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Photosystem I charge separation in the absence of center A and B. III. Biochemical characterization of a reaction center particle containing P-700 and F_X

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The Photosystem I reaction center is a membrane-bound, multiprotein complex containing a primary electron donor (P-700), a primary electron acceptor (A_0), an intermediate electron acceptor (A_1) and three membrane-bound iron-sulfur centers (F_X, F_B, and F_A). We reported in part I of this series (Golbeck, J.H. and Cornelius, J.M. (1986) Biochim. Biophys. Acta 849, 16–24) that in the presence of 1% lithium dodecyl sulfate (LDS), the reaction center becomes dissociated, resulting in charge separation and recombination between P-700 and F_X without the need for prereduction of F_A and F_B. In this paper, we report (i) the LDS-induced onset of the 1.2-ms 'fast' phase of the P-700 absorption transient is time-dependent, attaining a maximum 3:1 ratio of 'fast' to 'slow' kinetic phases; (ii) the 'fast' kinetic phase, corresponding to the $P\text{-}700^+ F_X^-$ backreaction, is stabilized indefinitely by dilution of the LDS-treated particle followed by ultrafiltration over a YM-100 membrane; (iii) without stabilization, the $P\text{-}700^+ F_X^-$ reaction deteriorates, leading to the rise of the long-lived P-700 triplet formed from the $P\text{-}700^+ A_O^-$ backreaction; (iv) the 'slow' kinetic phase correlates with the redox and ESR properties of F_A and/or F_B, which indicates that in a minority of particles the terminal iron-sulfur protein remains attached to the reaction center core; (v) the ultrafiltered reaction center is severely deficient in all of the low molecular-weight polypeptides, particularly the 19-kDa, 18-kDa and 12-kDa polypeptides relative to the 64-kDa polypeptide(s); (vi) the stabilized particle contains 5.8 mol labile sulfide per mol photoactive P-700, reflecting largely the iron-sulfur content of F_x, but also residual F_A and F_B, on the reaction center; and (vii) the apoproteins of F_A and F_B are physically removed from the reaction center particle as indicated by the presence of protein-bound zero-valence sulfur in the YM-100 filtrate. These results are interpreted in terms of a model for Photosystem I in which F_A and F_B are located on a low-molecular-weight polypeptide and F_X is depicted as a [2Fe-2S] cluster shared between the two high-molecular-weight polypeptides Photosystem I-A1 and Photosystem I-A2.

Abbreviations: PS I, Photosystem I; Chl, chlorophyll; ESR, electron spin resonance; F_X, synonym for center X (ESR nomenclature) and A_2 (optical nomenclature); F_B, synonym for center B (ESR nomenclature); F_A, synonym for center A (ESR nomenclature) and P-430 (optical nomenclature); CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate; DCIP, 2,6-dichlorophenolindophenol; EXAFS, extended X-ray absorption fine structure.

Introduction

Recent studies have indicated that as many as five electron acceptors function on the reducing

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side of Photosystem I: a chlorophyll primary acceptor, A_0, a quinone intermediate acceptor, A_1, and three membrane-bound iron-sulfur centers, F_X, F_B and F_A (see Refs. 1 and 2 for a review). This sequence of electron carriers stabilizes the otherwise short-lived charge separation between the primary reactants, P-700 and A_0, thereby permitting soluble proteins plastocyanin and ferredoxin to interact with transient, membrane-bound donor and acceptor molecules.

The properties of iron-sulfur center F_X have been probed mainly after chemical reduction of F_A and F_B, followed by measurement of the P-700$^+$ F_X^- transient after a short duration flash. This technique introduces a block in forward electron flow, forcing the photochemically reduced acceptor, F_X^-, to backreact with the primary electron donor, P-700$^+$. While these studies have been extremely fruitful in elucidating the nature of F_X, evidence now exists to indicate that close proximity of reduced F_A or F_B may affect certain properties of F_X, such as the midpoint potential and backreaction kinetics with P-700$^+$ [2].

We recently found that a Photosystem I particle treated briefly with 1% LDS shows light-induced charge separation and recombination between P-700 and F_X without the need for prereduction of F_A and F_B (Refs. 3 (part I of this series) and 4). This technique allowed us to characterize the reducing side of Photosystem I in the absence of F_A and F_B, and to correlate the acceptor found by optical methods, A_2, with that found by ESR methods, X (the nomenclature 'F_X' is now used to denote this acceptor). We found that several characteristics of F_X were altered by removal of F_A and F_B: (1) the backreaction kinetics between P-700$^+$ and F_X^- were slowed from 250 μs to 1.2 ms; and (2) the linewidth of the ESR spectrum of F_X broadened 30–35% relative to the untreated (control) particle.

One further consideration is that the terminal electron acceptors F_A/F_B interfere with instrumental techniques such as ESR, Mössbauer, and EXAFS spectroscopy in the study of F_X. The isolation of a reaction center particle devoid of F_A/F_B but retaining F_X would obviously allow physical and biochemical measurements otherwise unavailable in Photosystem I research.

In this paper, we report further characterization of a Photosystem I particle capable of sustained P-700$^+$ F_X^- reaction. We show that after LDS addition, F_X can be stabilized by dilution followed by exhaustive ultrafiltration or by K^+-induced precipitation of the LDS followed by centrifugation. In the former case, the ultrafiltration step results in a significant loss of small peptides, including the apoproteins of F_A and F_B, from the Photosystem I reaction center core.

Materials and Methods

Photosystem I reaction center particles containing 40 Chl/P-700 were isolated with Triton X-100 from EDTA-washed spinach thylakoid fragments [5]. The Photosystem I particles were treated with 1% LDS for 120 s at 250 μg/ml chlorophyll in 0.05 M Tris buffer (pH 8.3) followed by one of two methods for stabilization. In the first method, the LDS-treated particles were diluted 20-fold in Tris buffer followed by repeated washing (3-fold) of the supernatant over an Amicon YM-100 membrane. In the second method, the LDS-treated particles were diluted 10-fold in Tris buffer containing 0.2 M KCl to precipitate the LDS, and centrifuged at 12000 × g for 10 min prior to concentration over a YM-100 membrane. The final ultrafiltration buffer in both instances contained 0.02% CHAPS to minimize a film of Photosystem I particles from forming on the surface of the YM-100 membrane. The stabilized Photosystem I particles were concentrated to 1 mg/ml chlorophyll and stored in 20% glycerol at −80°C. The YM-100 filtrate was collected and concentrated over a YM-30 membrane to recover the low-molecular-weight peptides. Labile sulfide and zero-valence sulfur were determined according to Ref. 6.

