A Mössbauer Analysis of the Low-Potential Iron–Sulfur Center in Photosystem I: Spectroscopic Evidence That F_X Is a [4Fe-4S] Cluster†

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ABSTRACT: We report the results of a Mössbauer study of the low-potential iron–sulfur cluster F_X in the Photosystem I core protein of *Synechococcus* 6301. The Mössbauer spectrum of F_X in the oxidized state shows an isomer shift of 0.42 mm/s, which is in good agreement with the 0.43 mm/s isomer shift found in [4Fe-4S] proteins but not with the isomer shift of 0.26 mm/s found in [2Fe-2S] proteins. In the reduced state the spectrum is asymmetrically broadened at 80 K, indicating the presence of two very closely spaced doublets with an average isomer shift of 0.55 mm/s, which is also in agreement with [4Fe-4S] proteins. At 4.2 K, the spectrum exhibits broadening and magnetic splitting similar to what is observed for [4Fe-4S] proteins and quite unlike [2Fe-2S] proteins. Given the assumption that the iron atoms of F_X are tetrahedrally coordinated with sulfur ligands, the data strongly support the assignment of F_X as a [4Fe-4S] cluster.

The photosystem I reaction center is a membrane-bound, multiprotein complex which catalyzes the light-induced transfer of an electron from plastocyanin to soluble ferredoxin [for review, see Golbeck (1987)]. In this process, a photon is absorbed by 1 of 200 antenna chlorophyll molecules, resulting in exciton transfer among the antenna chlorophylls and excitation of the primary electron donor P700. The excited state of P700 is highly electronegative and reduces the primary electron acceptor A_0, leading to the $P700^+A_0^-$ charge-separated state. The electron is then transferred through a series of tightly bound electron acceptors (A_1, F_X, F_B, and F_A) to ferredoxin and ultimately to $NADP^+$.

The identity of the electron transport components is largely known. The primary electron donor, P700, is most likely a chlorophyll *a* dimer and may be similar to the bacteriochlorophyll *a* "special pair" observed in the bacterial reaction center; the primary electron acceptor, A_0, is a chlorophyll monomer; A_1 may be phylloquinone (vitamin K_1); F_X is an iron–sulfur cluster; and the terminal electron acceptors F_B and F_A are both [4Fe-4S] clusters. Iron–sulfur center F_X is particularly interesting in that it has an $E_{1/2}$ close to −700 mV (Ke et al., 1977; Parrett et al., 1989) and thus has the most negative midpoint potential of any known iron–sulfur cluster. Its ESR[1] spectrum is similar to those of [2Fe-2S] and [4Fe-4S] ferredoxins but with a more anisotropic *g* tensor. Its structural identity as a [2Fe-2S] or a [4Fe-4S] cluster is in dispute and is the topic of this study.

The photosystem I core protein is composed of two high molecular mass (82 and 83 kDa) polypeptides which are coded for by the light-inducible genes *psaA* and *psaB* (Fish et al., 1985; Kirsch et al., 1986). These high molecular mass (core) proteins are responsible for binding ~120 antenna chlorophyll *a* molecules, P700, and the electron acceptors A_0, A_1, and F_X. In addition, eight to nine low molecular mass (<19 kDa) polypeptides are associated with the photosystem I complex in higher plants (not including LHCI) and cyanobacteria. Of these low molecular mass polypeptides, an 8.9-kDa polypeptide coded for by the *psaC* gene has been shown to bind the terminal electron acceptors F_B and F_A (Ohyama et al., 1987; Wynn & Malkin, 1988).

