Low-Potential Iron–Sulfur Centers in Photosystem I: An X-ray Absorption Spectroscopy Study†

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ABSTRACT: We have measured the X-ray absorption spectra of Fe in photosystem I (PS I) preparations from spinach and a thermophilic cyanobacterium, *Synechococcus* sp., to characterize structures of the Fe complexes that function as electron acceptors in PS I. These acceptors include centers A and B, which are probably typical [4Fe-4S] ferredoxins, and X. The structure of X is not known, but its electron paramagnetic resonance (EPR) spectrum has generated the suggestions that it is either a [2Fe-2S] or [4Fe-4S] ferredoxin or an Fe–quinone species. The iron X-ray absorption K-edge and iron extended X-ray absorption fine structure (EXAFS) spectra reveal that essentially all of the 11–14 Fe atoms present in the reaction center are present in the form of Fe–S centers and that not more than 1 atom out of 12 could be octahedral or oxygen-coordinated Fe. This suggests that, besides A and B, additional Fe–S clusters are present which are likely to be X. Our EXAFS spectra cannot be simulated adequately by a mixture of [4Fe-4S] ferredoxins with typical bond lengths and disorder parameters because the amplitude of Fe backscattering is small; however, excellent simulations of the data are consistent with a mixture of [2Fe-2S] ferredoxins and [4Fe-4S] ferredoxins, or with unusually distorted [4Fe-4S] clusters. We presume that the [2Fe-2S] or distorted [4Fe-4S] centers are X. The X-ray absorption spectra of PS I preparations from *Synechococcus* and spinach are essentially indistinguishable.

Photosystem I (PS I),[1] found in green plants, algae, and cyanobacteria, catalyzes light-driven electron-transfer reactions from weak oxidants to electron acceptors that are among the strongest reductants known in biological systems. An excited electronic state of a Chl species in PS I called P700 produces the reducing equivalents which are ultimately used to reduce CO_2 and form carbohydrates. The electron-transfer reactions from P700 to the substrates are initially mediated by a number of PS I bound electron acceptors, including A_0, which is thought to be a chlorophyll species, A_1, which may be a phylloquinone, two ferredoxins, called centers A and B, and another species called X which is probably also a ferredoxin [for recent reviews, see Evans (1982) and Rutherford and Heathcote (1985)]. The path of electron transfer among these acceptors is not well understood. Subsequent to the involvement of the membrane-bound acceptors, several soluble mediators are involved, including a ferredoxin and $NADP^+$. The midpoint potentials (E_m, pH 10, vs SHE) of the acceptors in PS I are −530 mV for center A, −590 mV for center B, −705 mV for X, and presumably lower for A_0 and A_1 (Ke et al., 1973; Evans et al., 1974; Chamorovsky & Cammack, 1982). In the hydrophobic environment of the membrane protein complex these electron mediators can accumulate reducing equivalents without reducing water or oxygen.

The EPR spectra of centers A and B strongly resemble those of the [2Fe-2S], [4Fe-4S], and [8Fe-8S] ferredoxins, having anisotropic signals with $g_{av} < 2$. Mössbauer spectra suggest that both center A and center B are [4Fe-4S] ferredoxins (Evans et al., 1979). Recently, an 8-kDa protein in PS I has been identified that has a significant homology to the [8Fe-8S] bacterial ferredoxins (Oh-oka et al., 1987). This protein is presumably the binding site of centers A and B, and its sequence provides strong evidence that centers A and B are a [8Fe-8S] ferredoxin. Centers A and B are both found in a 1:1 stoichiometry with respect to P700 (Bearden & Malkin, 1972; Williams-Smith et al., 1978; Heathcote et al., 1978).

The structure of X is less clear. The EPR spectrum of X is similar to those of [2Fe-2S] or [4Fe-4S] ferredoxins, but *not* [1Fe], [3Fe-3S], or [3Fe-4S] ferredoxins. Like [2Fe-2S] and [4Fe-4S] ferredoxins, X has a low midpoint potential and is paramagnetic in the reduced state. Bolton (1977) has suggested that X may be an Fe–quinone species (Fe–Q) by analogy with the Fe–Q acceptor species in the reaction centers of purple nonsulfur bacteria, on the basis of the observation that its EPR spectrum is somewhat broader than those of the [2Fe-2S] and [4Fe-4S] ferredoxins. This suggestion was supported by EPR power saturation studies by Rupp et al.

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[1] Abbreviations: Chl, chlorophyll; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; Fd, ferredoxin; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; PMS, *N*-methylphenazonium methosulfate; PMSF, phenylmethanesulfonyl fluoride; PS I, photosystem I; PS II, photosystem II; P700, photosystem I reaction center chlorophyll; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SHE, standard hydrogen electrode; Tris, tris(hydroxymethyl)aminomethane.

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(1979). Two phylloquinones per P700 are found in PS I reaction centers (Takahashi et al., 1985; Malkin, 1986; Schoeder & Lockau, 1986). It has been suggested that these phylloquinones are associated with A_1 (Thurnauer et al., 1987; Brettel et al., 1986; Mansfield & Evans, 1986); however, this assignment is currently disputed (Palace et al., 1987; Ziegler et al., 1987). On the other hand, Mössbauer spectra of PS I preparations have been interpreted to suggest that X is a [4Fe-4S] ferredoxin (Evans et al., 1981). Concomitant with the reduction of A, B, and X, 65% of the Fe in the Mössbauer spectrum of PS I changed in a way that is consistent with an assignment as a [4Fe-4S] ferredoxin but not a [2Fe-2S] ferredoxin, while 35% of the Fe did not change. Center X is difficult to reduce quantitatively, and the portion of Fe which was not reduced may include X. This possibility was addressed by the authors, and a model involving two distinct X species per P700 was subsequently suggested (Evans et al., 1981; Bonnerjea & Evans, 1984). However, the proposal of four [4Fe-4S] centers per P700 is inconsistent with the stoichiometry of 10–14 Fe atoms and 10–13 acid-labile sulfides per P700 (Golbeck, 1980; Lundell et al., 1985).

