

Electrogenic reduction of the primary electron donor P700 by plastocyanin in photosystem I complexes

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Abstract An electrometric technique was used to investigate electron transfer between spinach plastocyanin (Pc) and photooxidized primary electron donor P700 in photosystem I (PS I) complexes from the cyanobacterium *Synechocystis* sp. PCC 6803. In the presence of Pc, the fast unresolvable kinetic phase of membrane potential generation related to electron transfer between P700 and the terminal iron–sulfur acceptor F_B was followed by additional electrogenic phases in the microsecond and millisecond time scales, which contribute approximately 20% to the overall electrogenicity. These phases are attributed to the vectorial electron transfer from Pc to the protein-embedded chlorophyll dimer P700⁺ within the PsaA/PsaB heterodimer. The observed rate constant of the millisecond kinetic phase exhibited a saturation profile at increasing Pc concentration, suggesting the formation of a transient complex between Pc and PS I with the dissociation constant K_d of about 80 μ M. A small but detectable fast electrogenic phase was observed at high Pc concentration. The rate constant of this phase was independent of Pc concentration, indicating that it is related to a first-order process. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Photosystem I; Plastocyanin; Electron transfer; Proteoliposome; Electrogenic phase; Flash-induced kinetics; *Synechocystis* sp. PCC 6803

1. Introduction

Photosystem I (PS I) complex is an iron–sulfur type of reaction center (RC) which consists of 11–13 subunits and catalyzes the photoinduced oxidation of plastocyanin (Pc)/cytochrome (cyt) c_6 and reduction of ferredoxin/ flavodoxin [1,2]. The following cofactors are bound to the PsaA/PsaB heterodimeric (82–83 kDa) polypeptides: P700 (a pair of chlorophyll molecules), A₀ (monomeric chlorophyll), A₁ (phyloquinone) and F_X (4Fe–4S center). The terminal acceptors F_A and F_B (4Fe–4S centers) are located in the PsaC (8.9 kDa) subunit.

The light-driven steps within the RC complex involve electron transfer from P700 to F_A/F_B via A₀, A₁ and F_X. On the oxidizing side of PS I, Pc (or cyt c_6) is known to function as an electron donor to the photooxidized P700. In higher plants only Pc is utilized as an electron transfer protein between cyt f in the cyt bf complex and PS I, whereas in algae and cyanobacteria, Pc or cyt c_6 is used, depending on the availability of either copper or iron in the culture medium, respectively [3]. Pc is a small (~ 10 kDa) type I blue copper protein; a copper ion is located at the northern end of the protein. It is generally believed that the hydrophobic patch on the northern surface of Pc surrounding the copper ligand His 87 is crucial for the interaction with PS I, whereas the electron transfer would take place via the surface-exposed imidazole ring of His 87 [4,5].

The three-dimensional structure of PS I from the cyanobacterium *Synechococcus elongatus* has been determined by X-ray crystallography at a resolution of 4 Å [6,7]. The primary donor P700 is localized near the luminal surface of the thylakoid membrane and is therefore accessible to the luminal electron donor proteins Pc and cyt c_6 .

The interaction between Pc and PS I was studied by laser-flash absorption spectroscopy in a variety of photosynthesizing organisms, including higher plants [4,5,8], green algae [9], and cyanobacteria [10–12]. Different reaction mechanisms for PS I reduction were suggested to explain the observed dependences of the electron transfer kinetics on Pc concentration [13].