The photosystem I complex can be isolated from the thylakoid membrane with a variety of nonionic or zwitterionic detergents. These detergent-based preparations have been used extensively for structure/function studies of the reaction center and its individual components. However, since the complex contains all three iron–sulfur centers, it has been difficult to study the low-potential iron–sulfur center, F_X, without any influence from iron–sulfur centers F_B and F_A. Earlier Mössbauer studies of the photosystem I complex were interpreted to suggest that F_X is a [4Fe-4S] cluster (Evans et al., 1981). These studies were based on the fact that 65% of the Mössbauer spectrum changed in a way that was consistent with a [4Fe-4S] cluster. However, F_X is difficult to reduce, and questions were raised whether the remaining 35% of the iron might represent the majority of F_X. Subsequent studies involving EXAFS spectroscopy (McDermott et al., 1988) and ^{19}F NMR based core extrusion (Golbeck et al., 1987) had indicated that among F_A, F_B, and F_X there may be [2Fe-2S] as well as [4Fe-4S] clusters [see also Bertrand et al. (1989)]. Since F_A and F_B were known to be [4Fe-4S] clusters, the [2Fe-2S] clusters were assigned to F_X. There were, however, difficulties in both sets of experiments: the EXAFS data could be fit either to 2[4Fe-4S] and 2[2Fe-2S] clusters or to three distorted [4Fe-4S] clusters, and core extrusion experiments had not been performed previously in iron–sulfur proteins lacking the characteristic amino acid sequence elements (CysXXCysXXCysXXXCysPro) found in more traditional proteins. In addition, both approaches suffered from the weakness that the spectra of F_A and F_B must be "deconvoluted" from the composite iron–sulfur spectrum to obtain the spectrum of F_X.

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[1] Abbreviations: PS I, photosystem I; Chl, chlorophyll; ESR, electron spin resonance; Tris, tris(hydroxymethyl)aminomethane; DCPIP, 2,6-dichlorophenolindophenol; Tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid; IS, isomer shift; QS, quadrupole splitting.

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More recently, a photosystem I core preparation became available which was totally devoid of the low molecular mass polypeptides, including the 8.9-kDa, F_A/F_B polypeptide (Golbeck et al., 1988a; Parrett et al., 1989). This photosystem I core protein retained the 82- and 83-kDa reaction center polypeptides and the electron transfer components P700 and F_X. Flash kinetic studies and ESR studies indicated that F_X was fully functional in accepting electrons both at room temperature and at 4.2 K. The complete retention of F_X, and the total lack of F_A and F_B, made this an appropriate preparation for further structural studies of F_X.

In this paper we report the results of a Mössbauer study of F_X in the photosystem I core protein from *Synechococcus* 6301. These results indicate that the structure of F_X is incompatible with the properties of a [2Fe-2S] cluster and strongly support its assignment as a [4Fe-4S] cluster.

Materials and Methods

Photosystem I reaction centers with ^{57}Fe-substituted iron–sulfur clusters were prepared by phototrophic growth of the cyanobacteria *Synechococcus leopoliensis* in medium C (Kratz & Myers, 1955) which had been 90% enriched with ^{57}Fe. A 20-mg sample of Fe_2O_3 (90% ^{57}Fe isotope) was refluxed over stream in 3 mL of 11.6 N HCl for 15 min to completely dissolve the solid material. This was evaporated under vacuum to near dryness, and a solution Na_2EDTA was added in slight molar excess over the amount of Fe. The pH was adjusted to 6.5 with NaOH to produce a 0.01 M stock solution of Fe(III)-EDTA. An identical procedure was used to convert iron-57 foil to soluble EDTA chelate.

Liquid stock cultures of *S. leopoliensis* 6301, equivalent to *Anacystis nidulans* TX 20, were grown in medium C in a continuous turbidostat at a cell density equivalent to 10 μg of Chl/mL. Aliquots of culture were inoculated into 200-mL cylindrical glass culture tubes of 38-mm diameter and then diluted with medium C devoid of the Fe component, to a cell density equivalent to 0.5–0.6 μg of Chl/mL. Iron was supplied as the ^{57}Fe-EDTA stock solution, to a final concentration of 10 μM. Inocula were grown at 39 °C while aerated with 1.5% CO_2-enriched air. Continuous illumination was provided with cool white fluorescent lamps, gradually increased from a mean photon flux density (PFD) at the culture surface of approximately 50 $\mu E \cdot m^{-2} \cdot h^{-1}$ to a PFD of 150 $\mu E \cdot m^{-2} \cdot h^{-1}$ as the cell density increased to an equivalent of 12 μg of Chl/mL over a 48-h growth period.