Extended X-ray absorption fine structure (EXAFS) is capable of discriminating between [4Fe-4S] clusters, [2Fe-2S] clusters, and six-coordinate Fe with oxygen and nitrogen ligands such as is found in the Fe–Q acceptor complex of bacterial reaction centers. A variety of Fe–S inorganic complexes and proteins have been studied by EXAFS spectroscopy (Teo et al., 1979). In [2Fe-2S] and [4Fe-4S] ferredoxins, the Fe has four S neighbors at about 2.25 Å. The EXAFS spectra of these compounds have a frequency component corresponding to the four S atoms, and this component is very similar in [2Fe-2S] ferredoxins and [4Fe-4S] ferredoxins. In both [2Fe-2S] ferredoxins and [4Fe-4S] ferredoxins every Fe atom also has an Fe neighbor(s) at about 2.74 Å; in the [2Fe-2S] ferredoxins each Fe has only one Fe neighbor, whereas in the [4Fe-4S] ferredoxins each has three. The difference in the number of Fe neighbors for the two clusters gives rise to a difference in the amplitude of the Fe backscattering and has a pronounced effect on the oscillation patterns. No scattering atoms other than Fe and S contribute significantly to the Fe EXAFS spectrum of Fe–S centers. EXAFS studies of the Fe–Q acceptor complex in bacterial reaction centers demonstrated that the Fe is coordinated by six ligands at 2.1 Å which are a mixture of oxygen and nitrogen (Bunker et al., 1982; Eisenberger et al., 1982). These conclusions were confirmed by the X-ray crystal structure of the bacterial reaction center which indicated that the Fe has two oxygen ligands from a glutamate and four nitrogen ligands from histidines (Deisenhofer et al., 1985). Such a structure is readily distinguished from an Fe–S center by EXAFS.

The structure of the absorption spectrum near the X-ray absorption edge also gives information about the structures of metal sites. The probabilities for the bound-state transitions depend on ligand field symmetry and ligand types via the mixing of the hydrogenic one-electron orbitals. For example, the 1s to 3d transition, which is formally unallowed, becomes prominent in complexes with noncentrosymmetric geometries because of p–d mixing and is systematically larger in tetrahedral complexes than in octahedral complexes (Roe et al., 1984; Srivastava & Nigam, 1972). While the K-edge spectra of [2Fe-2S] and [4Fe-4S] complexes are quite similar, the K-edge spectrum may be used to discriminate between Fe–S complexes, an Fe–Q complex, or a mixture of the two.

In this paper we present the X-ray absorption spectra of PS I preparations containing A, B, and X. These spectra are used to address whether X may be an Fe–Q, a symmetric [4Fe-4S] cluster, or one or more [2Fe-2S] clusters.

Experimental Section

Preparation of PS I from Spinach. Procedures were developed for purifying large quantities of PS I from spinach and *Synechococcus*. The preparation from spinach is similar to a previous PS I preparation (Ke et al., 1975), and the preparation from *Synechococcus* is somewhat similar to another preparation (Nechushtai et al., 1983). Unless otherwise specified, the procedures were carried out near 4 °C. Chloroplasts were prepared by grinding deveined market spinach leaves in a buffer containing 0.4 M sucrose, 50 mM Tris, pH 7.5, 20 mM NaCl, 5 mM EDTA, and 5 μM PMSF, filtering through eight layers of cheesecloth and two to four layers of Miracloth, and then centrifuging the chloroplast filtrate at 5000*g* for 6 min. The chloroplasts were resuspended in the same buffer, filtered again through Miracloth, and again centrifuged. The chloroplasts were washed free of ribulosebisphosphate carboxylase by suspension at 0.1 mg of Chl mL^{-1} in 10 mM sodium pyrophosphate, pH 7.5, with 1 mM EDTA and 5 μM PMSF and pelleting at 10000*g* for 10 min. This wash was performed twice. The membranes were then washed to remove coupling factor by suspension at 0.2 mg of Chl mL^{-1} in 2 M NaBr, 20 mM Tris, pH 7.5, 1 mM EDTA, and 5 μM PMSF, incubation for 20–30 min, dilution to twice the volume with water, and pelleting at 11000*g* for 15 min. The thylakoids were resuspended in a buffer containing 5 mM $MgCl_2$, 20 mM Tris, pH 7.5, and 5 μM PMSF, at roughly 1 mg of Chl mL^{-1}, and incubated for 30–60 min to ensure stacking. The thylakoids were pelleted (10000*g*, 10 min) and resuspended in the same buffer to remove excess bromide. Triton X-100 (Sigma) was added to a concentration of 5 mg of Triton (mg of Chl)$^{-1}$. PS II and most of the PS I were then pelleted by centrifugation at 40000*g* for 30 min. The supernatant was discarded. It contained most of the cytochrome b_6/f complex and roughly 10% of the PS I. The pellet was resuspended in the same buffer again at about 1 mg of Chl mL^{-1}, and 20 mg of Triton (mg of Chl)$^{-1}$ was added. This mixture was again centrifuged at 40000*g* for 45 min to separate PS I and PS II. The supernatant of this spin contained a crude preparation of PS I.

PS I was then purified on sucrose density gradients. Roughly 5 mg of Triton X-100 (mg of Chl)$^{-1}$ was added to the supernatant, and the suspension was centrifuged at 160000*g* for 8 h to concentrate PS I. The pellet typically contained 100–130 Chl per P700. This pellet was resuspended to a concentration of approximately 1 mg of Chl mL^{-1}. Digitonin and lauryl maltoside [1% (w/v) each] were added. Any remaining clumps were removed by centrifugation at 30000*g* for 15 min. The suspension (1.5–3 mL) was layered over a 30-mL 6–22% sucrose gradient. The gradients were made in a buffer containing 0.2% lauryl maltoside and 10 mM Tris, pH 7.5. The gradients were centrifuged at 27000 rpm in an SW28 rotor for 18–30 h. After centrifugation, two major green bands could be seen which contained PS I (the lower band) and the dissociated PS I antenna proteins with trace amounts of PS II nearby (the upper band). The upper band was highly fluorescent, while the lower band was not fluorescent to the eye. The lower band was siphoned in 1–2-mL fractions. Those tubes with Chl to P700 ratios less than about 50 and those lacking cytochromes and antenna proteins (as determined by peptide analysis) were pooled and saved. The preparation contained four obvious bands by SDS–PAGE: a band at 60 kDa which includes P700 binding proteins and

three peptides between 16 and 22 kDa.

