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Photosystem I charge separation in the absence of centers A and B. II. ESR spectral characterization of center '*X*' and correlation with optical signal 'A_2'

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The Photosystem I electron acceptor complex was characterized by optical flash photolysis and electron spin resonance (ESR) spectroscopy after treatment of a subchloroplast particle with lithium dodecyl sulfate (LDS). The following properties were observed after 60 s of incubation with 1% LDS followed by rapid freezing. (i) ESR centers A and B were not observed during or after illumination of the sample at 19 K, although the P-700$^+$ radical at $g = 2.0026$ showed a large, reversible light-minus-dark difference signal. (ii) Center '*X*', characterized by *g* factors of 2.08, 1.88 and 1.78, exhibited reversible photoreduction at 8 K in the absence of reduced centers A and B. (iii) The backreaction kinetics at 8 K between P-700, observed at $g = 2.0026$, and center *X*, observed at g = 1.78, was 0.30 s. (iv) The amplitudes of the reversible $g = 2.0026$ radical observed at 19 K and the 1.2 ms optical 698 nm transient observed at 298 K were diminished to the same extent when treated with 1% LDS at room temperature for periods of 1 and 45 min. We interpret the strict correlation between the properties and lifetimes of the optical P-700$^+$ A_2^- reaction pair and the ESR P-700$^+$ center X^- reaction pair to indicate that signal A_2 and center *X* represent the same iron-sulfur center in Photosystem I.

Introduction

According to electron-spin resonance experiments performed at cryogenic temperatures, photochemical charge separation proceeds between the primary electron donor P-700 (ESR Signal 1) and a primary electron acceptor complex consisting of A_0 and A_1 and three membrane-bound iron–sulfur centers (see Ref. 1 for review). After the initial charge separation has occurred, the electron is proposed to flow from A_1 to an intermediate electron acceptor, center *X*, and finally to the terminal electron acceptors, centers A and B (however, see also Refs. 2 and 3). Optical absorption measurements performed at room temperature indicate a similar pattern of events: a primary electron acceptor, A_1, is hypothesized to reduce an intermediate acceptor, A_2, which in turn donates an electron to the terminal electron acceptor, P-430 [4]. With the exceptions of P-700 and Signal 1 [23] and center A and P-430 [5], a strict correlation between the optical and ESR components has not been made. In particular, center *X* has been suggested to correspond to either optical signal A_2 [6] or P-430 [7].

The electron acceptor components in Photosystem I have been probed mainly through chemical

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; LDS, lithium dodecyl sulfate; P-700, Photosystem I reaction center chlorophyll; Signal 1, ESR resonance assigned to P-700$^+$.

reduction of the more oxidized species followed by measurement after pre-illumination (see Ref. 8). Optical signal A_2, for example, becomes visible with its characteristic backreaction with P-700$^+$ only after P-430 has been reduced chemically prior to illumination. The same technique is applicable to electron spin resonance spectroscopy, where chemical reduction of a specific component is achieved before freezing and subsequent illumination. This technique, although powerful, does have a potential liability: close proximity of a reduced acceptor species may distort the properties of the more primary electron acceptors, including spectroscopic properties and backreaction times with P-700$^+$. Golbeck and Cornelius [9] concluded recently that LDS dissociates the small peptides containing P-430 from the 64-kDa peptide(s) which contain P-700 and the iron–sulfur cluster A_2. The removal of P-430 allows photochemical accumulation of A_2^- without the need for prior reduction of P-430.

In this paper, we investigated the low-temperature ESR properties of P-700 and its reaction partner after treatment of a Photosystem I particle with 1% LDS at room temperature. We were particularly interested in determining the identity of the electron acceptor to P-700 in the absence of centers A and B, in investigating the properties of this acceptor, and in correlating the electron acceptor found by electron-spin resonance techniques with that found by optical flash techniques.

