the g values, reduction potentials, or the ability to photooxidize cytochrome b_{559} upon the further removal of the 33-kDa polypeptide and Mn.

It is difficult to establish the significance of each of these forms of cytochrome b_{559}—which forms arise due to nonspecific damage to the PSII samples and which reflect the in vivo forms of cytochrome b_{559} and the structural changes resulting from specific treatments. An alternate interpretation is that cytochrome b_{559} is actually 100% HP in vivo and that the IP and LP forms observed in PSII membranes are due to polypeptide-depleted sites or to some type of nonspecific damage that does not remove the polypeptides. However, we have shown that highly active PSII membranes contain approximately one HP and one IP cytochrome b_{559} and that this composition is not due to damage during the titration or the titration setup. Thylakoid membranes also appear to contain about 50% HP cytochrome b_{559}, but more extensive studies are needed to rule out the possibility that the IP form is caused by damage during the PSII preparation. Most of the past studies of thylakoid and PSII membranes that have included titrations over the full range of potentials have reported significant fractions of lower potential forms, consistent with the possibility of an in vivo composition of 50% HP cytochrome b_{559}. Indeed, Heber et al. (1976) reported an approximately 1:1 ratio of high- to low-potential cytochrome b_{559} in highly active CO_2-fixing chloroplasts. The IP and LP forms we observe do not appear to be heterogeneous, as might be expected if either form is due to nonspecific damage.

The reduction potentials that we report appear consistent with other titration studies of cytochrome b_{559} in PSII membranes and thylakoids. The high-potential form is generally accepted to have an E_m = 350–400 mV in thylakoid membranes (Cramer & Whitmarsh, 1977), and a pH-independent E_m = 380 mV has been reported in a recent titration study of PSII membranes (Ortega et al., 1988). The so-called low-potential form has been reported to have an E_{m7} = 80 mV, with a pH dependence of −40 mV/pH unit (pH 6.0–8.0) in thylakoids (Fan & Cramer, 1970) and an $E_{m7.6}$ = 140 mV with a pH dependence of −72 mV/pH unit (pH <7.6) in PSII membranes (Ortega et al., 1988). This form may be equivalent to our IP form: a more typical pH dependence of −60 mV/pH unit predicts E_{m6} = 140 mV (for E_{m7} = 80 mV) or 236 mV (for $E_{m7.6}$ = 140 mV), which is fairly consistent with our observation of E_m = 170–230 mV for the IP form at pH 6.0. The intermediate-potential form that has been reported in a study of NH_2OH-treated thylakoids ($E_{m7.8}$ = 240 mV; Horton & Croze, 1977) probably corresponds to the IP form we observe, although if this form is pH dependent, this E_m is somewhat higher than expected from our data. Perhaps a higher E_m for the IP form in NH_2OH-treated thylakoids results from the fact that these samples were not exposed to high ionic strength or to detergent or because the dark NH_2OH treatment does not completely remove the extrinsic polypeptides.

Although we have not measured the pH dependence of the reduction potentials we observe, past studies suggest that the HP form is pH independent and the IP form is pH dependent. If the LP form that we observe is also pH dependent, then at the high pH usually used in studies of thylakoids the reduction potential would be less than zero, and its titration curve would be complicated by overlap with cytochrome b_{563} in thylakoid membranes (Horton & Croze, 1977). If the LP form is pH independent, then at pH >7.0 the IP and LP forms will not be well resolved, and the titration curve could look like a heterogeneous mixture of forms. At the lower pH generally used in studies of PSII membranes a very broad titration curve would be observed, because at pH 6 the E_m of the IP form is shifted to a value between those of the HP and LP forms. Our studies suggest that the reported heterogeneous low-potential cytochrome b_{559} may actually consist of two distinct species, IP and LP. These ideas can be tested in future titration studies of PSII by examining the pH dependence of each potential form of cytochrome b_{559}.