Flash-induced absorption transients were determined at 700 nm [3]. The data were captured and averaged on a Nicolet 4094A Digital Oscilloscope and transferred to a Macintosh Plus computer for display, manipulation, and storage. The recovery time of the d.c.-coupled amplifier (EG&G model 113) following a saturating flash was approx. 10 μs; photoactive P-700 is defined for the purpose of this study as any 700 nm absorption transient with a lifetime of 50 μs and greater. For measurements of the P-700 triplet, a more than 10 MHz bandwith, a.c.-coupled amplifier

(Hewlett-Packard model 8447) was used. The fast and slow phases of the absorption transient were evaluated by extrapolating the logarithmic plot of the slow phase to time zero and peeling the resulting curve to extract details of the fast phase. The optical measurements were performed in a 1 cm pathlength cuvette containing 5 μg/ml chlorophyll, 0.033 mM DCIP and 1.7 mM ascorbate in 50 mM Tris (pH 8.3); this concentration of reductant was sufficient to ensure complete rereduction of P-700$^+$ within 10 s. The pH stability was determined in 0.1 M buffer (Hepes, pH 7.0–8.0; glycylglycine, pH 8.2–9.0; glycine, pH 9.2 and above).

Redox titrations were performed anaerobically by the addition of sodium dithionite to an aliquot of Photosystem I particles at a pH value capable of supporting the potential. A combination platinum/silver-silver chloride redox electrode was used to sense the potential. The electrode was calibrated with saturated quinhydrone at pH 5.0 and 6.0. The following mediators (Plant Protection Ltd., Bracknell, U.K.) were added to a concentration of 0.33 μM each: methyl viologen ($E_0' = -445$ mV); 1,1′-trimethylene-2,2′-bipyridylium dibromide ($E_0' = -548$ mV); 4,4′-dimethyl-1,1′-trimethylene-2,2′-bipyridylium dibromide ($E_0' = -740$ mV). The experiment followed a protocol in which an aliquot of sodium dithionite was added, several minutes were allowed for the system to reach equilibrium, the potential was recorded, and the flash-induced absorption transient was measured. Although only reductive titrations were performed, oxygen was admitted to the cuvette after measurement to verify normal P-700 activity after reoxidation of the bound iron-sulfur proteins.

ESR studies were performed on a Varian E-109 spectrometer equipped with an Air Products LTD liquid-helium-transfer cryostat. The spectrometer was interfaced to a Nicolet 4094 digital oscilloscope for averaging and baseline subtraction. Sample temperatures were monitored with a gold chromel thermistor situated directly below the sample tube. Light-minus-dark difference spectra were obtained by illuminating the sample with a 400-Watt tungsten lamp (Cary). Specific ESR operating conditions are specified in the figure legends.

Polyacrylamide gel electrophoresis was performed in a 13 cm × 1.5 mm slab gel containing a linear 10–20% polyacrylamide gradient. The control and LDS-treated Photosystem I particles (1 μg/μl protein) were incubated in a buffer system containing 0.0625 M Tris (pH 6.8), 2% SDS, 10% glycerol and 5% 2-mercaptoethanol for 2 min at 70 °C. The samples were cooled to ice temperature and applied to the stacking gel at a protein concentration of 20 μg/well. Electrophoresis was carried out at 10 °C at a constant current of 25 mA for 2000 Vh. Gels were stained with Coomassie Brilliant Blue, overstained with silver [7], and scanned with an LKB laser densitometer.

Results

Heterogeneity in Photosystem I and the response to LDS

When 1% LDS is added to a Photosystem I particle and flashed with a short duration pulse, two recovery phases are evident at 700 nm: a 1.2-ms, 'fast' phase that accounts for a maximum 60–70% of the initial absorption change, and a 'slow' phase that accounts for the remainder of the absorption change (Fig. 1). We reported earlier [3] that the fast phase represents the P-700$^+$ F_X^- backreaction that occurs in the absence of functional F_A^- and F_B^-; the slow phase was not characterized. (The optical nomenclature of P-430 and the ESR nomenclature of centers A and B are operational definitions of the terminal electron acceptor(s) in Photosystem I. We use the terminologies interchangeably as F_A/F_B.)

A detailed time-course of the LDS effect on the two phases of P-700 recovery is shown in Fig. 2a. On first addition of 1% LDS, there is an apparent loss of 12% in the initial amplitude of the absorption change (measured with a resolution of 50 μs) coupled with the appearance of 'fast' kinetic phase which corresponds to 25% of the absorption transient. This is followed by a time-dependent rise in the percentage of fast phase and a decline in the amplitude of the total absorption change. After 180 s of exposure to LDS, the percentage of fast phase reaches a maximum of 60–70% of the amplitude of the remaining P-700 absorption change.

The decay in amplitude of the P-700 absorption change follows first-order kinetics and is accelerated by the presence of Mg^{2+} (Fig. 2b). However, the onset of the fast kinetic phase of the

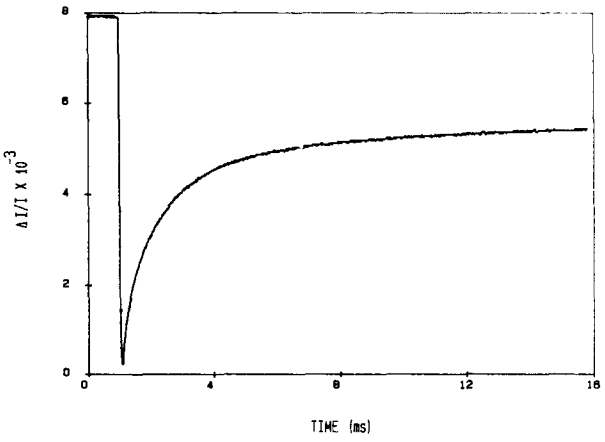

Fig. 1. Detailed properties of the P-700 absorption transient after LDS addition. Flash-induced absorption change at 700 nm 120 s after addition of 1% LDS and stabilization by dilution and ultrafiltration (see text). The monophasic, 1.2-ms event is termed the 'fast' phase and the remainder of the absorption change is the 'slow' phase. All measurements were made at 5 μg/ml chlorophyll in Tris buffer (0.05 M, pH 8.5) containing 0.033 mM DCIP and 1.7 mM ascorbic acid.

absorption transient is accelerated to a similar degree in the presence of Mg^{2+}. In Fig. 2c the ratio of fast to slow kinetic phases is plotted against the % absorption change remaining after timed LDS incubation with various concentrations of Mg^{2+}. The data show the maximum 3 : 1 ratio of fast to slow kinetic phases is reached after loss of about 35% of the total absorption change, and that the ratio is independent of Mg^{2+} concentration. In all cases, the ratio of fast to slow kinetic phases declines with further loss in P-700 amplitude.

