Biochimica et Biophysica Acta 891 (1987) 286–292
Elsevier

BBA 42521

Electron-spin resonance studies of the bound iron-sulfur centers in Photosystem I. II. Correlation of P-700 triplet production with urea/ferricyanide inactivation of the iron-sulfur clusters

Joseph T. Warden [a] and John H. Golbeck [b]

[a] *Department of Chemistry, Rensselaer Polytechnic Institute, Troy, NY and* [b] *Department of Chemistry, Portland State University, Portland, OR (U.S.A.)*

(Received 18 September 1986)
(Revised manuscript received 16 January 1987)

Key words: Photosystem I; ESR; P-700; Triplet; Iron-sulfur protein; (Spinach subchloroplast particle)

Photosystem I charge separation in a subchloroplast particle isolated from spinach was investigated by electron spin resonance (ESR) spectroscopy following graduated inactivation of the bound iron-sulfur centers by urea-ferricyanide treatment. Previous work demonstrated a differential decrease in iron-sulfur centers A, B and X which indicated that center X serves as a branch point for parallel electron flow through centers A and B (Golbeck, J.H. and Warden, J.T. (1982) Biochim. Biophys. Acta 681, 77–84). We now show that during inactivation the disappearance of iron-sulfur centers A, B, and X correlates with the appearance of a spin-polarized triplet ESR signal with $|D| = 279 \cdot 10^{-4}$ cm^{-1} and $|E| = 39 \cdot 10^{-4}$ cm^{-1}. The triplet resonances titrate with a midpoint potential of $+380 \pm 10$ mV. Illumination of the inactivated particles results in the generation of an asymmetric ESR signal with $g = 2.0031$ and $\Delta H_{pp} = 1.0$ mT. Deconvolution of the P-700$^+$ contribution to this composite resonance reveals the spectrum of the putative primary acceptor species, A_0, which is characterized by $g = 2.0033 \pm 0.0004$ and $\Delta H_{pp} = 1.0 \pm 0.2$ mT. The data presented in this report do not substantiate the participation of the electron acceptor A_1 in PS I electron transport, following destruction of the iron-sulfur cluster corresponding to center X. We suggest that A_1 is closely associated with center X and that this component is decoupled from the electron-transport path upon destruction of center X. The inability to photoreduce A_1 in reaction centers lacking a functional center X may result from alteration of the reaction center tertiary structure by the urea-ferricyanide treatment or from displacement of A_1 from its binding site.

Introduction

Contemporary studies of the Photosystem I (PS I) reaction center have revealed a multipeptide complex comprised of a primary electron donor P-700 and an electron-acceptor complex including two transient intermediates, A_0 and A_1, and three membrane-associated iron-sulfur clusters: centers X (A_2), A and B (see Ref. 1 for a review). Elec-

Abbreviations: A_0, transient primary electron acceptor in Photosystem I; A_1, transient secondary electron acceptor in Photosystem I; A_2, Photosystem I electron acceptor assigned to iron-sulfur center X; AIMS, aminoiminomethanesulfinic acid; P-700, Photosystem I reaction center chlorophyll; PS I, Photosystem I.

Correspondence: J.T. Warden, Department of Chemistry, Rensselaer Polytechnic Institute, Troy, NY 12180-3590, U.S.A. BITNET: usera89v@rpitsmts.

tron spin resonance (ESR) studies have provided considerable insight regarding the thermodynamic, quantitative and structural features of these acceptors; however, kinetic analysis of electron transfer on the reducing side of PS I remains ambiguous. Similarly, magnetic interactions between the iron-sulfur clusters complicate quantitative and structural analysis of these clusters and no adequate theory has been presented to account for the resulting variations of lineshape and line position.