Methods and Materials

Tris buffer, Triton X-100, ascorbic acid, and DCIP were purchased from Sigma (St. Louis, MO). LDS was obtained from British Drug Houses. All other chemicals were from Fisher. Fresh spinach was obtained from a local distributor. Photosystem I particles were isolated as described in Ref. 9.

Flash-induced absorption changes were measured as described previously [9]. ESR spectra were obtained with a Varian E-9 spectrometer equipped with an Air products LTD-3-110 liquid-helium transfer cryostat. Sample temperatures were monitored with a calibrated carbon resistor situated directly below the 3 mm inner-diameter quartz sample tube. Light-minus-dark difference spectra were obtained with a PDP-11/23 minicomputer interfaced directly to the spectrometer and illumination was provided by 1000 W tungsten-halogen lamp (Oriel). The Photosystem I particle was treated with 1% LDS at a chlorophyll concentration of 800 μg/ml in darkness for 60 s. Ascorbic acid (1.7 mM) and DCIP (33 μM) were added to the sample 2 min prior to the addition of the LDS. The reaction was stopped by rapid freezing in the ESR tube. Specific ESR operating conditions are provided in the figure legends.

Simulation of the center *X* ESR spectrum was performed utilizing an algorithm that conformed to the criteria described by Aasa and Vånngard [22]. Linewidth anisotropy was approximated as an unresolved, first-order hyperfine interaction. Simulations were obtained using a Digital Equipment Corporation PDP 11/23 processor or a Macintosh 512 K (Apple computer).

Results

Electron spin resonance studies of Photosystem I electron donors and acceptors at 19 K

The proposal that LDS dissociates centers A and B from the Photosystem I reaction-center core [9] was tested by examining the LDS-treated particle using electron spin resonance spectroscopy. The ESR spectra of photoreduced centers A and B are shown in a control Photosystem I particle in Fig. 1a. The sample was prereduced for 2 min with ascorbate-DCIP, taken to 19 K in darkness, and illuminated in the ESR cavity with a 1000 W lamp for 1 min. In agreement with earlier studies [10,11], the particle shows irreversible photoreduction of center A ($g = 2.05$, 1.94 and 1.86) and partial (less than 10%) reduction of center B ($g = 2.07$, 1.92 and 1.89). Under identical conditions, Signal 1, the $g = 2.0026$ radical P-700$^+$, is created in the light and remains irreversibly oxidized during the dark measuring period (note the large $g = 2.0026$ resonance in Fig. 1a). The light-minus-dark difference spectrum (Fig. 1b) therefore only shows a small amount of reversible P-700.

The ESR spectrum of the Photosystem I particle after 60 s incubation with 1% lithium dodecyl sulfate is shown in Fig. 1c. There is no significant photoreduction of either center A or center B when measured during illumination at 19 K or in darkness following illumination for 1 min. A

