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ELECTRON SPIN RESONANCE STUDIES OF THE BOUND IRON-SULFUR CENTERS IN PHOTOSYSTEM I

PHOTOREDUCTION OF CENTER A OCCURS IN THE ABSENCE OF CENTER B

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The Photosystem I acceptor system of a subchloroplast particle from spinach was investigated by optical and electron spin resonance (ESR) spectroscopy following graduated inactivation of the bound iron-sulfur proteins by urea/ferricyanide solution. The chemical analysis of iron and sulfur and the ESR properties of centers A, B and X are consistent with the participation of three iron-sulfur centers in Photosystem I. A differential decrease in centers A, B and X is observed under conditions that induce $S^{2-} \rightarrow S^0$ conversion in the bound iron-sulfur proteins. Center B is shown to be the most susceptible, while center 'X' is the least susceptible component to oxidative denaturation. Stepwise inactivation experiments suggest that electron transport in Photosystem I does not occur sequentially from X→B→A, since there is quantitative photoreduction of center A in the absence of center B. We propose that center A is directly reduced by X; thus, X may serve as a branch point for parallel electron flow through centers A and B.

Introduction

Stabilization of charge separation in PS I depends, in part, on a complex of electron carriers (A, B and X), which function between the primary electron acceptor, A_1 [1], and the mobile electron carrier, ferredoxin. ESR studies indicate at least two Fe-S clusters on the reducing side of PS I in chloroplasts [2]. Center A (*g* values 1.86, 1.94 and 2.05; $E_{m,7} - 530$ mV) is reduced by illumination at cryogenic temperature when chloroplasts are prepared in the dark [3]. Center B (*g* values 1.89, 1.92 and 2.07; $E_{m,7} - 590$ mV) is photochemically reduced when chloroplasts are prepared under normal room illumination and frozen in the light [3]. Both centers are chemically reduced by dithionite in the dark [4]. Centers A and B are present in equal amounts [5] and show spin-spin interaction in the reduced state; for example, reduction of center B causes the *g* 1.86 line of center A to broaden or shift, becoming inseparable from the *g* 1.89 line of center B.

Chloroplasts illuminated in the presence of dithionite show not only the reduced Fe-S centers, but also signals at *g* 1.76 and 1.86 [6]. The component responsible, named X, is detectable only in samples in which centers A and B are reduced prior to illumination. The identity of X is somewhat uncertain: it probably is an Fe-S protein, although its *g* values are outside the known range for ferredoxins [7].

The sequence in which this assembly of electron acceptors is assumed to function, suggested by redox potentials and order of photoreduction at

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; Chl, chlorophyll, PS, photosystem.

cryogenic temperature, is

$$\text{P-700} \xrightarrow{h\nu} [\text{I}] \rightarrow \text{X} \rightarrow \text{B} \rightarrow \text{A} \rightarrow \text{ferredoxin}$$

where [I] is identical to the optical signal, A_1. Recent work with barley chloroplasts [8], however, indicates that a significant amount of photochemically reduced center B can accumulate at cryogenic temperature, and that chemical reduction of center B can occur before the reduction of center A. Accordingly, the different behavior of centers A and B between spinach and barley chloroplasts suggests a scheme where either center A or center B can serve as electron acceptors from the earlier acceptor 'X' [8].

We undertook the present study in order to clarify the sequence of electron flow in PS I. In this paper, we report an investigation of the bound Fe-S proteins by ESR spectroscopy in spinach PS I particles that have undergone various degrees of S^{2-} to S^0 conversion. Our experimental data indicate a differential sensitivity of centers A, B and X to oxidative denaturation. We use these findings to show that at cryogenic temperature photoreduction of center A can occur in the absence of center B.

Materials and Methods

PS I subchloroplast particles (A-III) were prepared as described in Ref. 9 except that elution from the DEAE-agarose column occurred in buffer containing 0.1% rather than 1% Triton X-100. Prior to inactivation, particles were dialyzed against Tris-HCl buffer (0.05 M, pH 8.8) and 0.1% Triton X-100 to remove the 0.2 M KCl.