Increasing the LDS concentration to a maximum of 10% and raising the LDS/Chl ratio by a factor of 10–100 resulted in little or no improvement in the ratio of fast to slow kinetic phases. Readdition of 1% LDS to a pretreated and stabilized reaction center (see below) did not result in a

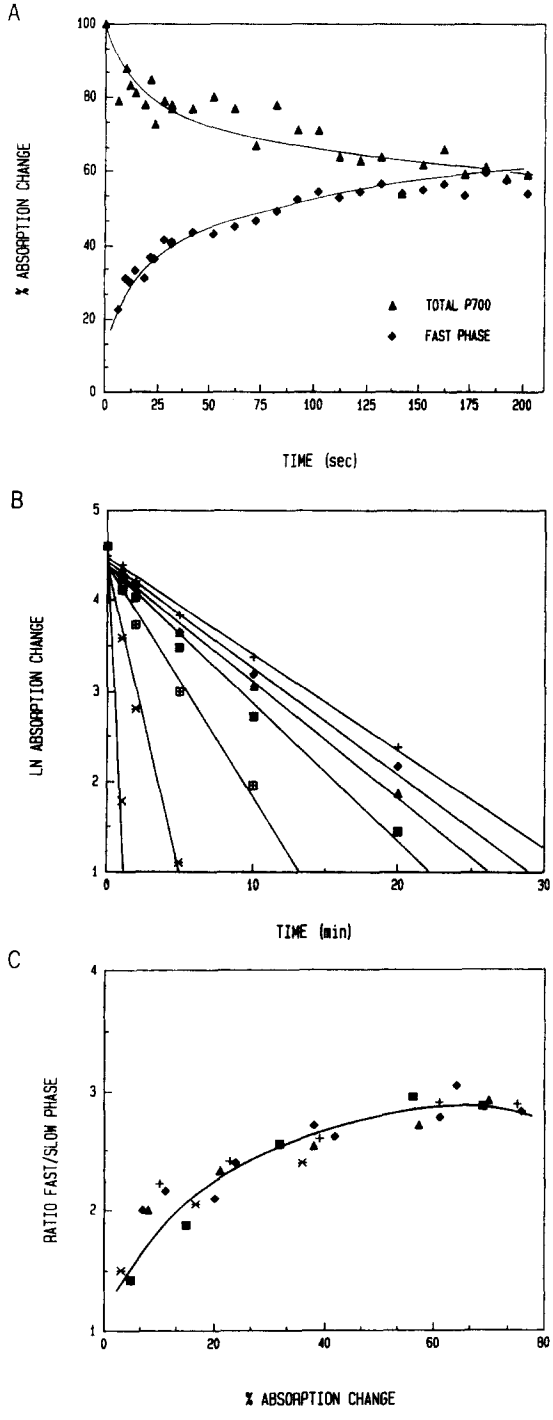

Fig. 2. Effect of incubation time and Mg^{2+} concentration on the total P-700 absorption change after LDS addition. (A) Time-course of the LDS-induced onset of the fast kinetic phase and loss of photochemical P-700 (measured with a resolution of 50 μs). (B) Time-course of the LDS-induced loss of P-700 amplitude (measured with a resolution of 50 μs) as a function of Mg^{2+} concentration. (C) Ratio of fast to slow kinetic phases as a function of P-700 amplitude remaining after incubation in various concentrations of Mg^{2+}. All measurements were made at 5 μg/ml chlorophyll in Tris buffer (0.05 M, pH 8.5) containing 0.033 mM DCIP and 1.7 mM ascorbic acid. The % fast and slow kinetic phases were determined by computer-aided curve peeling and extrapolation to time zero. Symbols: +, no Mg^{2+}; ◆, 0.1 mM Mg^{2+}; ▲, 1.0 mM Mg^{2+}; ■, 2.0 mM Mg^{2+}; ⊞, 5.0 mM Mg^{2+}; *, 10 mM Mg^{2+}; ×, 20 mM Mg^{2+}.

greater percentage of fast to slow kinetic phases; instead, both phases were induced to decay according to the kinetics of Fig. 2b. The LDS-induced appearance of the P-700$^+$ F_X^- reaction therefore appears to be in direct competition with the destruction of F_X during the 3-min induction period and thereafter. We have found that the 60–70% fast phase represents an upper limit to the LDS-induced effect under every experimental condition attempted to date.

Identity of the electron acceptor functioning after F_X destruction

The inactivation of F_X after prolonged treatment with LDS results in the loss of stable charge separation in Photosystem I (Fig. 2b). Under these conditions, there should be an enhancement of the charge-recombination process either by direct backreaction from the earliest acceptor, P-700$^+$ $A_0^- A_1$ or by a charge recombination of the P-700$^+$ $A_0 A_1^-$ radical pair. In the former case, one would observe a 5 μs transient at room temperature and an 800 μs transient at 77 K; in the latter case, one would observe a 120 μs transient at 77 K [8].