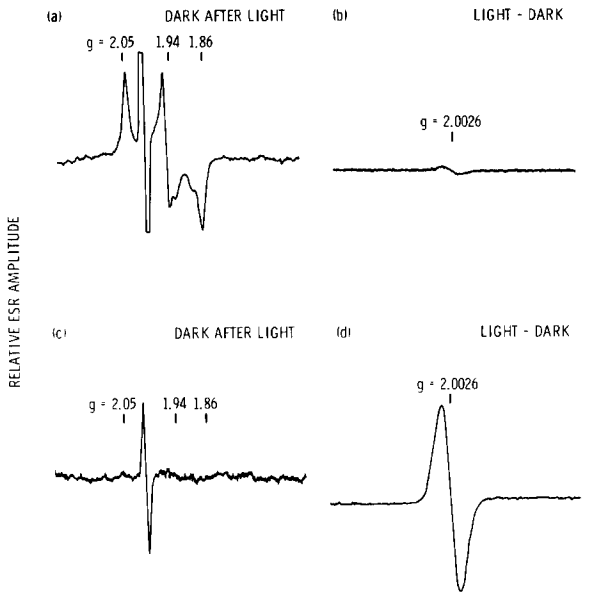

Fig. 1. ESR Spectra of the P-700 radical and centers A and B in Photosystem I particle before and after LDS treatment. (a) Control particle in Tris buffer (0.025 M, pH 8.5) after illumination for 30 s; (b) Light-minus-dark difference spectrum of the P-700$^+$ radical in a control particle; (c) LDS-treated particle in Tris buffer (0.025 M, pH 8.5) after illumination for 30 s. The particle was incubated for 1 min with 1% LDS at 800 μg/ml chlorophyll before rapid freezing; (d) Light-minus-dark difference spectrum of the P-700$^+$ radical in the LDS-trated particle. ESR conditions for centers A and B (a & c): microwave power, 10 mW; microwave frequency, 9.249 GHz; receiver gain, 5000; modulation amplitude, 25 G; scan width, 1000 G; scan time, 4 min; time constant, 0.3 s; temperature, 19 K. ESR conditions for P-700$^+$ radical (b & d): microwave power, 100 μW; microwave frequency, 9.249 GHz; receiver gain, 500; modulation amplitude, 2.5; scan width, 100 G; scan time, 4 min; time constant, 0.3 s; temperature, 19 K.

light-induced, reversible Signal 1 was found, however, which represents about 70% of the P-700 present in the control particle (Fig. 1d). The absence of photoreducible centers A and B, but the presence of reversible P-700 photooxidation indicates that primary photochemistry still occurs between P-700 and an unidentified acceptor species at 19 K.

ESR spectrum of the reaction partner to P-700 in LDS-treated particles

The ESR spectrum of a component which forms reversibly in the light at 8 K and has the characteristics of an electron acceptor from P-700 is shown

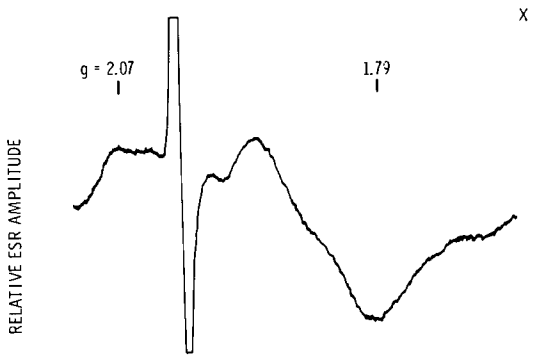

Fig. 2. Light-minus-dark ESR Spectrum of the reaction partner to P-700$^+$ in LSD-treated Photosystem I particle. The particle was treated at 800 μg/ml chlorophyll for 1 min prior to rapid freezing. ESR conditions: microwave power, 49 mW; microwave frequency, 9.249 GHz; receiver gain, 5000; modulation amplitude, 25 G; scan width, 1000 G; scan time, 16 min; time constant, 1 s; temperature, 8.6 K.

in Fig. 2. The highly anisotropic ESR signal with features near $g = 2.08$, 1.88 and 1.78 is representative of center X [12–16], and may reflect an unusual distortion of an iron-sulfur cluster. The experimentally observed g factors in Fig. 2 are similar to those observed in chemically reduced Photosystem I particles ($g = 2.08$, 1.88 and 1.76); however, the linewidth for each of the peaks is significantly greater than observed previously [12,15,16]. Quantitative studies indicate that this amount of center X represents 70% of that found in the control particle after reduction at pH 10.0 with dithionite. Fig. 3 shows the light-off kinetics of the $g = 2.0026$ species (P-700$^+$) and the $g = 1.78$ species (the high-field peak of center X). Both P-700 and center X show a 0.3 s light-off transient, which is consistent with a direct charge recombination between the two components. No evidence for other kinetic components for either P-700$^+$ or center X was observed. Care was taken to insure that the kinetics obtained were not influenced by incident microwave power.