In an earlier publication we reported an investigation of PS I particles which were subjected to a graduated inactivation utilizing a combination of urea and ferricyanide [2]. This study focussed on the iron-sulfur clusters in these partially inactivated preparations and detailed a differential susceptibility of centers X, A and B to destruction via oxidation of the cluster to elemental sulfur. Center X was shown to be most resistant to oxidative denaturation, whereas center B was most susceptible. In addition, these sequential inactivation experiments demonstrated that center A could be photoreduced in the absence of center B, thus suggesting that center X serves as a branchpoint intermediate for electron transport on the reducing side of PS I.

In the present study we have extended our investigation of urea/ferricyanide inactivated Photosystem I to examine the relationship between intact iron-sulfur cluster content, primary charge separation and subsequent electron transfers in the reaction center. We demonstrate that progressive destruction of the iron-sulfur clusters is accompanied by the formation of the reaction-center triplet (attributed to ^{3}P-700); however, total inactivation by the denaturant does not affect the microenvironment of the PS I primary donor-acceptor pair. Additionally, we present a redox titration of the PS I triplet, which is consistent with the assignment of this species to P-700, the primary donor of PS I.

Materials and Methods

Triton Photosystem I subchloroplast particles (A-III) were prepared as described previously [2]. Membrane-bound Fe-S centers were inactivated by addition of aliquots of 8 M urea and 0.1 M potassium ferricyanide to the A-III particles with subsequent column chromatography [2]. Acid-labile sulfide was assayed by a modification of the methylene blue protocol [3] and chlorophyll was determined by the method of Arnon [4].

Transient optical absorption changes for P-700$^+$ were measured at 800 nm with a purpose-designed, single-beam spectrophotometer as described in Ref. 5. Flash photolysis was performed on a 3 ml cuvette containing Photosystem I particles at 20 $\mu g \cdot ml^{-1}$/1 mM ascorbate/100 μM 2,6-dichlorophenolindophenol/100 μM methyl viologen. Quantitation of the P-700$^+$ absorption transients was performed in the millisecond time domain, thus excluding interferences from absorption transients associated with ^{3}P-700 and P-700$^+$-A_2^- recombination.

Electron spin resonance spectra were obtained with a Varian E-9 spectrometer equipped with an Air Products LTD-3-100 cryostat [2]. Sample temperatures were monitored with a calibrated carbon resistor situated directly below the 3-mm inner diameter quartz sample tube. Data acquisition and analysis were performed via a hardware interface to a PDP 11/23 computer. Triplet state measurements were made by the actinic light modulation technique [7]. A Princeton Applied Research 5204 Lock-in Analyzer in combination with a Rolfin programmable chopper was utilized for phase-sensitive detection of the photo-excited triplet. Actinic light for ESR experiments was provided by a 1000 W tungsten-halogen source (Oriel) or by a Varian Eimac 300 W xenon arc lamp.

Simulations of ESR spectra were performed with either a PDP 11/23 computer or Sun Microsystems 2 workstation as reported previously [6]. The simulation protocol assumed that the spectra for P-700$^+$, A_0^- and A_1^- could be generated by specifying a Gaussian lineshape and an isotropic g-tensor. Data files containing simulated and experimentally obtained spectra were transferred to a Macintosh 512 K computer (Apple Computer Inc.) for analysis.

Redox titrations were performed aerobically in a lab-constructed titration vessel, fitted with an Ingold combination Pt|Ag/AgCl electrode. The electrode was calibrated utilizing saturated quinhydrone. Potentials were adjusted with freshly

prepared solutions of 0.1 M potassium ferricyanide or 0.1 M ascorbate.

Results and Discussion

As documented previously [2], urea-ferricyanide inactivation of PS I particles results in a progressive loss of P-700 photochemistry with a concomitant loss in ESR-detectable iron-sulfur centers. Fig. 1 illustrates the disappearance of Fe-S centers A and B after nearly complete inactivation of the A-III particles. The control sample displays the characteristic photoreduction at cryogenic temperatures of center A ($g = 2.05, 1.94, 1.86$) and partial reduction (approx. 14%) of center B ($g = 2.07, 1.92, 1.89$). In contrast the spectrum of the inactivated preparation indicates a significant diminution of the Fe-S resonances, consistent with the observation that only 8% of the control P-700 photooxidation could be observed in the millisecond time domain.