Preparation of PS I from Thermophilic Synechococcus. Thermophilic *Synechococcus*, a generous gift of Prof. S. Katoh, University of Tokyo, was grown on a medium similar to that described by Dyer and Gafford (1961) except that the Fe and Cu concentrations were 75 μM. The cells were collected in a Sharples continuous-flow centrifuge, then washed in a medium containing 50 mM Hepes, pH 7.5, 10 mM NaCl, and 1 mM PMSF, and pelleted at 16000g for 12 min. The cells were resuspended (rejecting bacteria and dead cells) in 0.4 M mannitol, 5 mM EDTA, 50 mM Hepes, pH 7.5, and 1 mM PMSF. Lysozyme was added to approximately 3 mg (g of cells)$^{-1}$. After 30-min incubation at 37 °C, the cells were pelleted at 39000g for 15 min. They were resuspended in approximately 100 mL/30 g of cells in a medium containing 50 mM MES, pH 6.2, 10 mM NaCl, and 1 mM PMSF and broken with a French pressure cell operating at 435 atm. Approximately 0.5 mg of DNase was added to the solution, $MgCl_2$ was added to make 5 mM, and the solution was stirred over ice in the dark for 40 min. EDTA was added to make 10 mM. Unbroken cells were spun at 12000g for 15 min and discarded. Thylakoid fragments were then centrifuged from the supernatant at 300000g for 30 min. The resulting pellet was resuspended in 0.5 M sucrose with 50 mM MES, pH 6.2, and 5 mM NaCl (medium A) and stored at −80 °C. PS II was extracted with approximately 0.65% octyl β-glucoside at 1 mg of Chl mL^{-1} in medium A. The extracted thylakoids were then centrifuged at 300000g for 1 h. The thylakoid pellet was resuspended to 2.5 mg of Chl mL^{-1} in 50 mM Tris, pH 7.5, and 10 mM NaCl (medium B) with 1% Triton X-100, and the washed thylakoids were collected in a pellet by centrifugation as in the previous step. The PS I was then extracted by resuspending the pellet to 1–2.5 mg of Chl mL^{-1} in medium B, adding 20 mg of Triton X-100 (mL of Chl)$^{-1}$, and then centrifuging the insoluble material from solution at 40000g for 30 min. The supernatant was then purified on sucrose density gradients by the same procedure as for spinach PS I.

Characterization of the PS I Preparation. Chl concentrations were determined by extraction into 80% acetone, centrifugation to remove protein, and measurement of the optical absorbance as described by Arnon (1949).

The analysis for P700 was done by monitoring reversible photobleaching. The sample was diluted to between 5 and 30 μM Chl in a medium containing 20 mM Tris, pH 7.5, 0.1% Triton X-100, 0.5 μM PMS, 10 μM methyl viologen, and 0.1 mM sodium ascorbate. The sample was illuminated in a Cary 14 or an Aminco DW-2 spectrophotometer at right angles to the measuring beam with a tungsten filament lamp, the light from which was filtered through a water (IR) filter and a 400–560-nm band-pass filter and was focused on the sample. A 700-nm interference filter was placed in front of the photomultiplier tube detector to prevent light leaks from the side illuminator to the photomultiplier tube. The bleaching at 700 nm was measured under saturating actinic illumination. The extinction coefficient determined by Hiyama and Ke (1972), 64 mM^{-1} cm^{-1} at 700 nm, was then used to calculate the concentration of P700.

The preparations were analyzed for Fe content by atomic absorption. The sample was mixed with twice its volume of Ultrex 70% nitric acid and digested by boiling until it became clear (approximately 10 min). The samples were diluted in distilled water and analyzed with a Perkin-Elmer Model 303 flameless graphite tube atomic absorption spectrometer. Typical final Fe concentrations ranged from 0.5 to 5 μM. The samples were compared with commercially available Fe standards diluted in a similar medium.

Analysis for acid-labile sulfide was carried out by a modification of the method of Fogo and Popowski (1949) similar to that described by Golbeck and San Pietro (1976). The sample was taken into cold 80% acetone under nitrogen in a sealed microfuge tube. The tubes were then centrifuged at about 20000g for 10 min to separate the denatured protein pellet from the Chl extract. The pellet was then rinsed under nitrogen with cold 80% acetone. The pellet was analyzed by the method of Fogo and Popowski (1949); the methylene blue product was partitioned quantitatively into hexanol, and its concentration was monitored at 660 nm.

Peptide analysis by SDS–PAGE was done according to Laemmli (1970), except that slab gels were used and the separating gels were 15% acrylamide. When the PS I preparation from *Synechococcus* was analyzed, 6 M urea was included in the gels and samples.

PS I samples were reduced in a degassed 0.1–0.2 M glycine buffer at pH 10 with 10 mM dithionite and 10 μM each of phenazine methosulfate and methyl viologen. The potential under these conditions was −0.62 V vs SHE when measured with a Pt electrode and a Ag/AgCl reference. The sample was then frozen in liquid nitrogen while being illuminated by a focused 400-W tungsten filament lamp through a water filter.

For X-ray absorption experiments, PS I pellets were placed in lucite holders in which EPR, illumination, and X-ray absorption measurements could be made. Typical Fe concentrations were 5 mM for PS I as well as for the reference ferredoxins. The inorganic complexes were ground with approximately a 50-fold excess of $LiBF_4$ by weight in a dry oxygen-free environment and placed in a sealed container for X-ray absorption experiments.