The results of our EPR studies of cytochrome b_{559} are also generally consistent with past experiments. Photooxidation of cytochrome b_{559} in Tris-washed thylakoid preparations has been observed by both optical spectroscopy (Knaff & Arnon, 1969) and EPR (Malkin & Vänngård, 1980; Nugent & Evans, 1980). These results can now be explained specifically: photooxidation does not require the presence of the 17-, 23-, or 33-kDa extrinsic polypeptides or Mn, and the IP form of

cytochrome b_{559} can be photooxidized. The g values we observe are consistent with the values reported for untreated thylakoids, but not for Tris-treated thylakoids and isolated cytochrome b_{559}, $g_z = 2.94$ (Bergström & Vänngård, 1982; Babcock et al., 1985) vs $g_z = 3.01$ (this work). Perhaps our treatments have not perturbed the structure of cytochrome b_{559} as much as the Tris treatment of thylakoids or the procedure for the isolation of cytochrome b_{559}.

The shift in g value that we observe upon warming a low-temperature photooxidized sample was previously observed in Tris-treated thylakoid preparations (Malkin & Vänngård, 1980; Nugent & Evans, 1980). These g-value shifts reflect a change in structure, which can be explained as follows: 77 K photooxidation of cytochrome b_{559} traps it in a "reduced conformation" (higher g_z), which relaxes to an "oxidized conformation" (lower g_z) when it is warmed to 0 °C. A chemically oxidized (or dark-oxidized) sample relaxes to the oxidized conformation (lower g_z) before it is frozen. Such a conformational change upon oxidation is not unexpected: a comparison of the crystal structures of cytochrome c in the oxidized and the reduced state reveals small changes in structure that result in a more hydrophilic heme environment in the oxidized protein (Takano & Dickerson, 1981). Perturbations in the visible absorption spectrum of cytochromes c and c_2 have also been used to show that the heme is more exposed to the solvent in the oxidized proteins (Schlauder & Kassner, 1979). These structural changes would be expected to stabilize the positively charged oxidized heme. It is likely that upon oxidation cytochrome b_{559} undergoes a conformational change which results in a more hydrophilic heme environment. This conformational change also results in a very small structural change in the heme ligands, which is reflected in the small g-value change of 0.03.

An important aspect of this study is the ability to correlate EPR and redox titration data to obtain new insight into the structure of cytochrome b_{559} in PSII. One obvious conclusion from this comparison is that, since PSII samples typically contain at least two forms of cytochrome b_{559}, one cannot assign the observed g values to particular potential forms. The redox titrations also provide insight into the identity of the form that is photooxidized in polypeptide-depleted PSII membranes. The LP form (5 mV) will not be reduced by ascorbate (E_m = 50 mV), consistent with the observation of 20–40% dark-oxidized cytochrome b_{559} in the ascorbate-treated EPR samples. Therefore, the photooxidized species in polypeptide-depleted PSII is the IP form. It may be difficult to test whether the LP form can be photooxidized, because the reduction of this form will also reduce Q_A ($E_m \approx 0$ V; Diner & Delosme, 1983), which will prevent stable charge separation reactions. The results of early redox titrations of the photooxidation of cytochrome b_{559} (Erixon et al., 1972), which showed $E_m \approx 230$ mV in Tris-treated thylakoids and E_m = 450 mV in untreated thylakoids, also suggest that polypeptide-depleted PSII photooxidizes the IP form and that untreated PSII photooxidizes the HP form. de Paula et al. (1985) demonstrated photooxidation of up to 1.5 cytochrome b_{559} per PSII center in untreated PSII, which also indicates that the IP (230 mV) form can be photooxidized. The observation of the same g value in PSII samples with one photooxidized or two chemically oxidized cytochrome b_{559} indicates that the g values of the two forms must be similar enough that the average g value (observed in a sample with both oxidized) is indistinguishable from the g value of the photooxidized species. We conclude that the two forms of cytochrome b_{559} present in a sample [HP (375 mV) and IP (230 mV) or IP (170 mV) and LP (5 mV)] have identical or very similar ($\Delta g_z \leq 0.02$) g values.