Previous studies from our laboratory have established that although PS I subchloroplast particles, when incubated with an oxidant and urea, exhibit a progressive loss in P-700 photochemistry, P-700 content, as assayed by chemical difference spectroscopy, is relatively insensitive to the inactivation treatment (see Table II in Ref. 2). These data indicate that the peptide conformation in the region of P-700 is relatively insulated from the inactivation agents. If inactivation of the PS I particles disrupts secondary electron transport to centers A, B, X and possibly A_1, then concomitant with inactivation one would predict the loss of stable charge separation in PS I with the consequent enhancement of the recombination process, either via (a) direct back reaction from the acceptor, A_0 ($P\text{-}700^+ \cdot A_0^- A_1$) or by (b) annihilation of the $P\text{-}700^+ \cdot A_0 A_1^-$ radical pair. Thus parallel with inactivation of PS I and the loss of the iron-sulfur clusters, one should observe the development of the spin-polarized triplet signal if case (a) is predominant [15]. However, a marked enhancement of the triplet yield would not be expected if case (b) is operative [15]. Fig. 2 illustrates the appearance of the triplet arising from P-700 upon inactivation of PS I particles by urea-ferricyanide treatment. The zero-field splitting constants of the

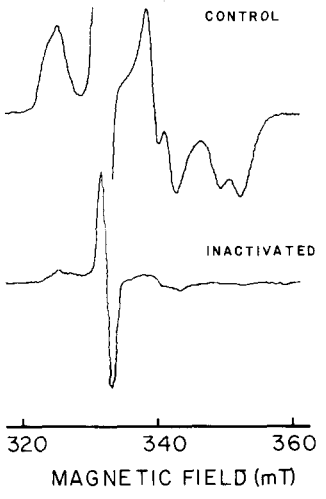

Fig. 1. ESR spectra of iron-sulfur centers A and B in control and inactivated PS I preparations. Chlorophyll, 400 μg ml^{-1} (0.05 M Tris, pH 7.8). ESR parameters: microwave power, 10 mW; microwave frequency, 9248 MHz; modulation amplitude, 2.0 mT; scan time, 4 min; time constant, 0.3 s; temperature, 20 K. Photoreduction of the iron-sulfur centers was performed at 20 K immediately prior to analysis.

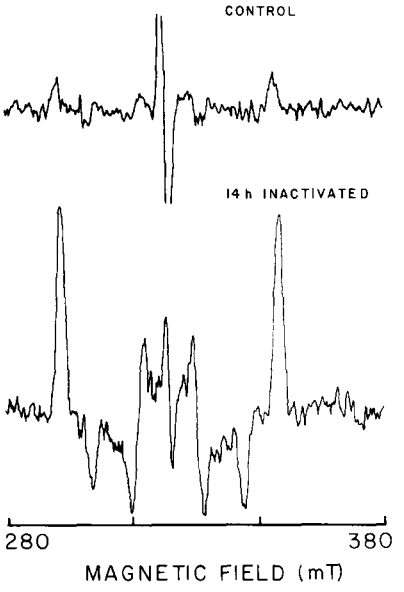

Fig. 2. ESR spectra of the spin-polarized triplet in control and inactivated PS I samples. Chlorophyll, 400 μg ml^{-1} (0.05 M Tris, pH 7.8). ESR parameters: microwave power, 100 μW; microwave frequency, 9248 Mhz; modulation amplitude, 2.5 mT; scan time, 16 min; time constant, 1 s; light modulation frequency, 31 Hz; phase, 299°; temperature 10 K.