Low-temperature EPR spectra were obtained on a Varian E-109 spectrometer operating at X-band using a TE-102 cavity with 100-kHz field modulation. An Air-Products Helitran cryostat was used to cool the sample to between 6 and 11 K.

EXAFS and Edge Data Collection. The EXAFS and X-ray edge spectra reported here were collected at the Stanford Synchrotron Radiation Laboratory on unfocused "wiggler" beam lines VII-3 and IV-1. Si⟨111⟩ monochromator crystals provided by SSRL were used. Electron energies were 3.0 GeV, and ring currents were between 20 and 90 mA. The EXAFS and edge data are fluorescence excitation spectra (Jaklevic et al., 1977), collected with a NE104 plastic scintillator array similar to that described by Powers et al. (1981). Between the sample and the detector, Mn filters and Soller slits were used to select for fluorescent photons from Fe (Stern & Heald, 1979). The signal from each photomultiplier was processed by a separate constant fraction discriminator. The photomultiplier counting rates were roughtly 10^4 to 5×10^4 s^{-1}; at these counting rates the response of the detector assembly is linear. An absorption spectrum of $K_3Fe(CN)_6$ was simultaneously monitored, and the sharp feature at 7130.1 eV was used as an energy calibration marker.

The total data collection time was about 2–3 h for each sample. The samples were cooled to −85 to −100 °C during data collection to prevent X-ray damage. All protein samples were analyzed for structural integrity both before and after beam exposure. Chl to P700 ratios and EPR spectra were used to characterize PS I; the preparations were not degraded by beam exposure within our accuracy. Assays for Fe and acid-labile sulfide, UV/vis spectra, and EPR were used to characterize the soluble spinach [2Fe-2S] ferredoxin and soluble *Clostridium pasteurianum* [4Fe-4S] ferredoxin. In

the soluble spinach [2Fe-2S] ferredoxin, greater than 94% of the Fe was in intact Fe–S clusters, and in the soluble *C. pasteurianum* [4Fe-4S] ferredoxin, approximately 83% of the Fe was in intact cores before and after beam exposure (as indicated by UV/vis spectra and Fe analysis).

EXAFS Analysis. For each spectrum, the X-ray fluorescence was normalized point by point to the incident beam intensity. The data were weighted by the incoming intensity and the collection time, and individual scans were added to improve the signal-to-noise ratio. After addition, the preedge background was fit to a linear function and subtracted, the scan was normalized with the edge height defined as unity, and then the data were divided by the free atom contribution (Teo & Lee, 1979). The scattering contribution, the effects of the manganese filters, and various other background components were subtracted. Since these generally cannot be calculated analytically, we subtracted a second- or third-order polynomial function that approximates the background curvature and then subtracted a running average of the data, averaged over a broad range (greater than 100 eV or 2 $Å^{-1}$) so that background features but not EXAFS oscillations were removed. The validity of the separation of background from EXAFS was checked by analysis of spectra of Fe-free buffers and of reference compounds.

The data were then converted from a function of incoming X-ray energy E to a function of outgoing photoelectron wave vector k with a threshold energy for electron escape E_0 of 7130 eV. This value for E_0 was chosen by varying E_0 while monitoring the quality of simulations. Only the region between 3.5 and 12.0 $Å^{-1}$ was analyzed. Below this region there are contributions from physical processes other than EXAFS, and eq 1 is not valid. Above this region the signal-to-noise ratio deteriorates. The data were then weighted by k^3 to emphasize the backscattering of the Fe shell, which peaks at higher k values than the S backscattering. Finally, we Fourier filtered the data before fitting, using a window that is wide compared to the obvious Fourier components ($R + \Delta = 0.5$–3.5 Å). This filtering, like the smooth background removal, can greatly improve the convergence of the fitting procedure.

The Fourier filtered, k^3-weighted data were then fit with parameters determined by Teo and Lee (1979) according to eq 1. In this equation, χ is the normalized EXAFS spectrum

$$\chi(k) = \frac{\mu(k) - \mu_s(k)}{\mu_0} = S_f \sum_j N_j |f_j(\pi,k)| \frac{\sin [2kR_j + \alpha_j(k)]}{kR_j^2} e^{-2\sigma_j^2 k^2} e^{-2R_j/\lambda(k)} \quad (1)$$

as a function of k, the photoelectron wavevector; μ is the measured X-ray absorption; μ_s is the smooth background in the absorption; μ_0 is the absorption of the free atom; S_f is a chemically independent scaling factor for the scattering amplitudes which is empirically derived; N_j is the number of atoms in the jth scattering atom group; $f_j(\pi,k)$ is the calculated backscattering amplitude for the jth scattering neighbors; R_j is the distance from the absorbing atom to the jth scattering atom(s); α_j is the photoelectron wave phase shift due to interactions with the absorbing and scattering atoms; σ_j, the Debye–Waller factor, is the root mean square deviation in the absorber/scatterer bond distance; and λ is the photoelectron mean free path.

The fits incorporated two or three types of ligands, with four adjustable parameters per ligand: the bond distance R; the number of atoms N; the Debye–Waller factor σ; and a parameter, ΔE_0, which shifts E_0 to match the phases of the experimental and calculated backscattering waves. In addition, the chemical identity of the ligand is specified, which determines the functional form of $\alpha_j(k)$ and $f_j(\pi,k)$. The Debye–Waller factor was chosen by comparison with models and by the quality of fit, as described under Discussion, and ΔE_0 was varied within the range +10 to −10 eV. The calculated spectrum was multiplied by a scaling factor, S_f, as has been previously described (Teo & Lee, 1979). EXAFS spectra of reference compounds containing [2Fe-2S] and [4Fe-4S] clusters and soluble spinach [2Fe-2S] ferredoxin were also analyzed to check the validity of the fits and to determine the scaling factor, S_f, for the EXAFS amplitudes.

Other details of the data analysis methods are described elsewhere (Goodin, 1983; Kirby, 1981).