Twenty of the 200-mL cell cultures grown through four to five doublings in ^{57}Fe were combined for membrane preparations. Cells were pelleted and resuspended to approximately 0.6 mg of Chl/mL in medium A (0.05 M Tris-HCl, pH 7.5; 2 mM EDTA). Cells were again pelleted and resuspended to 0.3 mg of Chl/mL in medium A. This suspension was forced at a pressure of 110 MPa through a French pressure cell at ambient temperature into a chilled collection tube. All previous steps were at room temperature; subsequent procedures were at ice temperature.

The broken cell suspension was centrifuged at 1000*g* for 4 min to pellet unbroken cells, which were discarded. NaCl was added to the supernatant from a 2.0 M stock solution to a final concentration of 0.25 M. This suspension was centrifuged at 40000*g* for 1 h to pellet the membrane fragments. The pellet was resuspended in medium A to a concentration equivalent to 1.5 mg of Chl/mL and immediately frozen to −20 °C. Several such membrane fragments were combined and maintained at −80 °C.

The photosystem I core protein was isolated from a *Synechococcus* photosystem I complex in a procedure similar to that described in Parrett et al. (1989). The photosystem I complex (250 μg/mL) was incubated with 6.8 M urea at room temperature for 15 min to dissociate the low molecular mass polypeptides; the reaction was terminated by diluting the sample 2-fold in Tris buffer (50 mM, pH 8.3) followed by overnight dialysis against the same buffer. The high molecular mass polypeptides were concentrated over an Amicon YM-100 ultrafiltration membrane and further purified by sucrose density ultracentrifugation (0.1–1 M sucrose in 50 mM Tris, pH 8.3, and 0.05% Triton X-100) for 36 h at 24000 rpm (SW-27 rotor). The lower chlorophyll-containing band was collected and dialyzed against 50 mM Tris, pH 8.3. After 12 h, the dialysis solution was replaced with 50 mM Tris, pH 8.3, containing 5 mM Tiron (to remove residual iron from the destruction of F_A and F_B). After 24 h, the dialysis solution was replaced with 50 mM Tris, pH 8.3, containing 0.05% Triton X-100 and dialyzed for an additional 12 h. The photosystem I core protein was concentrated over a YM-100 membrane to a chlorophyll concentration of ~5 mg/mL and stored at −80 °C.

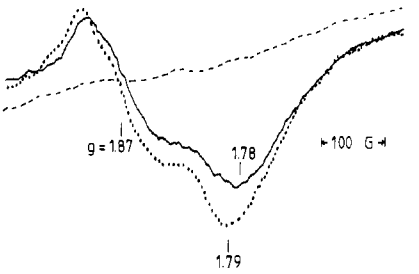

FIGURE 1: ESR spectra of the F_X center in the photosystem I core protein: (—) freezing under continuous illumination; (···) after storage at 77 K in the dark for 45 h; (– –) after subsequent dark adaptation for a few minutes at 0 °C and refreezing in darkness. The sample contained 1 mM DCPIP and 10 mM sodium ascorbate in 50 mM Tris, pH 8.3. Spectrometer conditions: temperature, 9 K; microwave power, 39 mW; amplitude modulation, 40 G; receiver gain, 1.25×10^5.

Mössbauer spectra were obtained with a constant-acceleration spectrometer and a ^{57}Co(Rh) source. For homogeneous illumination, samples at approximately 4.6 mg of Chl/mL, 1-mL volume, were used to fill five disc-shaped holders of 1-mm thickness each which were piled up at low temperature for the Mössbauer experiments.