PS I triplet ($|D| = 279 \cdot 10^{-4}$ cm^{-1} and $|E| = 39 \cdot 10^{-4}$ cm^{-1}) are consistent with previously reported values [8–11]. The values of these spectroscopic constants remain invariant throughout the time course of inactivation. As indicated by the upper trace in Fig. 2 the control sample exhibits a small triplet complement, even in the absence of reductant. We attribute this observation to a small fraction of reaction centers in which forward electron transport to the iron-sulfur clusters is inhibited, either by destruction of the centers during particle isolation and storage or by photoreduction of the centers during sample handling prior to freezing. However, at no time during our studies have we observed the acceptor radical, A_1, or the putative antenna triplet signal described by McLean and Sauer [10]. Note that in contrast to their procedures, the inactivation technique described herein and previously [2] permits the triplet to be observed in the absence of reductants, denaturing detergents and extreme pH.

If the triplet reflects the P-700$^+A_0^-$ radical pair then preoxidation of P-700 should result in the abolition of the triplet resonances. In order to verify that the polarized triplet resonance is associated with primary photochemistry associated with P-700, the dependence of the ESR triplet amplitude on redox potential has been examined in an inactivated preparation (Fig. 3). As illustrated in Fig. 3, the resonance at 290 mT is significantly diminished at potentials higher than 400 mV. The complete oxidative redox titration of the triplet resonance is illustrated in Fig. 4. The titration is reversible (data not shown) with an apparent midpoint potential of $+380$ mV $\pm$ 10 mV. This value, although lower than that reported for the P-700$^+$/P-700 couple in chloroplasts or membrane preparations [12–14], is consistent with the midpoint potential that we observe for P-700 in highly fractionated subchloroplast particles (Warden, J.T., unpublished data) and also that reported by Evans [14].

In order to characterize more fully the relationship of the PS I triplet to secondary electron transfer in the reaction center, we have investigated the dependence of the triplet on acid-labile sulfur content, and the presence of centers A and B, as monitored by ESR and by optical detection of P-700 photo-oxidation. Fig. 5 demonstrates that

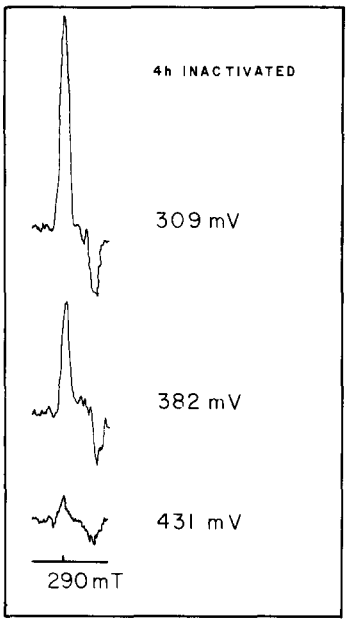

Fig. 3. Redox dependence of the low-field Z_1 resonance of the PS I triplet in inactivated particles. ESR conditions as in Fig. 2.

the decrease in acid-labile sulfur in the PS I preparation is strongly correlated with the induction of the P-700 spin-polarized triplet signal. The magnitude of the triplet resonance after 8 h of inactivation is 90% of that observed in a control sample prereduced with 10 mM AIMS (0.1 M glycine, pH 10.5). Thus although centers A, B and X have been destroyed, as verified independently by ESR, the central core of the reaction center remains essentially intact. This conclusion is fur-

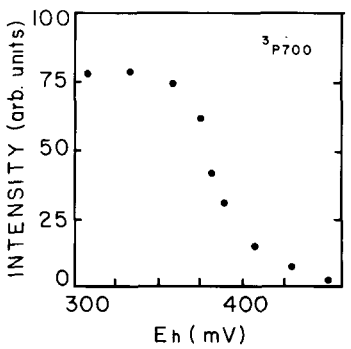

Fig. 4. Redox titration of the triplet signal in inactivated PS I particles. Experimental parameters as in Fig. 2.