Microwave power saturation characteristics of center X

In the LDS-treated particle, Center X at approx. 9 K exhibits significant microwave power saturation only at powers in excess of 200 mW ($P_{1/2} > 225$ mW). Comparison of these data (not shown) with those presented by Williams-Smith

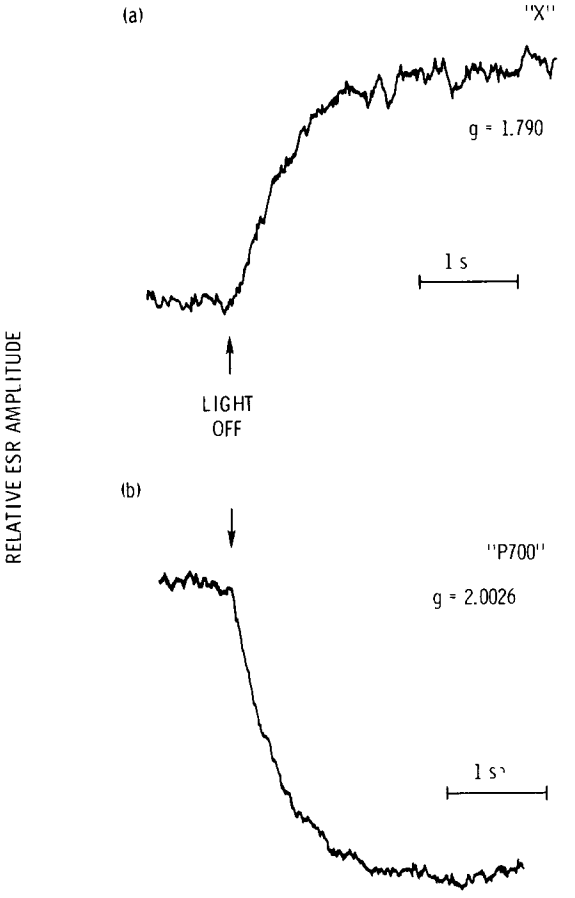

Fig. 3. Light-off kinetics of center X and Signal 1 in an LDS-treated Photosystem I particle. The particle was treated with LDS as described in Fig. 1. ESR conditions are given in Fig. 1 (Signal 1) and Fig. 2 (Center X). Spectrometer time constant was 0.06 s.

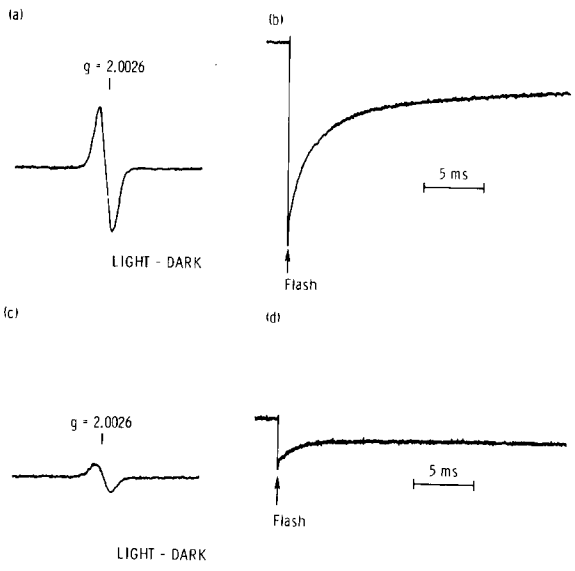

Fig. 4. Comparison of the ESR Signal 1 with the Optical P-700 Transient in an LDS-treated Photosystem I Particle. The particle was treated at 800 μg/ml chlorophyll in Tris buffer (0.05 M, pH 7.5) with 1% LDS at 0°C for 1 min (a, b) and 45 min (c,d). For ESR determinations (a, c), the treated particle was rapidly frozen to 19 K at the times specified; for optical determinations, the particle was diluted to 20 μg/ml chlorophyll. ESR conditions are identical to Fig. 1; optical measurements (b, d) were performed as described in Ref. 9.

and co-workers [15] reveal that, although we have obtained the spectrum for center X from a different preparation and under divergent pH and redox conditions than reported by these authors, the microwave power saturation behavior of center X is similar in both laboratories.