A Photosystem I particle was incubated with 5% LDS for 10 min to inactivate F_X, F_B and F_A and ultrafiltrered over a YM-100 membrane to remove the residual detergent. After this sustained exposure to LDS, only 15–20% of the P-700 absorption change could be observed at room temperature with a half-time exceeding 50 μs (Fig. 3a). The remaining absorption transient was found to be present with a half-time of 3 μs at 298 K, indicating the decay of the P-700 triplet state (not shown). At 77 K, the absorption change showed the same amplitude as the untreated control, but the monophasic decay showed a half-time of 800 μs (Fig. 3b). These data indicate that under the stated conditions destruction of F_X through prolonged incubation with LDS does not result in electron flow terminating at A_1; rather, charge recombination from A_0^- occurs through the P-700 radical-pair triplet. The decline in amplitude of the total P-700 absorption change following LDS addition (Fig. 1b) is therefore explained by the replacement of the ms-stable charge separation between P-700 and an iron-sulfur center for transient charge separation between P-700 and A_0. Similar behavior was found between P-700 and A_0

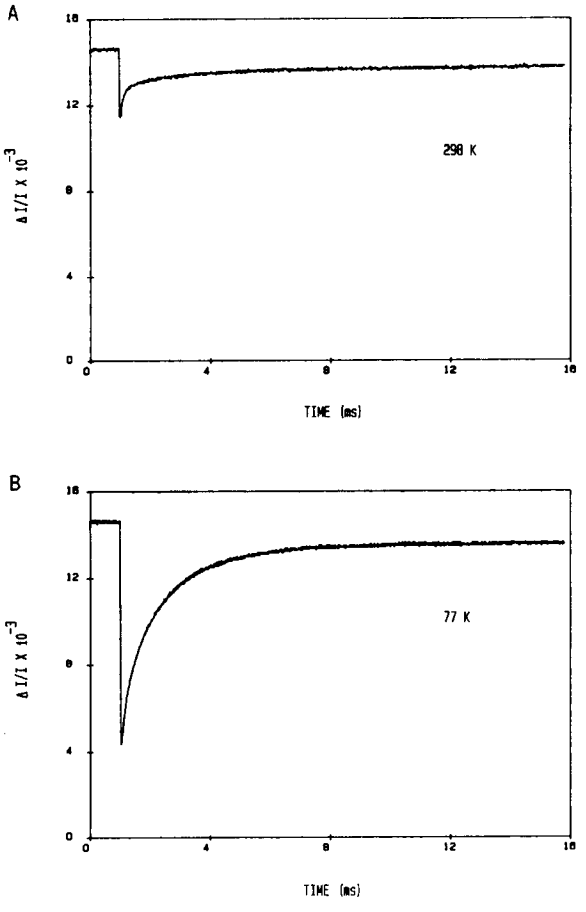

Fig. 3. Flash-induced absorbance changes in Photosystem I after inactivation of F_A, F_B and F_X with LDS. (A) Absorption transient at 298 K after 10-min incubation with 5% LDS, followed by 20-fold dilution in Tris buffer (0.05 M, pH 8.3) and ultrafiltration over a YM-100 membrane. (B) Same as (A), except at 77 K in the presence of 60% glycerol. The temperature was lowered in complete darkness over the course of 10 min to prevent sample cracking. The absorption changes were determined at 700 nm at a chlorophyll concentration of 5 μg/ml. The cuvette contained 0.033 mM DCIP and 1.7 mM ascorbic acid.

after urea-ferricyanide inactivation of F_X, F_B and F_A in spinach Photosystem I (Ref. 9: part II of this series).

Stabilization of the P-700$^+$ F_X^- reaction in LDS-treated Photosystem I

As indicated earlier, the absorption transient attributed to the P-700 F_X^- backreaction is unstable in the presence of LDS, having a half-time of about 10 min at pH 8.5 [3]. Some stabilization was

noted following 10-fold dilution of the treated particle in 0.05 M Tris buffer (pH 7.5), but no conditions were reported in which the destruction of F_X could be completely stopped. We have since developed two procedures for stabilizing F_X in the LDS-treated particles: (1) LDS present for 120 s followed by 20-fold dilution in 0.05 M Tris buffer (pH 8.3) and washing over a YM-100 membrane, and (2) LDS present for 120 s followed by addition of 0.2 M KCl, centrifugation of the potassium dodecyl sulfate precipitate, and concentration of the supernatant over a YM-100 membrane. As shown in Fig. 4a, both procedures were found to be extremely effective in preserving the P-700$^+$ F_X^- reaction. A particle treated in either manner and resuspended in Tris buffer (0.05 M, pH 8.3) showed no loss of P-700$^+$ F_X^- activity when stored for 2 weeks at 4°C.

The stabilized Photosystem I particle, however, appears to be severely stripped of detergent molecules, as indicated by the green film that collects on the surface of the YM-100 membrane. Attempts to resolubilize the aggregated particles completely while retaining P-700$^+$ F_X^- activity have not been successful. If the Photosystem I particles are redissolved in buffer containing 0.1% Triton X-100, solubilization is achieved but at the expense of stability: the fast phase of the absorption change is lost within 3 h. The particles are stable in 1% CHAPS, and dispersion with a Teflon homogenizer in Tris buffer (0.05 M, pH 8.3) containing 0.02%–0.1% CHAPS produces an almost optically transparent solution that is satisfactory for most spectroscopic and biochemical studies. Nevertheless, true solubilization is not achieved as indicated by the pellet formed after ultracentrifugation in 0.1–1.0 M sucrose gradient that contains 1% CHAPS. The same result was found with 0.1–1% octylglucoside.

Characterizing the properties of F_X depends on stability of the LDS-treated particles over a wide pH range. As shown in Fig. 4b, the fast phase of the P-700 absorption change in the LDS-treated and stabilized reaction center survives 5 min of incubation at pH values ranging from 7.0 to 11.0; a control particle is stable under similar conditions from pH 7.0 to 11.8 (the 5 min point was chosen as the minimum time needed for redox measurement: reduction with sodium dithionite