The bound Fe-S proteins were inactivated by addition of aliquots of 8 M urea and 0.1 M $K_3Fe(CN)_6$ to a 50-ml suspension of A-III particles at 90 μg/ml Chl. Incubations were terminated by passing the mixture over a Bio-Gel P-4 column previously equilibrated with Tris-HCl buffer (0.05 M, pH 8.8) and 0.1% Triton X-100. At this point, an aliquot was removed for chemical and photochemical analysis and the remainder was concentrated to approx. 400 μg/ml Chl on an Amicon P-30 membrane. All procedures were performed at 0–4°C under low level illumination.

P-700 absorption changes following a 5-μs flash were determined as described previously [10]. The cuvette contained 1 mM ascorbate, 0.076 mM DCIP, 0.1 mM methyl viologen and A-III particles at 40–45 μg/ml Chl. The P-700 chemical difference spectrum was measured with a Cary 14 spectrophotometer equipped with a 0–0.1 slide wire. After the ascorbate-minus-ferricyanide difference spectrum was obtained, excess ascorbate was added to the ferricyanide-containing cuvette and the desired wavelength range was rescanned. The P-700 concentration was calculated using the ΔA between two scans and an extinction coefficient of 64 $mM^{-1} \cdot cm^{-1}$ at 700 nm [11].

Acid-labile sulfide was assayed by a modification of the methylene blue method as described previously [12]. Spinach ferredoxin, a stable source of S^{2-}, served as a standard. Chlorophyll was determined as described in Ref. 13.

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 utilizing a Nicolet 1073 signal averager. Quantitative analysis of the relative amounts of centers A and B was performed by comparison of the experimental spectra with computer-simulated standards. Simulations of the individual powder spectra for centers A and B were performed with a PDP-11/23 apparatus, utilizing a modified version of a FORTRAN program provided originally by Dr. I. Goldberg (North American Rockwell Science Center). Simulation protocol was based on solution of the spin Hamiltonian for an anisotropic, spin = 1/2 system possessing rhombic symmetry ($g_x \neq g_y \neq g_z$). Table I presents the g value assignments for centers A and B utilized for the simulated spectra. The g values given for the 'noninteracting' situation in Table I are those observed in a reaction center when either center A or center B is reduced, but not both centers simultaneously. The case in which both nonheme iron centers are reduced within the same reaction center is described by the parameters given under the 'interacting centers' label. Accurate simulations of composite spectra from centers A and B, such as those presented later,

TABLE I

g VALUES FOR CENTERS A AND B OF PS I

	g_x	g_y	g_z
Noninteracting centers			
Center A	1.86	1.94	2.05
Center B	1.89	1.92	2.07
Interacting centers			
Center A	1.89	1.94	2.05
Center B	1.89	1.92	2.04

require spin-normalized contributions from both noninteracting as well as interacting centers.

Composite spectra of centers A and B in reaction centers exhibiting partial or total reduction or varying contributions from each of the iron-sulfur clusters were simulated via weighted convolution of the individual simulations for each center. The accuracy of these composite spectral simulations (i.e., 'standards') was confirmed by comparison of the computer-generated spectra with experimentally produced spectra in which the relative amounts of reduced centers A and B were known by redox poising. Details of the simulation process will be presented elsewhere (Warden, J.T., unpublished data).