The correlation of EPR with redox titration results can also aid in discriminating between the effects of the 17- and 23-kDa polypeptides and the effects of Mn on cytochrome b_{559}. As discussed above, Mn loss will occur during redox titrations of NaCl-treated PSII, because hydrophobic reductants, which must be present to act as mediators, will reduce and extract the Mn (Ghanotakis et al., 1984b). The apparent reversibility of the titration (see Figure 4b) does not rule out a change in reduction potential due to Mn loss, because it could occur before the start of the titration of the IP and LP forms; this is certainly possible since a significant concentration of hydroquinone will already be present at these potentials. Therefore, redox titrations alone cannot distinguish between changes in cytochrome b_{559} due to loss of the polypeptides and those due to loss of the Mn. In PSII samples depleted of the 17- and 23-kDa polypeptides but retaining the Mn, cytochrome b_{559} has been shown by EPR to be fully oxidized in the dark (de Paula et al., 1986). This suggests that both the HP (375 mV) and the IP (230 mV) forms of cytochrome b_{559}, which are reduced in untreated PSII (de Paula et al., 1985), have undergone a decrease in potential upon polypeptide depletion. The sample could then consist of two IP (170 mV) or one IP (170 mV) and one LP (55 or 5 mV) cytochrome b_{559}. The fact that the g value does not change upon removal of Mn from polypeptide-depleted PSII (see Figure 1) suggests that removal of Mn does not significantly alter the structure of cytochrome b_{559}. However, as discussed above, the g values of the IP (170 mV) and LP (5 mV) forms are probably quite similar. Thus, we cannot rule out the possibility that the removal of Mn causes the conversion of the IP to the LP form or the 50-mV decrease in the reduction potential of the LP form. We conclude that the Mn complex may perturb the average reduction potential of cytochrome b_{559} by at most 85 mV.

Finally, by utilizing both EPR and redox titrations to characterize cytochrome b_{559} in similarly treated PSII samples, we have obtained new insight into the structure of cytochrome b_{559} and its relation to the structure of the PSII complex. We have shown that the removal of the 17- and 23-kDa polypeptides causes a change in structure characterized by a small change in the g values (a decrease in g_z and an increase in g_y of 0.04–0.02) and a large decrease in the reduction potential (a decrease of 215 mV in the average E_m of the two forms). Changes in the ligand field of a low-spin heme cause g_z to vary substantially (2.41–3.05), while changes in the environment of the heme (the solvent in a model compound study) cause a change in g_z of about 0.03 (Walker et al., 1984). The small g-value change indicates that removal of these polypeptides does not significantly alter the ligand field of the heme; the decrease in the reduction potential must result from a change in the environment of the heme. Therefore, the unusually high potential of cytochrome b_{559} is probably not due to a strained conformation in which the His ligands are rotated away from a parallel configuration (Babcock et al., 1985). A structure with the imidazole rings in a perpendicular configuration is expected to give rise to a different EPR signal than is observed ($g_z > 3.3$ and g_y unresolved), and the change from a perpendicular to a parallel orientation of the His ligands should result in a decrease in g_z of about 0.4 and a reduction potential decrease of about 50 mV (Walker et al., 1986). Since the largest decrease in g_z that we observe, 0.07, is unlikely to correspond to a large decrease in the reduction potential, we conclude that the orientation of the His ligands is not a significant factor in modulating the reduction potential in the case

of cytochrome b_{559}. We propose instead that the unusually high potential of HP cytochrome b_{559} is due to an extremely hydrophobic environment, which would destabilize the positively charged oxidized state and, thus, increase the reduction potential. The removal of the 17- and 23-kDa polypeptides most likely lowers the reduction potential by increasing the solvent exposure of the heme. Both calculated (Stellwagen, 1978) and experimentally measured (Schlauder & Kassner, 1979) correlations between the solvent exposure and the reduction potential of hemes in various proteins suggest that an increase of about 10% in the solvent exposure of the heme would decrease E_m by 200 mV. The polypeptides could create a hydrophobic heme environment by direct screening or, since recent results indicate that they are on the opposite side of the membrane from the heme (Tae et al., 1988), they could stabilize an association between membrane-spanning α helices of certain proteins which results in a hydrophobic heme pocket on the opposite side of the membrane. An increase in the solvent exposure of the heme environment could also explain the probable pH dependence of the IP form: such a change in environment could shift the pK of nearby group(s) into the physiological range, which would result in a pH-dependent E_m. Since the g-value change observed when a low-temperature photooxidized sample is warmed is similar to that observed upon removal of the 17- and 23-kDa polypeptides, a similar small structural change, accompanied by an increase in heme solvent exposure, may be occurring in both cases. Thus, we suggest that the differences in structure among the different forms of cytochrome b_{559} and the changes in structure upon removal of the 17- and 23-kDa polypeptides and upon warming a photooxidized sample, all of which are characterized by small g-value differences, are due to differences in the solvent accessibility of the heme.