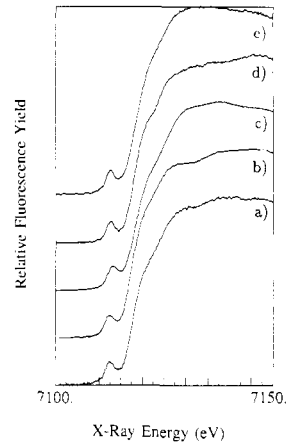

FIGURE 1: Iron X-ray absorption K-edge spectra of oxidized PS I and oxidized iron–sulfur compounds. From the bottom: (a) PS I from spinach, (b) PS I from *Synechococcus*, (c) $(Et_4N)_2Fe_4S_4(S\text{-benzyl})_4$, (d) spinach $[2Fe\text{-}2S]^{2+(2+,1+)}$ ferredoxin, and (e) $(Et_4N)_2Fe_2(S_2\text{-}o\text{-xyl})_2$. A linear background has been removed from all spectra. The preedge feature at approximately 7112 eV has been assigned as a 1s–3d transition and is a good indicator of noncentrosymmetric environments. The PS I acceptors from spinach and *Synechococcus* are quite similar to each other and to the soluble spinach [2Fe-2S] ferredoxin.

RESULTS

The PS I preparations from spinach and *Synechococcus* are very similar in terms of chemical and spectroscopic properties. Both have 35–60 Chl molecules, 11–14 Fe atoms, and 9–12 acid-labile S atoms per P700, in agreement with other determinations (Golbeck, 1980; Lundell et al., 1985). They have the following predominant polypeptides: 60-kDa subunits which are known to bind P700 and most of the Chl and three lower molecular weight peptides at approximately 18, 19, and 22 kDa. These PS I preparations have no EPR-detectable cytochrome, soluble spinach [2Fe-2S] ferredoxin, or Rieske Fe–S center and very little $g = 4.3$ Fe (much less than one atom per reaction center). The EPR spectra of both PS I preparations had signals due to centers A, B, and X similar to those previously reported (Evans, 1982). Samples prepared from the two organisms showed no significant differences in g values or line widths of the EPR signals.

Iron X-ray absorption K-edge spectra of PS I preparations in which A, B, and X are oxidized are shown in Figure 1, along with edge spectra for three reference compounds: $(Et_4N)_2Fe_4S_4(S\text{-benzyl})_4$, $(Et_4N)_2Fe_2S_2(S_2\text{-}o\text{-xyl})_2$, and spinach $[2Fe\text{-}2S]^{2+(1+,2+)}$ ferredoxin (Nomenclature Committee of the International Union of Biochemistry, 1979). Note that the shapes of the PS I edges and the edge positions are remarkably similar to that of the soluble spinach [2Fe-2S] ferredoxin, and are generally similar to all of the Fe–S spectra. In particular, the 1s to 3d transition in the preedge region is at least one-tenth of the total edge height in all cases. The

Table I: Bond Distances and Coordination Numbers of Iron–Sulfur Compounds, Proteins, and PS I[a]

sample	R_{Fe-S} (Å)	N_S	σ_{Fe-S} (Å)	R_{Fe-Fe} (Å)	N_{Fe}	σ_{Fe-Fe} (Å)
spinach [2Fe-2S] Fd	2.26	4.1	0.07	2.74	0.9	0.05
$(Et_4N)_2Fe_4S_4(S\text{-benzyl})_4$	2.25	4.0	0.08	2.74	2.9	0.08
C. pasteurianum Fd	2.23	4.0	0.08	2.75	2.8	0.09
PS I from spinach	2.27	3.9	0.07	2.78	1.9	0.08
PS I from *Synechococcus*	2.26	4.6	0.07	2.76	2.1	0.07

[a] Bond distances and Debye–Waller parameters determined by the Teo–Lee method (Teo & Lee, 1979). Error in the determination of R is 0.03 Å, and that in σ is 0.02 Å. The error in N is approximately 20% in all cases except for *C. pasturianum* ferredoxin for which it is 30%.

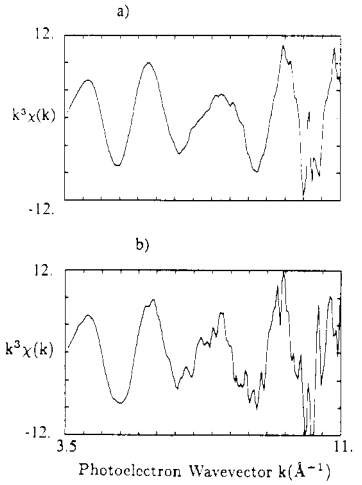

FIGURE 2: EXAFS spectra of PS I preparations, with background removed and weighted by k^3. The top spectrum (a) is PS I from *Synechococcus*; the bottom spectrum (b) is PS I from spinach. The spectra clearly contain more than one Fourier component.

data from *Synechococcus* and spinach PS I are also remarkably similar to one another. In our hands, the K-edge spectra of reduced ferredoxins and Fe–S clusters were not substantially different from those of the oxidized clusters when the reduction was conducted anaerobically (data not shown). The edge position shifted by a small amount (<0.5 eV) to lower energy, but no distinguishable changes were observed in edge shape. When the ferredoxins were irreversibly destroyed by oxidative damage, we saw dramatic changes in edge shape similar to those previously attributed to reduction (Teo & Shulman, 1982).