Correlation of ESR center X with optical signal A_2

At room temperature, the amplitude of the photo-inducible 1.2 ms absorption transient at 698 nm decreases with a half-time of 15 min at pH 7.5 [9]. Since both center X [14,15] and Signal A_2 [17,18] have been suggested to be iron–sulfur proteins, we attempted to correlate the amount of flash-induced P-700 formed at room temperature with the amount of reversible Signal 1 seen at 8 K. Fig. 4 illustrates the P-700$^+$ radical at $g = 2.0026$ observed at 19 K and the 698 nm P-700 transient observed at room temperature; 70% of the $g = 2.0026$ radical and 74% of the optical P-700 signal remain intact after incubation with 1% LDS for 1 min. After 45 min of incubation at room temperature, only 21% of the $g = 2.0026$ radical and 23% of the optical P-700 signal can be found. The close correspondence between these values indicates that center X and Signal A_2 are equally sensitive to LDS treatment, strengthening their identification as the third iron–sulfur center in Photosystem I.

Simulation of the ESR spectrum of center X

The ESR spectra of iron-sulfur clusters are characterized often by field-dependent, inhomogenous line broadening. This phenomenon, loosely termed 'g strain', is manifested by anisotropic linewidths which cannot be simulated readily by conventional algorithms [20]. The spectrum of center X presented in Fig. 2 is consistent with that

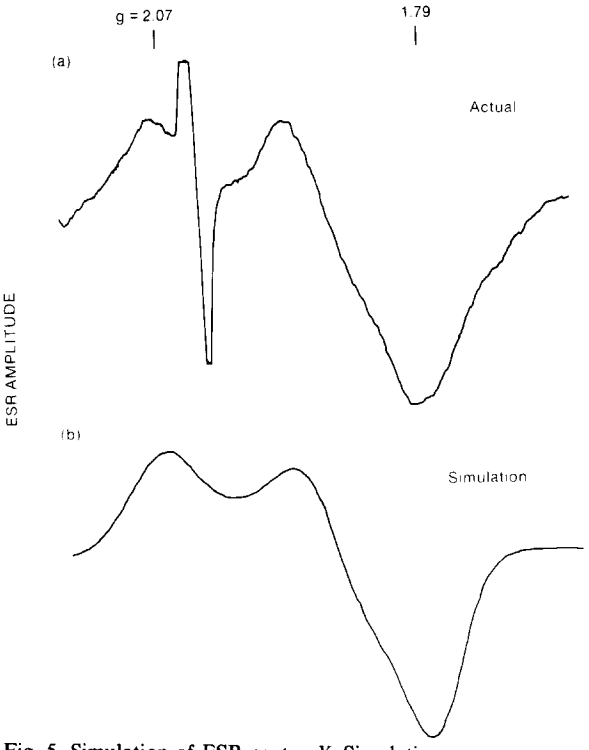

Fig. 5. Simulation of ESR center X. Simulation parameters: g (x, y, z), 1.786, 1.880, 2.075; principal linewidths (ΔH (x, y, z)), 105, 143, 135 G; microwave frequency, 9.25 GHz.

of a 'g-strained' powder spectrum in that the peak-to-peak linewidths associated with each of the principal components of the g tensor are non-equivalent. A simulation of the spectrum of center X is presented in Fig. 5. This 'best-fit' simulation, although reasonable for the low- and mid-field components, does not reproduce accurately the high-field feature. Our approach in simulating the ESR spectra of iron-sulfur clusters has been to use a linewidth tensor with a principal axis system that is coincident with the g tensor and a lineshape function (gaussian) that is symmetrical in magnetic field. However, the $g \approx 1.78$ component of center X clearly exhibits an asymmetrical lineshape, that cannot be fit faithfully by the symmetric lineshape utilized. To date there has been no rigorous simulation of ESR spectra from [4Fe–4S] clusters; however, the approach of Hagen et al. [21] should be beneficial in interpreting the linewidth variation observed in the center X spectrum.