Results

Golbeck et al. [10,14] reported that PS I subchloroplast particles treated with an oxidant, urea, and Triton X-100 showed a progressive loss of P-700 photochemistry with concomitant conversion of S^{2-} to S^0 in the bound Fe-S proteins. The properties of the progressively denatured PS I particles are tabulated in Table II. The control particle contains between 10 and 12 S^{2-}/P-700 and has a Chl/P-700 ratio of less than 30, which is in agreement with earlier results [10]. A comparison of the S^{2-} content and degree of P-700 photobleaching in the inactivated samples demonstrates the relationship between intact Fe-S clusters and long-lived charge separation in PS I. The data also show that P-700, assayed by chemical difference spectroscopy, has a slight sensitivity to urea/ferricyanide solution. We have not, however, corrected the P-700 photochemical data for this loss, since it is likely that the denatured P-700 is predominantly associated with a denatured acceptor system. As was observed earlier, the relationship between intact Fe-S protein and flash-induced P-700 oxidation is approximately linear, which led to the suggestion that inactivation of the bound Fe-S clusters (in a single photosynthetic unit) proceeds in an all-or-none manner [10]. However, our more refined measurements (below) indicate that the inactivation pattern may be more complex than that suggested by the chemical data alone.

The ESR spectra of the same samples obtained after freezing in subdued light and subsequent illumination at 19.5 K are shown in Fig. 1. The control particle (Fig. 1a) exhibits significant photoreduction of center A (g 2.05, 1.94 and 1.86) and partial photoreduction of center B (g 2.07, 1.92 and 1.89). The pronounced shoulder at g 2.07 is a component of center B which becomes apparent when there is no interaction between the two reduced Fe-S centers [8]. This condition is met at

TABLE II

CHEMICAL AND PHOTOCHEMICAL PROPERTIES OF PS I SUBCHLOROPLAST PARTICLES FOLLOWING INACTIVATION WITH UREA/FERRICYANIDE

[Chl], 40 μg/ml; Chl/P-700, 29; ALS/P-700, 11.2; CD, chemical difference; PC, photochemical oxidation; ALS, acid-labile sulfide.

Sample and conditions	$[P\text{-}700]^{CD}$ (μM)	$P\text{-}700^{PC}$ (% control)	[ALS] (μM)
Control	1.38	100	15.5
2 M urea, 2 h	1.49	76	11.7
4 M urea, 3 h	1.37	35	4.9
2 M urea, 5 mM $Fe(CN)_6^{3-}$, 4 h	1.13	30	4.1
4 M urea, 5 mM $Fe(CN)_6^{3-}$, 4 h	0.98	9	2.0