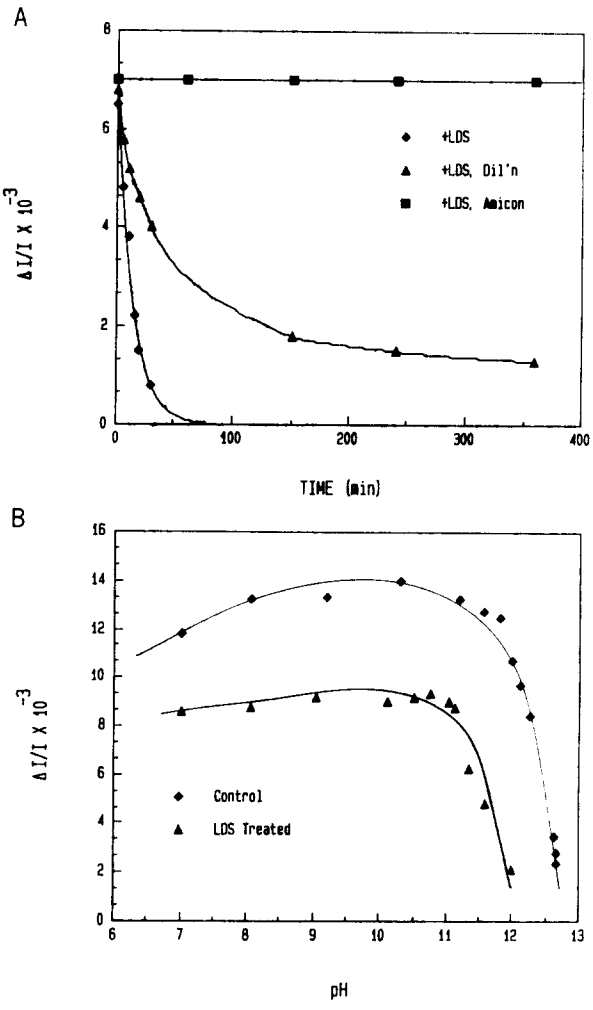

Fig. 4. Amplitude of the P-700 absorption change (measured with a resolution of 50 μs) after LDS-addition and stabilization. (A) Time-course of the total P-700 absorption change at pH 8.3 in 1% LDS for 120 s, in 1% LDS for 120 s and 20-fold dilution in Tris buffer (0.05 M, pH 8.3) and in 1% LDS for 120 s followed by 20-fold dilution and ultrafiltration over an Amicon YM-100 membrane (or K^+ precipitation of the LDS followed by centrifugation and ultrafiltration). (B) Magnitude of the total P-700 absorption change at various pH values in control and LDS-treated, stabilized reaction centers. The ultrafiltered, concentrated samples were diluted to 5 μg/ml chlorophyll in 0.1 M buffer at the specified pH value and flashed after 5 min of incubation. The sample included ascorbic acid (1.7 mM) and DCIP (0.033 mM).

and measurement of activity after a flash). After 40 min of incubation, the stabilized reaction center shows no deterioration in photochemical activity from pH 7.5 to 10.0 (data not shown). In contrast,

a particle containing LDS shows a steep drop in stability after only 2.5 min at pH 9.0 [3].

Characterization of the fast and slow kinetic phases by ESR and potentiometric titration

In control reaction centers at redox potentials increasingly more negative than −450 mV, the 30-ms absorption transient corresponding to the P-700$^+$ P-430$^-$ backreaction begins to be replaced with a 250 μs transient which corresponds to the P-700$^+$ F_X^- backreaction [10]. As shown in Fig. 5a, the experimentally determined midpoint potential of the 700 nm absorption transient is −500 mV, a value only slightly more negative than the midpoint of P-430 determined in digitonin Photosystem I particles [11]. In both titrations (Fig. 5a; see also Ref. 11), some drift occurred at potentials less than −550 mV, making the last few data points unreliable. The slow phase of the absorption transient in the LDS-treated reaction center (see Fig. 1a) titrated with a midpoint potential of −510 mV (Fig. 5b), a value nearly identical to that determined for the 30-ms transient in the control particle (Fig. 5a).

The ESR spectra of chemically induced F_A/F_B in control and LDS-treated and stabilized reaction center particles are shown in Fig. 6. The latter sample was treated for 60 s with 1% LDS to minimize destruction of the reaction center (see Fig. 2a); this particular sample retains approx. 50% of the slow kinetic phase. We find the presence of F_A and F_B in the treated particles in the amount corresponding to the percentage of slow phase remaining in the optical absorption transient (however, see Ref. 4). This correlation has been established in preparations containing varied amounts of fast and slow kinetic phases, and we conclude that the presence of F_A and F_B in the LDS-treated reaction centers is consistent with the redox titration of a −500 mV species as minority electron acceptor. Note also that the F_A resonance at $g = 1.86$ and the F_B resonance at $g = 1.89$ continue to show spin–spin interaction regardless of the degree of F_A/F_B removal. This indicates that unlike the inactivation induced by urea-ferricyanide solution [12], LDS treatment does not result in the differential loss of F_B relative to F_A.

Fig. 7 shows that the ESR signal of F_X is present after illumination of the stabilized particle in the presence of ascorbate and DCIP. The ESR signal of F_X (center X) and the 1.2-ms optical transient (center A_2) copyrify and correlate strongly with enrichment in the 64-kDa polypeptide (see below). These results are in substantial agreement with our earlier proposal [3] that F_X is located on the P-700-containing polypeptide.

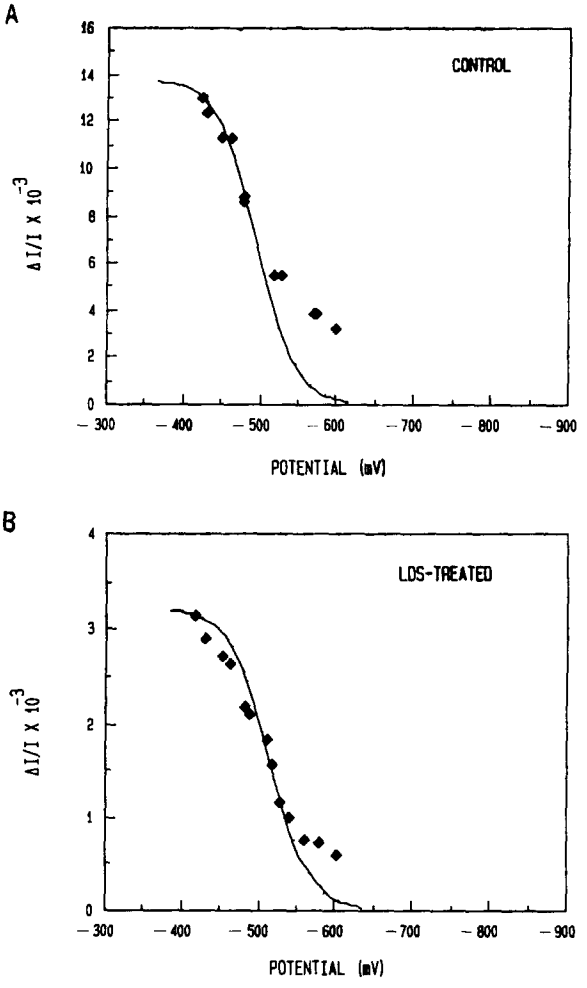

Fig. 5. Redox titration of the electron acceptors in control and LDS-treated stabilized Photosystem I reaction centers. (A) Amplitude of the 700 nm absorption change as a function of redox potential in a control Photosystem I particle. (B) Amplitude of the 700 nm absorption change in an LDS-treated, stabilized Photosystem I particle. The titration shown here represents only the species functioning as electron acceptor in the slow kinetic phase. The titrations were performed at 5 μg/ml chlorophyll; details are given in Materials and Methods.