Discussion

The electron spin resonance data presented in this paper support the hypothesis that a brief (cless than 10 s) treatment with 1% LDS dissociates the Photosystem I reaction center, thereby removing the peptide(s) containing centers A and B (P-430) from the reaction-center core. Since two molecules of P-430 exist per P-700 [23], the loss of both ESR centers A and B is entirely consistent with their identification as optical centers P-430. The ESR data indicate also that center X functions as the terminal electron acceptor in a Photosystem I particle that has been treated briefly with 1% LDS. The identification of center X is based on the spectrum (especially the high field peak at $g = 1.78$), microwave power saturation characteristics [15], and backreaction time with P-700$^+$ at low temperature [3]. The low-temperature properties of P-700 after LDS treatment resemble closely the properties of a control particle which in centers A and B are reduced chemically prior to illumination. In particular, the $g = 2.0026$ radical due to P-700$^+$ shows reversible photooxidation at 19 K with a half-time of 0.3 s. In a control particle with center A as the terminal acceptor, the photooxidation of P-700 is irreversible at 19 K.

In agreement with the optical P-700 determinations [9], the amplitude of the reversible ESR Signal 1 (as monitored at 19 K) was unstable and decreased progressively as the particle was incubated at room temperature with 1% LDS. The rates of disappearance of the optical P-700$^+$ A_2^- reaction pair and the ESR P-700$^+$ center X^- reaction pair were identical at the 1 min and 45 min points, providing additional evidence that Signal A_2 and center X represent the same component in Photosystem I. Since the spectrum of the 1.2 ms optical component is representative of an iron-sulfur protein, the correlation between optical signal A_2 and ESR center X supports the identification of the latter as an iron-sulfur cluster. The replacement of the 30 ms P-700$^+$ P-430$^-$ backreaction with a 1.2 ms transient, the insensitivity of the 1.2 ms component to methyl viologen, and the identical decay courses of the 1.2 ms optical transient and the low-temperature P-700$^+$ X^- reaction pair, are all inconsistent with the identification of center X as P-430.

Due to the low redox potential of center *X*, approx. −710 mV [24,25], all previous electron spin resonance studies of this component have been performed under conditions that result also in the reduction of centers A and B. Most commonly center *X* is observed after prior reduction of Photosystem I with dithionite at alkaline pH [13,26], although trapping of center *X* by strong illumination in the presence of a reductant has been reported also [27,28]. Of special interest for Photosystem I studies is the contention of Malkin and Bearden that the use of alkaline pH results in the diminution of P-700 photooxidation [29]. The data reported above, therefore, are significant in that they represent the first time that center *X* has been observed and its properties delineated in the absence of a reduced electron acceptor system.

The spectrum of center *X* is characterized by a large *g* anisotropy ($g_z = 2.075$, $g_y = 1.880$ and $g_x = 1.786$) with $g_{av} \approx 1.91$, a value smaller than that observed for other iron–sulfur clusters (e.g., center A, $g_{av} \approx 1.95$). In labeling the principal *g* factors in our experimental and simulated spectra, we have followed the protocol of Beinert and Albrach [30], in which $g_z = g_1$ if $(g_1 - g_2) > (g_2 - g_3)$ and $g_1 > g_2 > g_3$. However, we caution that assignment of the labels *x*, *y* and *z* to the molecular geometric axis system can only be validated by single crystal studies. The values for the *g* factors that we have observed (Fig. 2) and determined by simulation (Fig. 5) for center *X* in the LDS-treated particle are in good agreement with previous data for this component [13,15,16,26,28]. These observations indicate that LDS treatment does not perturb severely the geometrical structure of the iron-sulfur cluster of center *X*, since g_{av} and the corresponding *g* factors are sensitive to the composition and spatial arrangement of electronic excited states and the degree of delocalization of the unpaired electron over the atomic constituents of the iron–sulfur cluster [31]. Similarly, the close correspondence of the *g* factors of center *X* in the absence or presence of reduced centers A and B suggests that the unusual range of the *g* factors for this low-potential Photosystem I acceptor results from the inherent geometrical and electronic structure of this cluster and not from dipolar and/or spin-spin interactions with centers A and B.