The results of these EPR and redox titration experiments suggest several guidelines for future investigations of cytochrome b_{559}. The identification of an IP form of cytochrome b_{559} in PSII samples indicates that titrations must be performed before any conclusions regarding the reduction potential of cytochrome b_{559} can be drawn. As discussed above, the redox form of cytochrome b_{559} cannot be determined solely from the g value of the EPR signal. The common three-point titration procedure, which consists of determining the fractions of cytochrome b_{559} that can be reduced by hydroquinone, ascorbate, or dithionite, will not necessarily distinguish the IP from the other two forms and is meaningless unless the potentials set by the combinations of oxidants and reductants are measured. Ferrocyanide reduction will also not discriminate between the HP and IP forms: we observe E_m = 220 mV in a PSII solution containing 1 mM ferrocyanide. Another common assumption is that any cytochrome b_{559} which is found to be oxidized in the dark (in an EPR spectrum) is low potential. This conclusion depends on the ambient redox potential of the solution, which is not usually measured, and on prior exposure of the sample to light, since rereduction of photooxidized cytochrome b_{559} is quite slow in PSII membrane samples.

The other significant conclusion of this work is that low-potential forms of cytochrome b_{559} (IP and LP) are not necessarily damaged and nonfunctional. First of all, native PSII appears to consist of one HP and one IP cytochrome b_{559}, as we have discussed. We have also shown that the IP form can be photooxidized, a property which is most likely related to the function of cytochrome b_{559}, and that both the IP and LP forms exhibit well-defined reduction potentials. These results suggest that although the LP form may be nonfunctional, the IP form is a functional form of cytochrome b_{559}. Future titration studies should yield a consensus regarding which forms of cytochrome b_{559} are due to damage and which reflect the actual composition of PSII. This information will be important both for understanding the structural role of cytochrome b_{559} within the PSII complex and for evaluating various proposals for its function.

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EXAFS Structural Study of F_X, the Low-Potential Fe-S Center in Photosystem I†

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ABSTRACT: We present iron extended X-ray absorption fine structure (EXAFS) spectra of a photosystem I core preparation containing F_X, the very low potential iron–sulfur cluster in photosystem I. The preparation lacks F_A and F_B. The amplitude of Fe–Fe backscattering in the EXAFS spectrum indicates that F_X may be a [4Fe-4S] cluster and is not a [2Fe-2S] cluster or clusters.

There are contradictory data in the literature concerning the structure of an Fe-S cluster called F_X,[1] which serves as an electron acceptor in photosystem I of green plants and cyanobacteria. In its reduced state, F_X is characterized by an EPR spectrum similar to those of [2Fe-2S] and [4Fe-4S] ferredoxins but with a somewhat more anisotropic g tensor. This cluster operates at approximately −720 mV, in contrast to typical ferredoxins, which have reduction potentials of −250 to −450 mV. The properties of F_X have been reviewed (Evans, 1982). The fact that its reduction potential is so low makes it an interesting target for structural studies. In the present work we are concerned with identifying whether F_X is of cluster type [2Fe-2S], [4Fe-4S], or something other than these. Previous structural analyses by spectroscopic methods have been consistent with [4Fe-4S] (Evans et al., 1981) or [2Fe-2S] (Golbeck et al., 1987; McDermott et al., 1988; Bertrand et al., 1988) or a distorted [4Fe-4S] cluster (McDermott et al., 1988). Examination of the polypeptide sequences of psaA and psaB (Fish et al., 1985), which are the putative binding sites of F_X (Golbeck et al., 1988), reveals none of the characteristic sequence elements of [2Fe-2S] or [4Fe-4S] ferredoxins [reviewed in Stout (1982)]. A consideration of the stoichiometries of Fe, acid-labile sulfide, and cysteines per complex would argue against the presence of [2Fe-2S] clusters on the psaA and psaB polypeptides (Bruce & Malkin, 1988; Golbeck et

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[1] Abbreviations: EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; F_A, F_B, and F_X, ferredoxins in photosystem I, alternatively referred to as centers A, B, and X; kDa, kilodaltons; PS I, photosystem I; psaA and psaB, reaction center polypeptides in PS I, also referred to as PSI-A1 and PSI-A2; P700, primary electron donor in PS I; Tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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