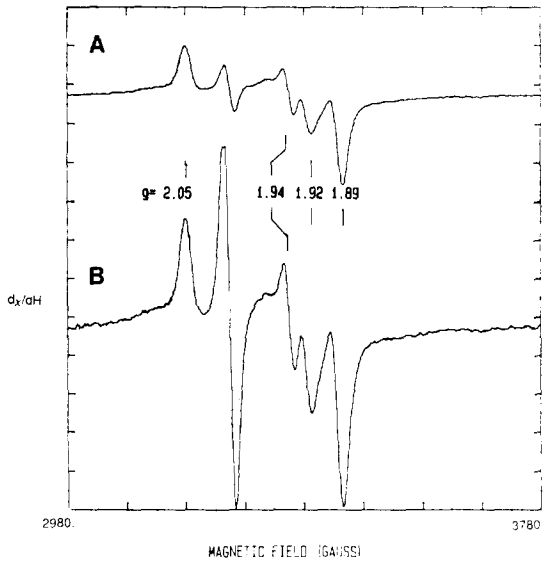

Fig. 6. X-band ESR spectra of F_A/F_B in control and LDS-treated Photosystem I. (A) LDS-treated (60 s incubation) and stabilized particle; after addition of 10 mM dithionite and 10 μM methyl viologen at pH 10.0. (B) Control reaction center particle; after addition of 10 mM sodium dithionite and 10 μM methyl viologen at pH 10.0. Amplitudes are corrected for difference in chlorophyll concentrations and tube volumes. Note that the LDS-treated spectrum shows evidence for interacting centers F_A and F_B. Spectrometer conditions: temperature, 12 K; microwave power, 1 mW; microwave frequency, 9.18 GHz; receiver gain, $12.5 \cdot 10^3$; modulation amplitude, 12.5 G at 100 kHz.

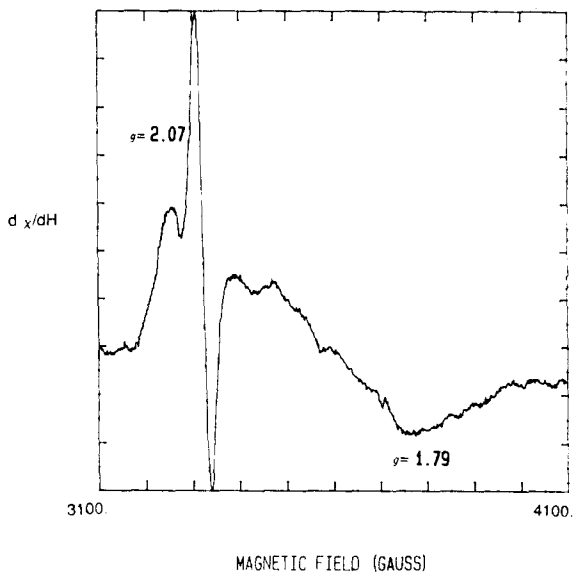

Fig. 7. Light-minus-dark (after light) X-band ESR spectrum of LDS-treated and stabilized Photosystem I particles. Sample contains 10 mM ascorbate and 10 μM DCIP at pH 7.5. Spectrometer conditions: temperature, 6 K; microwave power, 50 mW; microwave frequency, 9.25 GHz; receiver gain, $5 \cdot 10^3$; modulation amplitude, 40 G at 10 kHz. Spectra represent four 8-min sweeps each for the light and dark samples.

Polypeptide composition of the stabilized reaction center

Fig. 8a shows the polypeptide composition of the control Photosystem I particle as determined by SDS-polyacrylamide gel electrophoresis. The spinach preparation contains six major peptides, with molecular weights of 64, 19, 18, 17, 15 and 12 kDa; the 13-kDa peptide is highly variable and is considered a contaminant in this preparation. When the electrophoresis is performed at 10 °C in a 1% SDS gel, the majority of chlorophyll is found associated with a 64-kDa peptide, but a small amount remains bound to an approx. 120 kDa peptide.

The polypeptide composition of a reaction center particle treated for 120 s with LDS and stabilized to preserve $P\text{-}700^+ F_X$- activity is shown in Fig. 8b. The most salient feature of the stabilized particle is the differential loss of the low-molecular-weight polypeptides relative to the 64-kDa peptide (note that the integrator sensitivity in Fig. 8b and c is 2-fold that of Fig. 8a). When the areas under the peaks are normalized to the 64-kDa polypeptide (Table I), the ultrafiltered particle is found to retain less than 15% of the 19-kDa and less than 30% of the 18-kDa and 12-kDa peptides in Photosystem I. As shown in Fig. 8c, the missing polypeptides, especially the 12-kDa, 18-kDa and 19-kDa polypeptides, can be recovered from the YM-100 filtrate by concentration over a YM-30 membrane. Note also the presence of the 14-kDa and 17-kDa polypeptides in this fraction, which is a further indication that the stabilized particle has suffered some depletion in all of the low-molecular-weight peptides. We consistently observe this pattern of polypeptide removal from the Photosystem I core particle under conditions that lead to the loss of F_A and F_B.