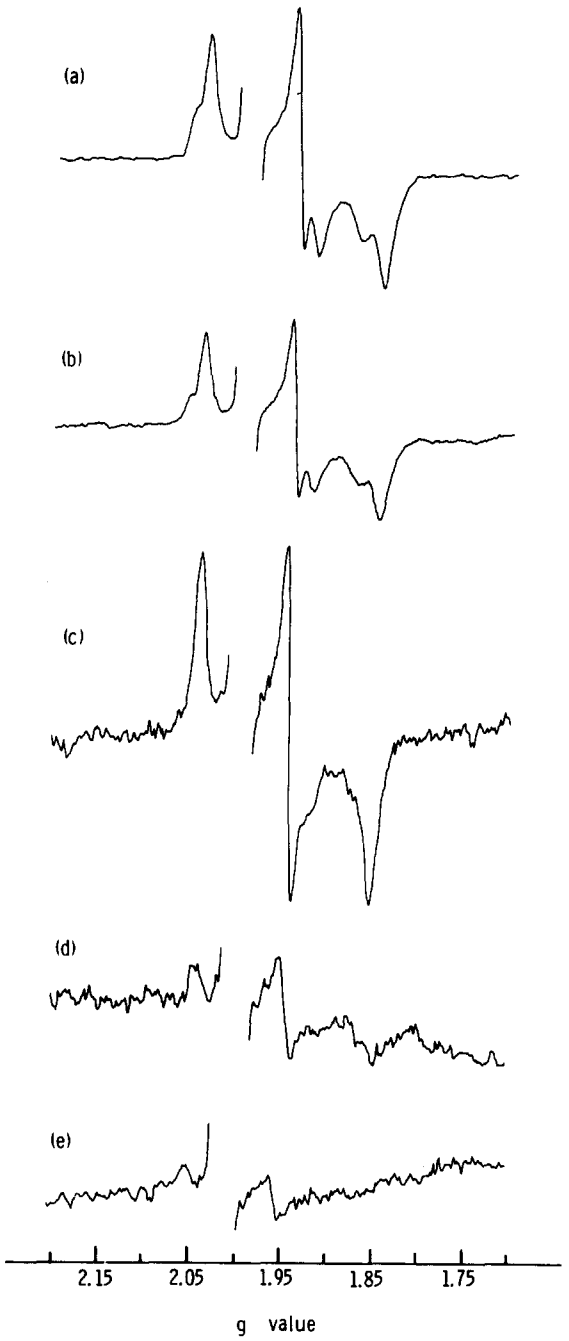

Fig. 1. ESR spectra of centers A and B in control and inactivated particles after illumination at 19.5 K. ESR parameters: microwave power, 10 mW; microwave frequency, 9.25 GHz; receiver gain, $2.5 \cdot 10^3$; modulation amplitude, 16 G; scan width, 2900–3900 G; scan time, 2 min; time constant, 0.3 s. (a) Control, [Chl] 433 μg/ml; (b) 2 M urea sample, [Chl] 386 μg/ml; (c) 4 M urea sample, [Chl] 414 μg/ml, gain×4; (d) 2 M urea, 5 mM FeCN sample, [Chl] 448 μg/ml, gain×4; (e) 4 M urea, 2 mM FeCN sample, [Chl] 417 μg/ml, gain×4.

cryogenic temperature where the one electron available in P-700 is donated to either center A or B in a given photosynthetic unit. A computer simulation of the ESR spectrum shows that 14% of center B is photoreduced, implying that 86% of center A is reduced. This observation indicates that either (1) center A and center B independently accept an electron directly from X or that (2) centers B and A function in series, but in a minority of instances, center B does not transfer its electron to center A.

The ESR spectra of the progressively inactivated PS I particles are presented in Fig. 1b–e. The most significant feature of this series is that the g 2.07, 1.92 and 1.89 lines decline at a faster rate than the g 2.05, 1.94 and 1.86 lines. Quantitative analysis, performed by comparing the spectra with computer-simulated standards, shows that the percentage of electrons trapped in center B decreases from 15% (2 M urea) to less than 10% (4 M urea) to approx. 0% (2 M urea, 5 mM FeCN; and 4 M urea, 5 mM FeCN).

The ESR spectra of the control and inactivated particles after chemical reduction with dithionite and methyl viologen are shown in Fig. 2. As indicated by the presence of the g 1.86 line in the control particle (Fig. 2a), the reduction of center B was not complete. Analysis of the spectrum showed that 45% of center B was chemically reduced. In these studies, we avoided conditions of extremely high pH; although this would have made reduction of center B easier, it could have jeopardized the integrity of a particle that had already been subject to an oxidant and a chaotropic agent. Fig. 2 also shows that the g 2.07 line has disappeared as expected when both Fe-S centers have been reduced in a single photosynthetic unit [8].

Progressing through the denaturation series, we see an unmistakable trend (Fig. 2b–e): center B is more sensitive to denaturation than center A. (The broad underlying resonance in Fig. 2c–e probably is due to Fe released from the bound Fe-S proteins that remains loosely attached to the particle; see Ref. 9.) Quantitative analysis of the data from Fig. 2, along with that of Fig. 1, is shown in Table III. If we assume that centers A and B are present in PS I in equal amounts [5], we find that the 2 M urea sample contains 78% of center A and only 39% of center B and the 4 M urea sample contains