The spectra of center *X* presented in Fig. 2 and 5 are free from interference from other iron-sulfur resonances (compare with Fig. 2 of Heathcote et al. [15]) and therefore are more tractable for analysis by computer simulation. The most salient characteristic of the experimental spectrum and its simulated counterpart is the anisotropy in the linewidth for the principal resonances ($\Delta H_z = 135$ G; $\Delta H_y = 143$ G; $\Delta H_x = 105$ G). Additionally, we observe that the linewidths for center *X* in the LDS-treated sample are approx. 30% broader than those observed in control particles [15,16]. Linewidth anisotropy in ESR powder spectra may arise from a number of contributions: unresolved hyperfine couplings, dipole–dipole interactions, *g* strain and anisotropic relaxation [32]. Hagen and Albracht have examined the ESR spectrum of spinach ferredoxin (a 2Fe–2S cluster) and have attributed the linewidth anisotropy of this spectrum to an anisotropic distribution of dislocation strains [32]. Extrapolation and application of this hypothesis to interpret the ESR spectrum of center *X* in LDS-treated samples indicate that the presence of the detergent results in an increased flexibility or conformational elasticity of the iron-sulfur cluster, which is expressed by the observed anisotropic broadening of the spectrum in comparison to the control. Alternatively, the broadening of the ESR spectrum in the LDS-treated particle as compared to the dithionite-reduced control particle might result from the absence of exchange-contributions to the linewidth from centers A and/or B. This hypothesis presupposes that the spectrum of center *X* in the presence of reduced centers *A* and *B* is exchanged-narrowed by coupling to one or both of these paramagnets [33]. Dissociation of the Photosystem I reaction center by LDS would disrupt this exchange interaction, thereby giving rise to the broadened spectrum observed in Fig. 2. Differentiation between these two hypotheses will require a detailed analysis of the ESR spectrum of center *X* over a range of microwave powers and temperatures in both control and LDS-treated samples.

The conclusive identification of center *X* with the optical component A_2 mandates that the kinetics of recombination between reduced center *X* and P-700$^+$ correspond to the kinetics of A_2 monitored under similar experimental conditions. The lifetime of center *X* as monitored directly or

indirectly (via P-700$^+$) by ESR at cryogenic temperatures has been reported as clustering in the range approx. 250 ms: 130 ms [34], 250 ms [27], 300 ms [3] and 800 ms [12,35]. In the LDS-treated Photosystem I particle we have observed a 300 ms recombination time for P-700$^+$ X^- (Fig. 3); these kinetics are first-order and monophasic. The similar lifetimes for the P-700$^+$ X^- pair in the control [27,34] and LDS-treated samples suggest that (1) centers A and B do not influence the P-700$^+$ X^- backreaction at cryogenic temperatures, and (2) the native structure of the reaction center is not drastically perturbed by the detergent treatment. The variance of the 300 ms lifetime for reduced center X reported here with the 100 ms lifetime for A_2^- at 77 K reported in the companion paper [9] and with the 130 ms kinetics for X^- and A_2^- at 9 K detailed by Shuvalov et al. [34], most probably has its origin in differences in sample preparation. There are likely to be variations in the observed lifetimes in various preparations due to the fact that electron tunneling is extremely sensitive to the distance between electron carriers.

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