ESR experiments for control purposes were performed with a Bruker ER 200D spectrometer (Athens) or a Varian E-109 spectrometer (Portland) equipped with an Air Products Ltd liquid helium transfer cryostat. Experiments were performed on diluted samples (0.8 mg Chl/mL) from the same batch used in the Mössbauer spectroscopy. Similar conditions were used in the Mössbauer experiments except that the DCPIP and sodium ascorbate concentrations were 2 times higher.

Results

Maximum photoreduction of F_X^- was obtained by simultaneously illuminating and freezing the photosystem I core protein in the presence of 1 mM DCPIP and 10 mM sodium ascorbate (Figure 1, solid line). The ESR signal of F_X^- produced as described was undiminished following storage at liquid nitrogen temperature in darkness for 45 h (Figure 1, dotted line), the length of a typical Mössbauer experiment. The slight variability in line shape and peak position, as well as the somewhat larger signal amplitude, may be due to changes in experimental conditions between the two runs. Variability in the ESR spectrum of F_X has been documented (Parrett et al., 1989). Because of this effect, and because the line shape of F_X is inherently broad, we observed no differences

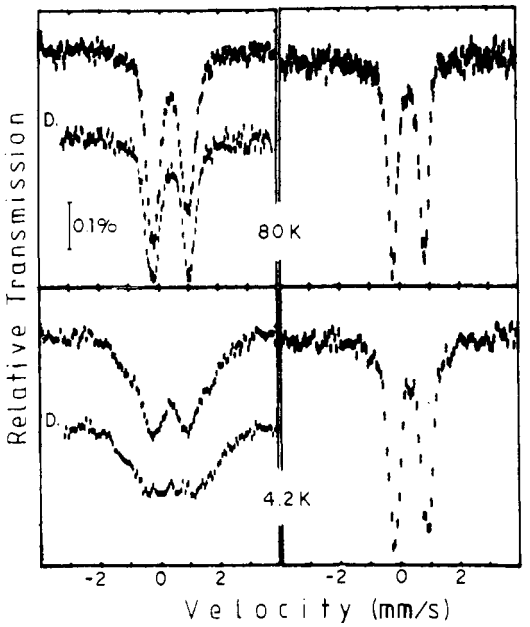

FIGURE 2: Mössbauer spectra of the F_X center in the photosystem I core protein. (Left) Spectra following F_X reduction. (Right) Spectra with F_X in the oxidized state obtained after subsequent dark adaptation at −8 °C for 10 min. Spectra labeled D are those on the left after subtraction of 15% of the spectra on the right. All measurements were obtained in the presence of a 1-kG magnetic field applied perpendicular to the γ-rays.

Table I: Mössbauer Parameters at 77 K of the F_X Center in the PSI Core Preparations

	IS (mm/s)[a]	QS (mm/s)
oxidized	0.43	1.06
reduced	0.55[b]	1.21

[a] With respect to metallic iron at room temperature. [b] Conservative estimate (see text).

in the ESR spectrum when compared with that of unenriched material. A few minutes of dark adaptation at −10 °C of the reduced sample resulted in complete elimination of the F_X^- signal (Figure 1, dashed line).

The Mössbauer spectra of the photosystem I core protein treated under conditions that result in the reduction of F_X are shown in Figure 2, left. The spectra following 10-min dark adaptation of the reduced sample at −8 °C (resulting in the oxidation of F_X) are shown in Figure 2, right; the latter spectra are identical with those of the untreated sample. The parameters of these spectra obtained by computer simulation with Lorentzian lines are given in Table I. The spectrum of the reduced sample at 80 K (Figure 2, left) is broad and asymmetric, probably due to the superposition of two closely spaced doublets in addition, perhaps, to remaining, nonreduced minority contributions. The spectrum of the same sample at 4.2 K, also in an applied field of 1 kG perpendicular to the X-rays (Figure 2, lower left), exhibits broadening and magnetic splitting limited between −2 and +3 mm/s. No notable differences were observed when the applied field was increased to 9 kG (spectrum not shown).