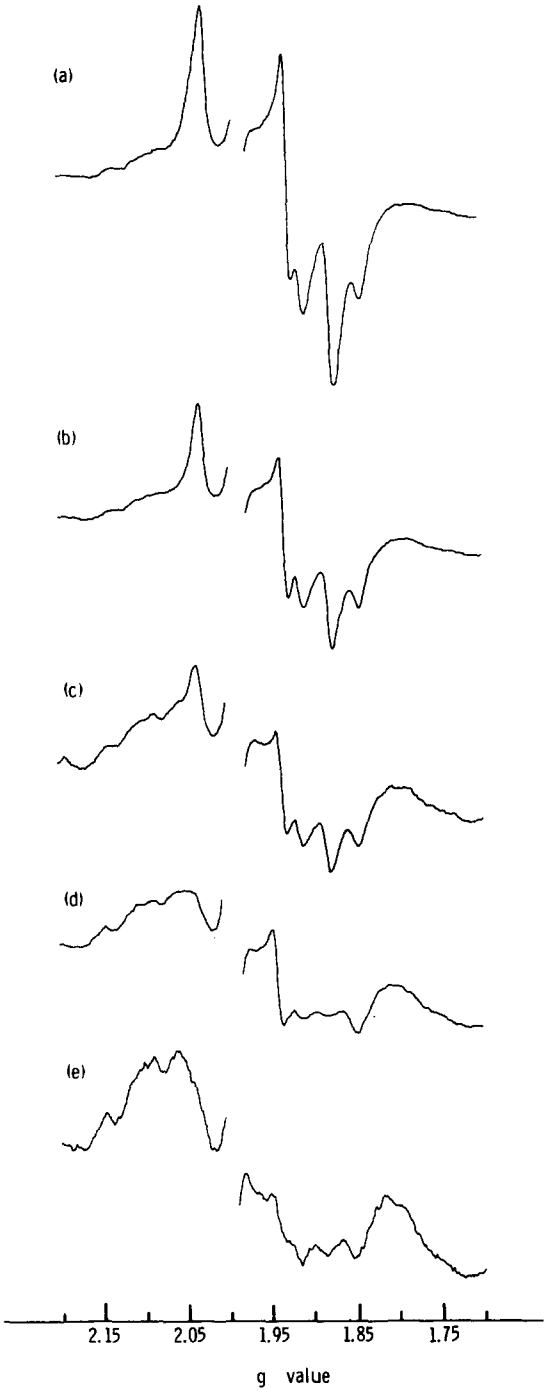

Fig. 2. ESR spectra of centers A and B in control and inactivated particles after chemical reduction with dithionite and methyl viologen at pH 10. ESR parameters identical to those of Fig. 1 except receiver gain was changed to $1.6 \cdot 10^3$. (a) Control; (b) 2 M urea sample; (c) 4 M urea sample, gain×2; (d) 2 M urea, 5 mM FeCN sample, gain×2; (e) 4 M urea, 2 mM FeCN sample, gain×4.