Non-heme iron and labile sulfide content of the stabilized reaction center

The control Photosystem I particle (Table II)

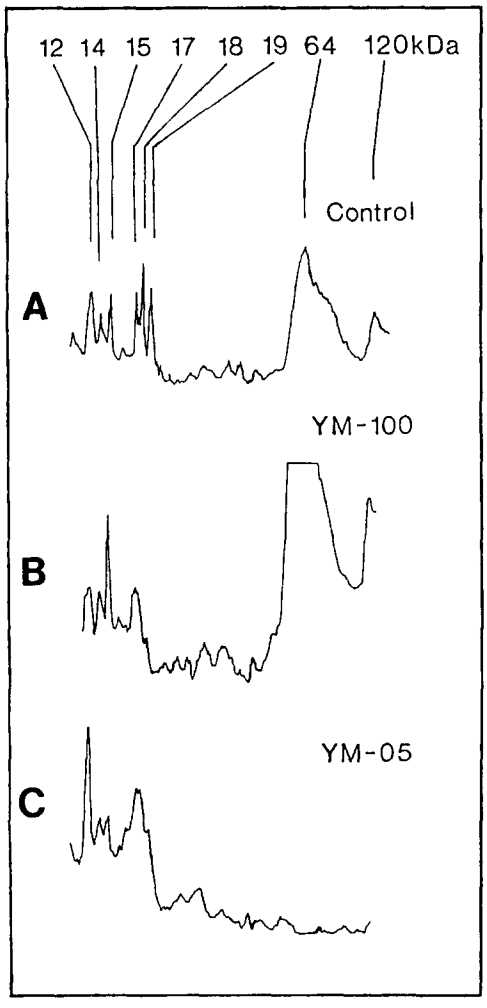

Fig. 8. Polypeptide composition of the Photosystem I particle after LDS addition. (a) Control reaction center particle, (b) after 120-s incubation with LDS and stabilization by 20-fold dilution in Tris buffer (0.05 M, pH 8.3) followed by concentration over a YM-100 membrane, (c) YM-100 filtrate collected and concentrated over a YM-30 membrane. The sample in (b) was washed and concentrated three times over the YM-100 membrane to ensure complete detergents and peptide removal. The integrator sensitivity of (b) and (c) is twice that of (a). Molecular weights are calculated on the basis of the R_f values of soluble proteins treated identically to the Photosystem I particles but run in adjacent wells. Details of the electrophoresis are given in Materials and Methods.

was found to contain 12 ± 2 mol each of non-heme iron and acid-labile sulfide per mol P-700, a value consistent with previous determinations [12–14]. The negligible content of zero-valence sulfur indicates that the control particle contains few, if any,

TABLE I

POLYPEPTIDE CONTENT OF CONTROL AND LDS-TREATED AND STABILIZED PHOTOSYSTEM I AFTER SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND NORMALIZATION OF INTEGRATED PEAK AREAS TO THE 64-kDa POLYPEPTIDE

For control values relative areas are associated with electrophoretic peaks after staining with Coomassie Brilliant Blue and overstaining with silver.

Polypeptide (kDa)	Control	YM-100
110	0.048	0.042
64	1.000	1.000
19	0.082	< 0.010
18	0.086	0.026
17	0.052	0.023
15	0.077	0.050
12	0.139	0.028

damaged iron-sulfur clusters (see Ref. 15). A particle treated with LDS for 30 s and stabilized by dilution and ultrafiltration retains 65% slow phase and contains 8.3 ± 2 mol labile sulfide and 10.0 ± 2 mol non-heme iron per mole of P-700. If F_A, F_B and F_X each contain four atoms of iron and labile sulfide (see Ref. 16), and if the sample is depleted 35% in F_A and F_B, the calculated 9.2 mol labile sulfide present per mol photoactive P-700 would be in quantitative agreement with the experimental determination. The slightly higher iron content than labile sulfide is most likely due to nonspecific attachment of the iron released from F_A and F_B during LDS treatment [15]. Indeed, the large reso-

TABLE II

NON-HEME IRON, LABILE SULFIDE AND ZERO-VALENCE SULFUR CONTENT OF CONTROL AND LDS-TREATED AND STABILIZED PHOTOSYSTEM I

Non-heme iron was determined by atomic absorption spectroscopy following digestion with nitric and perchloric acids. P-700 was determined photochemically during a saturating flash.

Sample	Non-heme iron/ P-700	S^{2-}/P-700	$(S^{2-}+S^0)$/ P-700
Control	12.0±2	12.1±2	11.3±2
35% Fast phase	10.0±2 [a]	8.3±2	12.7±2
65% Fast phase	7.0±2 [a]	5.8±2	8.3±2

[a] After 24 h dialysis against 1.0 mM Tiron in 0.025 M Tris buffer (pH 8.0).

nance at $g = 4.3$ due to non-specifically bound iron in the LDS particle, but not in the control, supports the conclusion that iron from the deterioration of F_A and F_B remains attached to the particle (data not shown). It is quite likely, therefore, that the iron-sulfur clusters in F_A and F_B sustain significant damage during the LDS treatment and stabilization protocol. The 12.7 ± 2 mol (labile sulfide + zero-valence sulfur)/mole photoactive P-700 indicates that the apoprotein(s) containing F_A and/or F_B remains attached to the Photosystem I core particle.

In an LDS sample which shows 65% fast phase, the experimentally determined labile sulfide content of 5.8 ± 2 mol/mol photoactive P-700 shows that only about one-half of the iron-sulfur complement is retained (Table II). Applying the same analysis as above, we would expect 6.8 mol labile sulfide/mol photoactive P-700 in a particle containing F_X but depleted in all but 35% of F_A and F_B. In contrast to the sample above, incubation with dithiothreitol caused only a slight increase in sulfide content to 8.3 mol (labile sulfide + zero-valence sulfur)/mol photoactive P-700, indicating that the apoprotein(s) of F_A and/or F_B have been partially lost from the particle. Analysis of the YM-100 filtrate after concentration over a YM-30 membrane showed that the missing zero-valence sulfur is associated with the low-molecular-weight peptides (see Fig. 7). The iron content showed 13 ± 2 mol non-heme iron per mol P-700; however, after 24 h dialysis against 1 nM Tiron, the non-heme iron content dropped to 7.0 ± 2 mol per mol P-700 without loss of P-700 photochemical activity. We conclude, based on the polypeptide content and iron-sulfur analyses, that LDS dissociates F_A and F_B physically, as well as functionally, from the Photosystem I reaction center, and that the apoprotein(s) of F_A and/or F_B are partially recovered in the YM-100 filtrate.