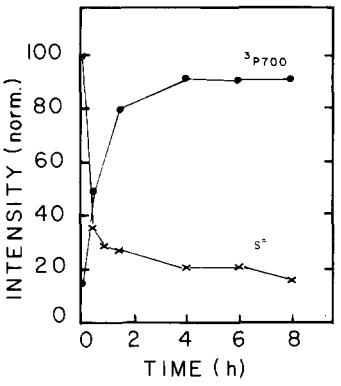

Fig. 5. Time dependence for the development of the PS I triplet signal (^{3}P-700) and the decrease in acid-labile sulfur (S^{2-}) content in particles during inactivation with urea-ferricyanide. Data points are expressed relative to a non-inactivated control sample (0 h).

ther supported by the comparative data illustrated by Fig. 6. The extent of P-700 photooxidation (observable in the millisecond time domain, e.g., electron transport to centers A and B), as monitored by optical flash photolysis, decreases in parallel with the loss of acid-labile sulfur during inactivation of the PS I particles (compare with Fig. 5). However, the magnitude of the ESR triplet resonance, induced by chemical reduction of the preparation, is only diminished slightly throughout the time course of inactivation.

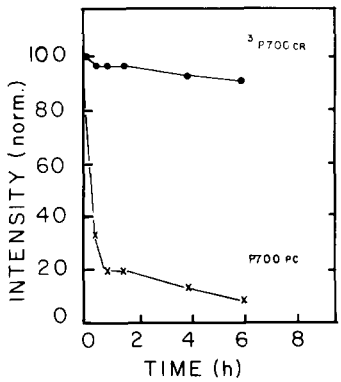

Fig. 6. Time dependence of the ESR-detected PS I triplet (^{3}P-700$_{CR}$) content (observed after prereduction of the particles with 10 mM AIMS/0.1 M glycine, pH 10.5) and photooxidizable P-700 (P-700$_{PC}$) during progressive inactivation with urea-ferricyanide. Data points are presented as in Fig. 5. P-700 transient absorption was monitored at 800 nm, 2 ms post actinic flash.

A closer examination of the $g = 2$ region of the ESR spectrum of the totally inactivated preparation (Fig. 7a) reveals an asymmetric resonance, that is detected by utilizing the light-modulation technique. In contrast to P-700$^+$, which in the control sample exhibits a peak-to-peak (ΔH_{pp}) linewidth of 0.8 mT with $g = 2.0025 \pm 0.0001$, this transient resonance is shifted downfield and is characterized by a ΔH_{pp} of circa 1.0 mT. If this spectrum is comprised solely of contributions from P-700$^+$ and a reduced acceptor, then deconvolution of this component into its constituent spectra is technically feasible. Such a spectral deconvolution assumes the existence of a reliable spectrum for P-700$^+$ (Fig. 7a) and requires that P-700$^+$ and the reduced acceptor occur stoichiometrically. Compliance with the latter criterion dictates that the areas defined by the second integral for each of the component spectra are equal. Deconvolution of the spectrum of the inactivated sample of Fig. 7a is performed by iterative and incremental

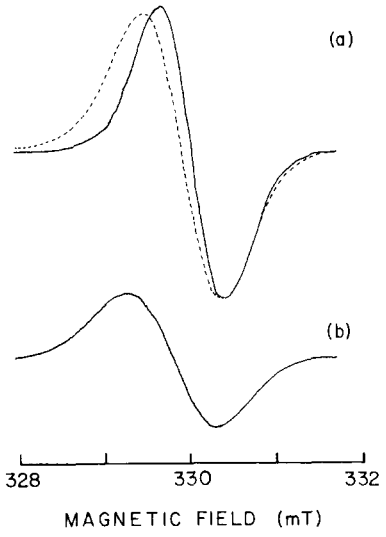

Fig. 7. (a) ESR resonances in the $g \approx 2$ region for control (solid line) and inactivated (dashed) PS I particles. Chlorophyll, 400 μg ml^{-1}. ESR parameters: microwave power, 50 μW; microwave frequency, 9248 Mhz; modulation amplitude, 0.32 mT; scan time, 16 min; time constant, 1 s; light modulation frequency, 31 Hz; phase, 299°; temperature 10 K. Note that the P-700$^+$ spectrum was obtained in the absence of illumination with inactivated particles oxidized with 2 mM potassium ferricyanide. (b) ESR spectrum of the acceptor species in inactivated particles as obtained via the deconvolution process described in the text.

subtraction of the P-700$^+$ resonance from the composite spectrum. The iterative process is terminated when the areas for each of the constituent spectra are equivalent. This deconvolution procedure produces a slightly asymmetric resonance with a linewidth (ΔH_{pp}) of 1.04 ± 0.02 mT and a *g*-factor of 2.0037 ± 0.0002 (Fig. 7b).