The k^3-weighted Fe EXAFS spectra of PS I preparations containing A, B, and X in the oxidized state are shown in Figure 2. The envelope and "beat" pattern clearly indicate the presence of more than one frequency component in the data. The modulus and the real part of the Fourier transforms of these spectra are shown in Figure 3. The Fourier transformed EXAFS spectra of PS I provide evidence for the presence of two classes of neighbor atoms with peak positions (or bond lengths) characteristic of Fe–S clusters. Curve fitting of the EXAFS spectra indicated that the closest neighbor is likely to be S and the other neighbor is Fe. Although EXAFS simulations cannot distinguish between Fe and some other transition metals, we have analyzed our PS I preparation for all other transition metals necessary for growth (Mo, Mn, Zn, Cu, and Co) by atomic absorption analysis and found less than 0.4 atom per P700 in all cases. Table I lists the Fe–S and Fe–Fe bond lengths, the number of neighbors, and the Debye–Waller parameters determined by EXAFS simulations. The bond lengths for the PS I preparations are nearly identical with those we determined for the [2Fe-2S] and [4Fe-4S] ferredoxins and the inorganic clusters. The data do not indicate any additional neighbors other than the Fe and S. In particular we found no evidence for first-shell nitrogen or oxygen ligands. There are no significant differences between

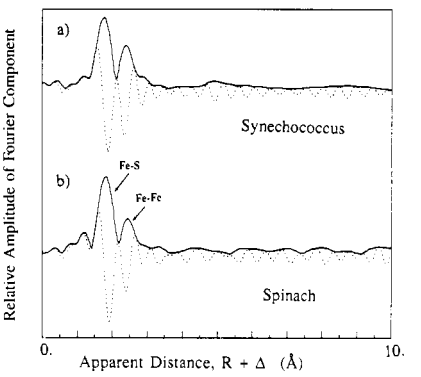

FIGURE 3: Fourier transformed EXAFS spectra of PS I: (a) from *Synechococcus* (top) and (b) from spinach (bottom). (Solid line) Modulus; (dotted line) real component. The first peak is due to backscattering from sulfur at 2.27 Å, and the second is due to backscattering from iron at 2.75 Å. The features appear at an apparent distance which is shorter than the actual distance by an amount, Δ, which may be approximated as $(1/2)/\langle \delta\alpha/\delta k \rangle$.

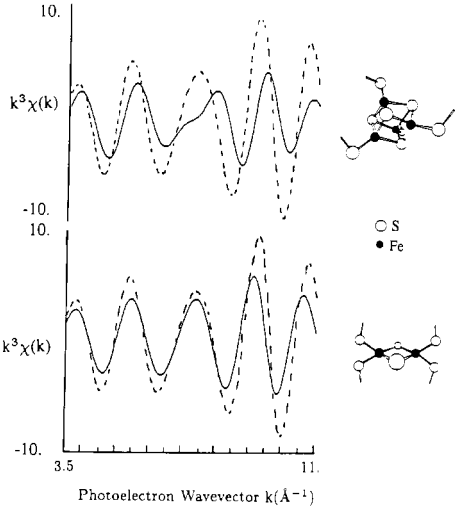

FIGURE 4: The Fourier filtered k^3-weighted spectrum of spinach PS I preparation (dotted spectrum) is plotted with the spectra of two reference samples (solid lines). The solid spectrum in the top panel is of $(Et_4N)_2Fe_4S_4(S\text{-benzyl})_4$, and that in the bottom panel is of spinach $[2Fe\text{-}2S]^{2+(1+,2+)}$ ferredoxin. The beat region around k = 7.5 Å^{-1} is more pronounced for [4Fe-4S] clusters than for [2Fe-2S] clusters, because the amplitude of the Fe backscattering is much greater for [4Fe-4S] clusters. Structures of [2Fe-2S] and [4Fe-4S] clusters are shown next to their respective EXAFS spectra; the terminal S ligands are thiolates or cysteine residues for the inorganic complexes and the ferredoxins, respectively. The beat region indicates that PS I is likely to contain some [2Fe-2S] ferredoxins.

the *Synechococcus* and the spinach PS I EXAFS spectra. There were also no significant differences between oxidized and reduced *Synechococcus* PS I EXAFS spectra (data not shown).

Figure 4 shows the Fourier-filtered spinach PS I EXAFS data plotted over data from soluble spinach [2Fe-2S] ferredoxin and data from $(Et_4N)_2Fe_4S_4(S\text{-benzyl})_4$. In the region of k = 7.5 Å^{-1} the [4Fe-4S] reference compound spectra differ

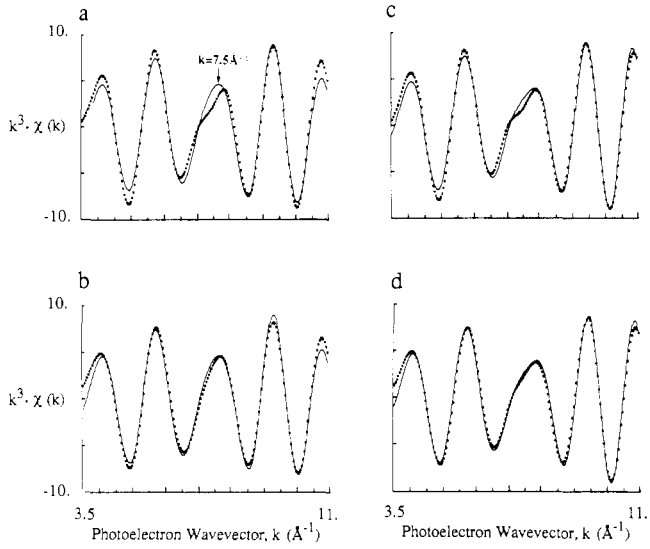

FIGURE 5: Fourier filtered, k^3-weighted EXAFS spectra of Fe in PS I (solid lines), and calculated simulations of the spectra (dotted). The fits are as follows: (a) fit of spinach data assuming all [4Fe-4S] ferredoxins, (b) fit of spinach data assuming an equal number of [2Fe-2S] and [4Fe-4S] ferredoxins, (c) fit of *Synechococcus* data assuming all [4Fe-4S] ferredoxins, and (d) a fit of *Synechococcus* data assuming an equal number of [2Fe-2S] and [4Fe-4S] ferredoxins. Note that the quality of fit in the beat region, where the data are quite sensitive to the ratio of Fe and S backscattering, indicates that PS I is likely to contain [2Fe-2S] as well as [4Fe-4S] ferredoxins. However, other Fe–S complexes in which there is one Fe neighbor or very disordered Fe neighbors at 2.7–2.8 Å would also be consistent with these EXAFS data.