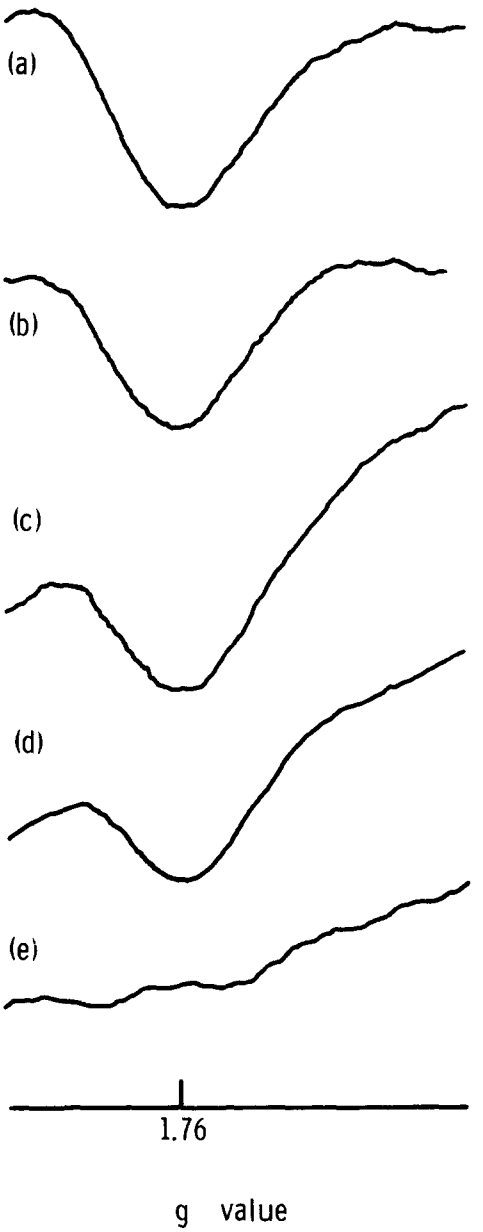

Fig. 3. ESR difference spectrum (light-minus-dark) of high-field peak of X in control and inactivated particles. ESR parameters: temperature, 8.5 K; microwave power, 70 mW; microwave frequency, 9.24 GHz; receiver gain, $4 \cdot 10^2$; modulation amplitude, 32 G; scan time, 2 min; time constant, 1 s. (a) Control; (b) 2 M urea sample, (c) 4 M urea sample, (d) 2 M urea, 5 mM FeCN sample; (e) 4 M urea, 5 mM FeCN sample. The sample was reduced with dithionite and methyl viologen at pH 10 prior to illumination.

40% of center A and only 19% of center B. Clearly, center B is preferentially inactivated by the urea/ferricyanide treatment relative to center A.

The ESR spectra of the high-field peak of X, obtained during illumination of chemically reduced control and inactivated particles, are shown in Fig. 3. Although X is susceptible to oxidative denaturation, it is less sensitive than either center A or B. In order to quantify the signal, we had to consider the fact that subsequent to chemical reduction 45% of center B is in the reduced state. Since the electron in P-700 will reside within component X or center B depending on whether or not B is already reduced, the percentage of intact X can be calculated assuming a 1:1:1 ratio of X:A:B [5]. As shown in Table III, a higher percentage of X survives than either center A or B regardless of the degree of inactivation. To check the assumptions used for calculating the data of Table III, we repeated the ESR experiments on the 2 and 4 M urea samples at pH 12 using aminoiminomethanesulfinic acid as reductant. Both centers A and B became completely reduced, eliminating the need for the correction factors described above. Illumination of the sample under these conditions produced the full extent of component 'X'. The calculated content of centers A, B and X in these two samples agreed with the data shown in Table III.

The complete ESR spectrum of X is best observed in the 2 M urea, 5 mM FeCN sample in which only 15% of centers A and B survives but 42% of X remains intact (Fig. 4). Except for a slight contribution by center A at g 2.05 and 1.94, the spectrum is almost entirely that of X; the g values are 2.08, 1.86 and 1.76. The sensitivity of component X to urea/ferricyanide is consistent with its identification as an iron-sulfur protein. Its greater resistance to inactivation compared to either center A or B indicates that it may be located deeper in the thylakoid membrane.

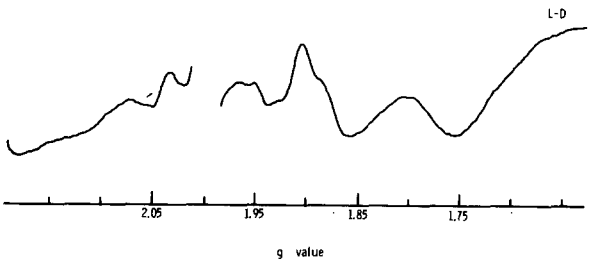

Fig. 4. Complete light-minus-dark (L-D) difference spectrum of X in 2 M urea, 5 mM FeCN sample following chemical reduction with dithionite and methyl viologen. Conditions are identical to those of Fig. 3.