An alternative approach for multi-component spectrum analysis is based on the synthesis of a composite spectrum via convolution of the constituent spectra. As a test of our deconvolution procedure for the composite spectrum in Fig. 7a we have attempted to synthesize the composite spectrum via convolution of the P-700$^+$ resonance with admixtures of the resonances for A_0 and A_1. For simulation of trial spectra for A_0 and A_1 we have incorporated a range of published literature parameters [15–18]. Utilizing gaussian lineshape functions and isotropic *g*-tensors for the contributing components, the composite spectrum of Fig. 7 can be constructed by mixing the P-700$^+$ resonance with a signal that is composed of a major component (80%) with a linewidth (ΔH_{pp}) of 1.01 ± 0.02 mT and a *g*-factor of 2.0033 ± 0.0002 and a minor component (< 20%) with a ΔH_{pp} of 1.08 ± 0.02 mT and a *g*-factor of 2.0054 ± 0.0002. The spectral parameters of the major component are consistent with the range of those attributed to A_0 [15–18]. Similarly the minor component can be assigned to A_1 [15,16]. The ESR signal that results from the convolution of the two acceptor resonances (A_0 and A_1) is identical within experimental error with that obtained by the deconvolution technique. In contrast with earlier studies [15], we are unable to obtain a satisfactory fit to the composite spectrum of Fig. 7 if we assign A_0 a *g*-factor of 2.0017.

We had expected to find evidence for predominant photoreduction of A_1 (i.e., appearance of a 1.1 mT resonance with $g \approx 2.005$ [15]) after complete destruction of centers A, B and X. However, the direct observation of the P-700$^+A_0^-$ pair indicates that A_1 does not function to a significant extent as an electron acceptor at cryogenic temperatures in the absence of the iron-sulfur clusters as produced by urea-ferricyanide inactivation. This suggestion is supported by the spectrum analysis data and further by the observed induction of the reaction-center triplet during inhibition (Fig. 5), since recent studies have correlated production of triplet P-700 with the presumed recombination of the P-700$^+A_0^-$ radical pair (and not that of P-700$^+A_1^-$) [15,16,19]. We suggest that A_1 and center X are in close association; destruction of the cluster corresponding to center X apparently leads to an extensive decoupling of A_1 from linear electron flow. Center X and A_1 (phylloquinone by inference) have been shown to reside on the P-700-Chl *a* protein [6,20,21]. The inability to photoreduce A_1 in reaction centers lacking a functional center X may result from either a displacement of A_1 from its binding site or a change in the configuration of the 64 kDa protein which results in the loss of A_1 function. Preliminary analysis of the phylloquinone content in urea-ferricyanide inhibited PS I particles indicates that disruption of electron transport to A_1 is not the result of a loss of the quinone from the reaction center, since control and inactivated particles assay for identical quinone content within experimental error. In this regard we have recently obtained similar evidence for the disfunction of electron transfer between A_0 and A_1 in PS I particles inactivated with lithium dodecyl sulfate (Warden, J.T., Csatorday, K. and Golbeck, J.H., unpublished data).

Acknowledgements

The authors acknowledge the analytical contributions of Gerard Palace for the determination of phylloquinone content in Photosystem I preparations. This research and manuscript preparation were supported by a grant from the National Institutes of Health (GM26133) to J.T.W. and in part by a grant from the National Science Foundation to J.H.G. (DMB-8517391).

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