from the PS I spectra. Around k = 7.5 Å^{-1} the dimer and tetramer oscillations are far out of phase, and the phase of the PS I spectrum resembles that of the dimer. Both the [2Fe-2S] and the [4Fe-4S] reference spectra appear to be damped relative to the PS I spectra; this may be due to a small amount of contaminating oxygen-coordinated Fe, larger Debye–Waller factors, self-absorption affects, or some combination of all of these. However, simulations are able to retrieve accurate numbers of scatterers for the ferredoxins (Table I). Curve fitting analysis demonstrates that the spectrum is better explained by a sum of [2Fe-2S] ferredoxins and [4Fe-4S] ferredoxins than by a sum of all [4Fe-4S] ferredoxins. The best fits of the PS I data involved two Fe neighbors and Debye–Waller factors similar to those of our reference compounds (Table I). We specifically addressed whether X might be a [4Fe-4S] ferredoxin or [2Fe-2S] ferredoxins by attempting to fit the EXAFS data with specified numbers of Fe neighbors. Figure 5 shows fits to the spinach PS I (a and b) and to the *Synechococcus* PS I (c and d) data. In each case the number of Fe neighbors used in the fit was constrained to either 2.3 (b and d) or 3.0 (a and c) either to simulate a mixture of two [2Fe-2S] and two [4Fe-4S] ferredoxins or to simulate all [4Fe-4S] ferredoxins. The Debye–Waller disorder parameter was *not* fixed for any simulations, and the values used give optimal simulations. The central portions of these fits are the most informative, because the shape of the beat region of the envelope is quite sensitive to the ratio of Fe and S backscattering. It is also the most reliable portion of the data, because the signal-to-noise is poor in the high k region, while the very low k region has features owing to processes other than EXAFS. For both the spinach and *Synechococcus* PS I spectra, the fits to a model with both [2Fe-2S] and [4Fe-4S] ferredoxins had lower least-squares errors by a factor of more than 2 than did the fits for an all-[4Fe-4S] model; the former fits also match the data much better in the region of k = 7.5 Å^{-1}.

Table II: EXAFS Simulation Parameters of PS I Data[a,b]

simulation	N_{Fe}	σ_{Fe-Fe} (Å)	error[c]
PS I from spinach simulated assuming all [4Fe-4S]	3.0	0.12	60
PS I from spinach, best fit	1.9	0.08	9
PS I from *Synechococcus*, simulated assuming all [4Fe-4S]	3.0	0.10	100
PSI from *Synechococcus*, best fit	2.1	0.07	50

[a] Values determined by the Teo–Lee method (Teo & Lee, 1979). [b] The errors for the best fits are smaller by a factor of 6 for spinach and by a factor of 2 for *Synechococcus* as compared with those for all-[4Fe-4S] simulations. The Debye–Waller factors of 0.08 and 0.07 Å for the best fits are similar to the values obtained from reference Fe–S complexes. [c] The error for a fit is the sum of the absolute value of the residuals, weighted by k^3.

Thus, even with a large Debye–Waller factor, fits using three Fe neighbors at 2.76 Å did not agree well with the data. Table II contains the parameters obtained from an all-[4Fe-4S] simulation and a best fit simulation of the PS I data. Assuming that 10 or 15% of the Fe is in a non-ferredoxin and oxygen-coordinated environment did not change our essential conclusion that including [2Fe-2S] clusters as well as [4Fe-4S] clusters (or using a smaller amplitude of Fe backscattering) achieves a better simulation than including [4Fe-4S] clusters alone (simulations not shown).

DISCUSSION

The Fe K-edge spectra of PS I are almost identical with that of the soluble spinach [2Fe-2S] ferredoxin including the size of the 1s to 3d transition, which is an indicator of the amount of tetrahedral Fe. This feature is systematically larger in tetrahedral than in trigonal-bipyramidal complexes, and smaller yet in octahedral complexes, as is predicted from symmetry considerations (Roe et al., 1984). Octahedral or distorted octahedral Fe complexes with mainly oxygen or nitrogen ligands have edge spectra which are very different from those of Fe–S complexes. On the basis of simulated spectra of mixed systems and spectra of ferredoxins containing small amounts of hexacoordinate iron, the presence of an approximately octahedral Fe complex in a primarily [Fe-S] sample is obvious even when it constitutes a small fraction (10–15%) of the Fe (McDermott, 1987). We conclude that among the 10–14 Fe atoms per P700 there may be one octahedral Fe atom, but it is unlikely that there are two or more. These X-ray absorption results are not consistent with a model in which the iron in PS I consists of only an [8Fe-8S] ferredoxin and one or more octahedral Fe atoms (as part of an Fe–Q complex). This suggests that there are additional Fe–S centers present besides A and B. The additional Fe–S clusters are likely to be X on the basis of its EPR spectrum. This conclusion that essentially all the iron in PS I is present as Fe–S clusters could not be reached from the Fe and S stoichiometries because of the considerable inherent errors in these chemical determinations.

The EXAFS spectra have the characteristic features of an Fe–S cluster, with S and Fe peaks at 2.27 and 2.74 Å, respectively and no other significant peaks. To discriminate between the two probable structures for X, [2Fe-2S] cluster(s) or a [4Fe-4S] cluster, we simulated the EXAFS spectrum to derive the average number of Fe neighbors. The [2Fe-2S] clusters and [4Fe-4S] clusters differ in the number of Fe neighbors by a factor of 3, so that discriminating between all [2Fe-2S], half [2Fe-2S] and half [4Fe-4S], and all [4Fe-4S] clusters is within the capabilities of EXAFS. However, N is correlated with two of the parameters in our simulations: S_f, a scaling factor for the overall backscattering, and σ, the