The spectrum of the reduced sample at 4.2 K (Figure 2, lower left) appears to have a contribution from the oxidized spectrum (Figure 2, right); this probably represents a minority of centers with F_X in the oxidized state. We have accordingly made attempts in subtracting part of the oxidized spectrum from the reduced spectrum at both 4.2 and 80 K; an upper limit of 25% in the contribution of the oxidized component could be placed on the basis of quantitative examination of the spectral shape; however, subtraction of 15% of the oxidized spectrum would result in the most reasonable shape of the spectra. The difference spectra in the latter case are labeled D in Figure 2.

The uncertainty in the amount of the nonreduced contribution does not alter the qualitative interpretation of the spectrum at 4.2 K. However, the apparent IS at 80 K increases with increasing amounts of subtracted oxidized component. At zero subtraction the IS is 0.52 mm/s, at 15% subtraction it is 0.55 mm/s, and at 25% subtraction it becomes 0.58 mm/s; this uncertainty does not affect the main conclusion in the next section.

In addition to the above, weak contributions could be observed outside the velocity range of Figure 2, at 5.2 and 4.8 mm/s—spectra were not recorded outside the range −7 to +7 mm/s—which were light insensitive. These apparently are part of a magnetic spectrum of impurity iron, having negligible contributions in the velocity range of Figure 2.

Discussion

A relatively large number of iron–sulfur proteins have been studied by spectroscopic techniques over the past 15–20 years. The class of proteins which are of interest for comparison with the F_X cluster are those having an even number of iron atoms, i.e., proteins with [2Fe-2S], [4Fe-4S], or 2[4Fe-4S] clusters. It is known that [2Fe-2S] clusters can be distinguished from [4Fe-4S] clusters on the basis of their Mössbauer properties. Unlike the [2Fe-2S] clusters, the [4Fe-4S] clusters are characterized by extensive charge delocalization which makes the individual iron atoms least distinguishable. More significantly, the average IS value for the oxidized state of the [4Fe-4S] clusters, 0.42 mm/s (Cammack et al., 1977; Mullinger et al., 1975), reflecting a $4Fe^{2.5+}$ ($2Fe^{2+}$,$2Fe^{3+}$) oxidation state, is significantly higher than the IS value for the [2Fe-2S] clusters, 0.26 mm/s (Cammack et al., 1977; Sands & Dunham, 1975), corresponding to a $2Fe^{3+}$ state. A similar trend in the average isomer shift values is also observed in the reduced states with 0.55 mm/s for the [4Fe-4S] clusters ($4Fe^{2.25+}$) (Cammack et al., 1977) vs 0.43 mm/s for the [2Fe-2S] clusters (Cammack et al., 1977; Sands & Dunham, 1975). Furthermore, in the reduced state, the spectra of [4Fe-4S] clusters at 4.2 K and in small applied magnetic fields are limited in a narrow velocity range (Mullinger et al., 1975) compared with the spectra of [2Fe-2S] clusters which extend over broad velocity limits (Cammack et al., 1977; Sands & Dunham 1975).

Comparison of the Mössbauer parameters of center F_X, see Table I, with those of other iron–sulfur proteins (Cammack et al., 1977) shows that in the oxidized state the isomer shift coincides within experimental error with that of the [4Fe-4S] clusters provided that the majority of the Mössbauer absorption results from this species. The analysis of the Mössbauer spectra in the reduced state indicates that over 75% (most likely 85%) of the iron is reducible under the conditions of the experiment. Under similar conditions the EPR spectra show the presence of only one component and no trace of F_A or F_B contributions which would have been the most likely iron contaminants in these preparations. The X-ray K-edge spectrum (McDermott et al., 1989) confirms the presence of an intact iron–sulfur cluster in the photosystem I core protein and indicates that it represents >80% of the iron present. For the purpose of this discussion, the present preparations can be therefore considered homogeneous.