Discussion

The optical absorption, electron spin resonance, and biochemical data presented in this paper support the hypothesis that LDS dissociates the Photosystem I reaction center, thereby removing the low-molecular-weight polypeptides containing F_A and F_B from the P-700 and F_X-containing reaction center core [3]. Since the destruction of F_X is slower than the liberation of F_A and F_B in the majority of the reaction centers, the photochemical activity corresponding to $P\text{-}700^+ F_X^-$ is manifest during the LDS-induced deterioration of the particle. Stabilization of the $P\text{-}700^+ F_X^-$ activity can be achieved by exhaustive removal of the detergent followed by ultrafiltration. This protocol has a significant advantage over other methods of stabilization: in addition to eliminating traces of LDS and Triton, the ultrafiltration step removes small polypeptides, including the apoproteins, of F_A and F_B, from the reaction center core.

There is, however, a time dependency to the LDS effect which is apparent by the continued presence of a slow kinetic phase at 700 nm in addition to the $P\text{-}700^+ F_X^-$ backreaction. This slow phase titrates with a midpoint potential of -500 mV, indicating that the event represents electron donation to the terminal acceptors, F_A and/or F_B. The slow kinetic phase of P-700 recovery also correlates with the presence of ESR-detectable F_A and F_B. The midpoint potentials of F_A and F_B have been measured by ESR techniques to be -530 mV and -580 mV, respectively [18,19]. These values were determined at cryogenic temperature after the potential was established at 298 K. If we accept these values for the redox potentials of F_A and F_B at room temperature (it must be assumed that the equilibrium and pH did not change during the course of freezing), it appears that the acceptor functioning in control and LDS-treated particles is only F_A. However, we might have expected the room temperature optical titration to represent the lower potential acceptor, F_B, which should function as electron acceptor upon the chemical reduction of F_A. Unfortunately, F_A and F_B are not distinguishable by optical spectroscopy, and it is not possible to determine which cluster serves as electron acceptor by this technique alone. We must conclude that either (1) only F_A functions as electron acceptor, or (2) the potentials of F_A and F_B are higher than expected at room temperature. In the latter case, the optical titration might indeed represent F_B.

The proposal that F_A and F_B are removed by LDS is supported by the loss of numerous small polypeptides from the reaction center core. These

small polypeptides can be fractionated from the supernatant by ultrafiltration with a YM-100 membrane and concentration over a YM-30 membrane. Although the presence of protein-bound zero-valence sulfur with the low-molecular-weight peptides indicates the presence of the apoproteins of F_A and/or F_B in the YM-30 fraction, we have not determined which low-molecular-weight peptide(s) contain the iron-sulfur clusters. The concerted loss of F_A and F_B, however, is consistent with the suggestion [20] that the iron-sulfur clusters are located on a single polypeptide. Lagoutte et al. [21] used ^{35}S labeling and carboxymethylation with [^{14}C]iodoacetate to identify an 8-kDa peptide in spinach as a possible iron-sulfur protein. The amino acid analysis of this peptide showed 7 ± 1 cysteine residue, enough for two [FE-S] clusters. This analysis agrees with the results of Høj and Møller [22], who found that an 8-kDa peptide in barley was the most sulfur- and cysteine-rich of the Photosystem I components. The 8-kDa peptide is difficult to resolve and may stain lighly with Coomassie brilliant blue or silver; in our polyacrylamide gels it may correspond to the 12-kDa polypeptide.

We had concluded earlier [3], based on kinetic data and on the presence of zero-valence sulfur with the 64-kDa peptide, that F_X is associated with the P-700 peptide in Photosystem I. This conclusion is now strengthened by the retention of light-induced ESR signal characteristic of F_X on the LDS-treated and stabilized reaction center. In spite of the loss of a significant fraction of the low-molecular-weight polypeptides from the particle, F_X, assayed by ESR and optical spectroscopy, follows enrichment in only the high-molecular-weight protein. Unfortunately, the continued presence of several low-molecular-weight polypeptides with the LDS treated and stabilized particle does not allow us to rule out categorically the involvement of a low-molecular-weight peptide with the formation or stabilization of F_X. Nevertheless, we surmise that the low-molecular-weight peptides on the LDS-treated and stabilized particle represent that minority fraction (approx. 25–30%) of reaction centers in which F_A and F_B remain attached to the Photosystem I core polypeptide(s). At our laboratory one is therefore continuing work on the purification of a minimum polypeptide reaction center particle that carries out photochemistry from P-700 to F_X.

We recently published a model for a Photosystem I subunit in which a [2Fe-2S] cluster is shared between the two high-molecular-weight polypeptides PS I-A1 and PS I-A2 [16]. This structure is supported by sequence data for the PS I-A1 and PS I-A2 genes from six organisms which show the presence of 3–5 cysteine residues in PS I-A1 and two cysteine residues in PS I-A2 [23]. Three cysteines are conserved in PS I-A1 across all six organisms, but in liverwort the fourth cysteine is missing. The two cysteines are conserved in PS I-A2 across all six organisms, and are analogous to two of the cysteines in PS I-A1. Since low potential iron-sulfur clusters are assumed to require four cysteine residues, the simplest structure would be an interpolypeptide [2Fe-2S] cluster held between the homologous cysteine residues on PS I-A1 and PS I-A2 [16]. We are less sanguine about the existence of a nitrogen- (or oxygen-) containing amino acid as the fourth ligand, since the substitution of a neutral N-containing ligand for an –SH group should raise, rather than lower, the midpoint potential of the iron-sulfur cluster. Indeed, only high-potential iron-sulfur clusters are considered to contain N- as well as –SH ligands [24].

Additional constraints on the structure of Photosystem I include quantitative data which indicates the presence of four high-molecular-weight polypeptides [14] and 12 ± 2 mol non-heme iron and labile sulfide per mol of P-700 in Photosystem I (see Ref. 2). These data suggest a model in which the reaction center consists of two closely associated Photosystem I subunits [16]. In this depiction, two [2Fe-2S] clusters from each of the subunits would undergo association to form F_X (see also Ref. 25). Indeed, recent ESR results have been interpreted to indicate that the signal corresponding to F_X may arise from either a [2Fe-2S] cluster with distorted bond angles in the reduced state and correspondingly weak antiferromagnetic coupling or from weak exchange between two nearby [2Fe-2S] clusters [13].

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