Debye–Waller disorder parameter. S_f was determined from analysis of spinach [2Fe-2S]$^{2+(1+,2+)}$ ferredoxin and other reference compounds and agrees with determinations from previous studies (Teo et al., 1979). Because the soluble ferredoxins are similar in structure to the PS I Fe, the determination of the number of neighbors is probably not subject to previously discussed systematic errors that arise from chemically dependent factors (Eisenberger & Lengeler, 1980). An erroneous value for σ can only partially compensate for an erroneous value for N, because the value of σ affects the envelope of the backscattering function while N affects the overall scaling of the backscattering. If an assumption is made that $N_{Fe} = 3.0$ as in Figure 5a,c, the best fits within this constraint involve high values for σ_{Fe-Fe} (ca. 0.12 Å) which partially compensate for the higher values for N. These simulations are not satisfactory because they disagree with the data in the diagnostic beat region near $k = 7.5$ Å^{-1}. Our best fits for the PS I data had 2.0 ± 0.4 Fe neighbors, with Debye–Waller factors more typical of Fe–S clusters (0.07–0.08 Å). These simulations agree much better with the data than do those which assume an all-[4Fe-4S] model for PS I (Table II and Figure 5). Thus, the EXAFS data suggest that some of the Fe in PS I has fewer than three iron neighbors at ca. 2.77 Å and a mixture of [2Fe-2S] and [4Fe-4S] ferredoxins is an acceptable model for PS I on the basis of the EXAFS results. Other simulations which contain additional disordered Fe shells that partially cancel upon addition are also consistent with the data; a highly distorted ferredoxin might give rise to such a spectrum.

It is logical to assign this binuclear or distorted multinuclear Fe–S cluster(s) as X, since centers A and B are probably [4Fe-4S] centers of standard structure. One model for the Fe in PS I which we considered to be likely, on the basis of biochemical precedent, is a mixture of two [2Fe-2S] clusters (which are X) and two [4Fe-4S] clusters (which are centers A and B). The simulation involving two [2Fe-2S] and two [4Fe-4S] ferredoxins is within the error of the parameters used for the best global fit, while the simulation using all [4Fe-4S] clusters is outside of that error range (Figure 5). Some biologically unprecedented structures are also consistent with these EXAFS data, such as the highly distorted tetranuclear or the linear trinuclear Fe–S clusters (Hagen et al., 1983; Kanatzidis et al., 1983). It is of note that the distorted tetranuclear cluster, which has one five-coordinate iron, has a considerable lower redox potential as compared with standard [4Fe-4S] clusters. It is unclear at present whether these biologically unprecedented structures would be consistent with the redox properties and EPR spectra of X. Ongoing EXAFS experiments with X isolated in the absence of centers A and B will further clarify the structure of X.

The presence of [2Fe-2S] ferredoxins in PS I and their assignment as X have been suggested by recent core extrusion ^{19}F NMR characterizations (Golbeck et al., 1987). Previous Mössbauer results were interpreted to indicate that X, A, and B are all [4Fe-4S] ferredoxins (Evans et al., 1981). In this EXAFS study we analyzed a composite of all of the Fe in the sample, while in the Mössbauer study the reducible portion only was analyzed. It is difficult to reduce X quantitatively by illumination in a concentrated sample. It is possible that the Mössbauer spectrum reflects mainly A and B, while the 35% of Fe that on reduction and illumination had an unchanged Mössbauer spectrum may include X, as has been suggested (Bonnerjae & Evans, 1984).

Recently there has been evidence that X is bound on the same peptides which bind P700, A_0, and A_1 (Golbeck & Cornelius, 1986; Warden & Golbeck, 1986). These are two highly homologous peptides called PSI-A1 and PSI-A2. It is not known how many copies of each peptide per P700 are present, but it is clear that one copy of each peptide (i.e., two peptides total) would not provide the eight cysteines required for binding two [2Fe-2S] clusters (Fish et al., 1985). If X is bound by less than eight cysteines, then it cannot be a pair of [2Fe-2S] clusters; either it has nitrogen or oxygen ligands like the Rieske centers, or it is a tetranuclear or trinuclear Fe–S cluster. Due to inherent limitations in sensitivity, EXAFS and edge data do not address whether X may have one N or O ligand, but the involvement of ligands which are less electron donating than S is unlikely on the basis of the unusually low midpoint potential of X. In the peptide sequences of [2Fe-2S], [4Fe-4S], [3Fe-4S], and [1Fe] ferredoxins, cysteines are found in a Cys-X-X-Cys pattern. Within each class of ferredoxin there are additional characteristic or invariant amino acid sequences [reviewed in Stout (1982)]. Exceptions to the Cys-X-X-Cys pattern occur in the Rieske Fe–S centers, all of which seem to contain a Cys-Pro-Cys-His and a Cys-Thr-His-Leu-Gly-Cys-Val pattern; these unique patterns may be related to the fact that the Fe in the Rieske Fe–S centers has one or more histidine ligands rather than all cysteine ligands (Pfefferkorn & Meyer, 1986; Beckmann et al., 1987; Gabellini & Sebald, 1986; Harnisch et al., 1985). Both the Cys-X-X-Cys and the characteristic Rieske sequence elements are missing in the protein sequence of PSI-A1 and PSI-A2 published by Fish et al. (1985). The absence of these sequence elements may lend support to the idea that X is an unprecedented type of Fe–S cluster.

We also conclude from this study that the structures of A, B, and X are preserved across the estimated two billion year evolutionary gap separating spinach and *Synechococcus*. We have found no significant differences between the two complexes by EPR, EXAFS, X-ray absorption edge spectroscopy, SDS–PAGE, or chemical analysis for Fe and sulfide. This conclusion, as well as the conclusion that A_0 and A_1 are similar in prokaryotes and eukaryotes, has been reached by other workers also (Smith et al., 1987). The structural similarity is not surprising on the basis of the great degree of homology for PS I peptides from the two classes of organisms (Bryant et al., 1987). The use of prokaryotes to accomplish genetic or isotopic substitutions which would be difficult in higher plant systems will probably become more common. The spectroscopic comparison of cyanobacterial and plant reaction centers validates the notion of generalizing results from prokaryotes to higher plant systems.

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