The differential sensitivity of centers A, B and X to oxidative denaturation allows us to determine the sequence of electron flow in PS I. If the electron pathway were linear and obligatory from X → B → A, the amount of photochemically reduced center A at cryogenic temperature (where only one turnover of P-700 is expected) should equal the amount of surviving (chemically reducible) center B. When we compare the photochemical and chemical reduction of the three centers (Table III), we find that the amount of photochemically reduced center A nearly equals the amount of surviving center A, not center B. This quantitative photoreduction indicates that center

TABLE III

QUANTITATIVE ANALYSIS OF ESR SPECTRA PRESENTED IN FIGS. 1–3

All data are normalized for chlorophyll concentration.

Sample and conditions	Photochemically reduced center A (% control)	Chemically reduced (% control)		
		Center A	Center B	X
Control	100	100	100	100
2 M urea	82	78	39	85
4 M urea	41	40	19	66
2 M urea, 5 mM $Fe(CN)_6^{3-}$	11	15	10	42
4 M urea, 5 mM $Fe(CN)_6^{3-}$	<5	<5	<5	<10

A can accept electrons directly from X without the mediation of center B.

Our data can be reconciled with the observation that center B is photoreduced in control chloroplasts following chemical reduction of center A by assuming a parallel operation of centers A and B. In this scheme, X would function as a branch point or 'redox switch'. Under oxidizing conditions, X would transfer its electron to the higher potential acceptor, center A, which, in turn, would reduce (presumably) soluble ferredoxin. When center A is reduced, X would transfer its electron to the lower potential electron acceptor, center B. These conditions might be met during high electron flux where the reduction rate of center A by PS I may be greater than the oxidation rate by its physiological partner.

Discussion

Measurements of bound iron-sulfur center A, center B and X indicate that the urea-induced inactivation pattern may be more complex than the all-or-none model envisioned earlier [10]. Our ESR-based experiments show that the iron-sulfur clusters have differing sensitivities to oxidative denaturation: center B was found to be the most sensitive component while X is the least sensitive. We now believe that the apparent linearity between P-700 photooxidation and labile sulfide content [10] may be the result of an unexpected pattern of denaturation in which the amount of surviving center A simply parallels the percentage of remaining bulk labile sulfide. The lower content of surviving center B is, for the most part, offset by the greater retention of X particularly in the 2 and 4 M urea samples. Since X is always present in amounts greater than is center A, it may be fair to assume that the surviving centers A are linked to functional components X.

In interpreting these results, we have to suppose that exposure to urea does not change the properties of the reaction center and its components to produce a set of unphysiological conditions. Evans and Heathcote [16] have reported that the redox properties of centers A and B as well as the back-reaction kinetics with P-700 are altered when particles are exposed to glycerol and ethylene glycol. Our experimental conditions, however, differ in that our samples are free of perturbant when measurements are taken. We must also assume that the majority of reaction centers participate in electron transport at low temperature, particularly since correlations are made with spectroscopic measurements obtained at room temperature. If not, the subset of reaction centers measured could have properties at low temperature quite different from those of the majority.

Given these cautions, we present the following conclusions. The chemically determined amounts of iron and sulfur, the amount of chemically and photochemically active P-700, and the ESR properties of centers A, B and X are consistent with the participation of three membrane-bound iron-sulfur centers in PS I. Sufficient nonheme iron and acid-labile sulfide remain in the PS I particle (Table II) to allow an additional [2Fe-2S] or [4Fe-4S] cluster if we assume centers A and B are each composed of [4Fe-4S] cores [15]. Both P-700 photochemical oxidation and the ESR signals of centers A, B and X decrease, although the latter does so in an unequal manner, under conditions which induce $S^{2-} \rightarrow S^0$ conversion in the bound iron-sulfur proteins. On the basis of stepwise denaturation, electron transport at cryogenic temperature in PS I does not occur sequentially, since there is significant photoreduction of center A in the absence of center B. Therefore, X must serve as a branch point at which centers A and B function as parallel electron acceptors:

$$\text{P-700} \xrightarrow{h\nu} [\text{I}] \rightarrow \text{X} \begin{matrix} \nearrow \text{B} \\ \searrow \text{A} \end{matrix}$$

Acknowledgements

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