The spectra in the reduced state indicate significant charge delocalization similar to what is observed in [4Fe-4S] clusters and unlike that in the [2Fe-2S] clusters. There is some un-

certainty in the IS value in the range 0.52 mm/s (no subtraction of oxidized component) to 0.58 mm/s (assuming maximum contribution of oxidized component), but a reasonable estimate is 0.55 mm/s (assuming 15% contribution of the oxidized component), which falls in the middle of the range of the reported values for the [4Fe-4S] clusters.

The spectra in the reduced state at 4.2 K in a small applied field are qualitatively similar to those of the [4Fe-4S] proteins. The spectrum of F_X^- is somewhat more narrow than the spectrum of the reduced [4Fe-4S] ferredoxin from *Bacillus stearothermophillus* (Mullinger et al., 1975), a fact which may be a reflection of a higher degree of electron delocalization in the present case.

The above results are incompatible with the properties of [2Fe-2S] clusters and strongly support the assignment of F_X to a [4Fe-4S] cluster. This identification is in agreement with a recent EXAFS study of F_X performed on the same preparation (McDermott et al., 1989). It might be questioned whether F_X in the photosystem I core protein is altered from its native form in the photosystem I complex. The ESR spectra of F_X in the photosystem I core protein are somewhat broader and shifted in the g values, but they still retain the properties of [4Fe-4S] or [2Fe-2S] clusters; other differences include a slight positive shift of the midpoint potential of F_X and an increased half-time for charge recombination between $P700^+$ and F_X^- [discussed in Parrett et al. (1989)]. However, the ability to restore electron flow between P700 and F_A/F_B after reconstitution of a functional photosystem I complex from the photosystem I core protein and the isolated 8.9-kDa, F_A/F_B polypeptide would indicate that the F_X has not been irreversibly damaged by chaotropic treatment (Golbeck et al., 1988b). Moreover, no conceivable alteration could convert a presumably [2Fe-2S] cluster in the photosystem I complex to a [4Fe-4S] cluster in the photosystem I core protein. We must assume therefore that F_X in the photosystem I core protein is a [4Fe-4S] cluster in its native form. This issue, however, will be the subject of future Mössbauer studies of intact photosystem I preparations.

The above discussion has inherently assumed that the iron atoms in F_X are tetrahedrally coordinated with sulfur ligands. If either of these restrictions is relaxed, the main conclusions of this paper can be invalidated. The identity of the ligands of F_X is indeed an open question; however, there is no precedent for ligands other than sulfur in a low-potential iron–sulfur protein. Assuming that the ligands to the iron atoms are sulfur, then the coordination of the iron is most likely tetrahedral for the following reasons. (1) There is no precedent of a 2Fe– or 4Fe–sulfur protein with other than tetrahedral coordination. (2) No site differentiation is observed in the Mössbauer spectra, which would justify the presence of a mixture of four-coordination and higher coordination sites, as is observed in model compounds [Kanatzidis et al., 1985; see also Golbeck (1987) and McDermott et al. (1989)]. (3) The QS values in Table I would be small for five-coordinated iron atoms, and the isomer shifts are small for six-coordinated iron atoms.

The characterization of iron–sulfur cluster F_X as a [4Fe-4S] cluster has additional consequences relative to the overall structure of the photosystem I reaction center. On the basis of the apparent requirement of four cysteine residues for ligating low-potential [2Fe-2S] and [4Fe-4S] clusters and the number of conserved cysteine residues on the *psaA* and *psaB* polypeptides (three on the former and two on the latter), it is most likely that F_X is a bridged [4Fe-4S] cluster ligated by a pair of homologous cysteine residues from each reaction center polypeptide [see Golbeck (1987)]. A heterodimer composed of the *psaA* and *psaB* polypeptides, therefore, constitutes a necessary and sufficient structure for the photosystem I core protein.

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