In addition to absorption flash spectroscopy, the electron transfer efficiency in this system can be studied by electrometric techniques. In our preceding works we studied the reduction of the photooxidized primary electron donor P700⁺ with the native secondary donor cyt c_6 [14], and artificial donors (redox dyes) using a direct electrometric technique [15]. In the presence of high concentrations of redox dyes, the fast generation of the membrane potential related to electron transfer between P700 and the terminal iron–sulfur clusters F_A/F_B was followed by a new electrogenic phase in the millisecond time domain, which contributed approximately 20% to the overall photoelectric response. This phase was attributed to the vectorial transfer of an electron from the redox dye to the protein-embedded Mg-porphyrin ring of P700⁺. Because in the presence of artificial donors the contribution of this phase to the overall electrogenic response was approximately equal to the contribution of the phase observed in the presence of cyt c_6 [14], it was concluded that electrogenic reduction of P700⁺ in vivo occurs as a result of vectorial electron transfer within

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Abbreviations: Pc, plastocyanin; PS I, photosystem I; cyt, cytochrome; P700, chlorophyll dimer; F_X, F_A, F_B, 4Fe–4S clusters; A₀, monomeric chlorophyll; A₁, phyloquinone; RC, reaction center; $\Delta\psi$, transmembrane electric potential difference

PsaA/PsaB heterodimer molecule rather than within the cyt c_6 -P700 complex.

The most typical secondary donor for P700⁺ is Pc, which is the only native donor in higher plants and algae, and also one of two alternative donors in cyanobacteria. So far, the electron transfer between Pc and P700⁺ was mainly studied by flash spectroscopy, but not by electrometric techniques. The goal of this work was to study the Pc → P700⁺ electron transfer reaction using time-resolved electrometry. These studies are expected to provide a new insight into molecular mechanisms of electron transfer in PS I.

2. Materials and methods

Soybean lecithin (type II S), Tris, HEPES, sodium cholate, sodium ascorbate, potassium ferricyanide, CaCl₂, Sepharose 4B, ammonium sulfate and Sephadex G-50 fine were purchased from Sigma (St. Louis, MO, USA). Other reagents were commercial products of the highest purity available.

Pc was isolated from fresh spinach leaves by the procedure of Morand and Krogmann [16]. The concentration of donor protein was determined spectroscopically using an extinction coefficient of 4.9 mM⁻¹ cm⁻¹ at 597 nm for the oxidized form [17].

PS I complexes from *Synechocystis* sp. PCC 6803 were obtained using β-dodecyl maltoside and sucrose gradient ultracentrifugation [18]. Incorporation of PS I complexes into liposomes has been described previously [14].

Photoelectric responses were measured as described in [14,15]. Saturating laser flashes were provided by a frequency-doubled Quantel Nd:YAG laser (wavelength, 532 nm; pulse half-width, 15 ns; flash energy, 40 mJ).

3. Results

In the absence of soluble electron donors and acceptors, the kinetics of dark reduction of P700⁺ is characterized by back reactions from one of the bound acceptors [19]. The addition of the donor, which can donate an electron to the photooxidized P700⁺ more rapidly than the photoreduced acceptor, gives rise to competitive substitution of recombination by direct electron transfer.

Fig. 1A shows flash-induced photoelectric responses of the proteoliposomes containing PS I complexes from *Synechocystis* sp. PCC 6803 adsorbed on the surface of a phospholipid-impregnated collodion film. In accordance with the previously obtained results, the formation of the transmembrane electric

potential difference ($\Delta\psi$) corresponded to the negative charging of the interior of the proteoliposomes [14,20]. $\Delta\psi$ developed within a time shorter than the instrument-limited response time constant of 200 ns and was ascribed to the charge separation between P700 and the terminal iron-sulfur acceptor F_B [20].

Fig. 1A (trace 1) shows the kinetics of $\Delta\psi$ decay in the absence of soluble acceptor. The main component with a lifetime (τ) of ~100 ms corresponds to the charge recombination between P700⁺ and (F_A/F_B)⁻, whereas the minor faster components are attributed to back reactions from F_X and A₁ in a fraction of PS I complexes with impaired forward electron transfer to F_A/F_B. The slowest component ($\tau \approx 700$ ms, 20% of the overall amplitude) is attributed to the passive discharge across the membrane as a result of oxidation of the terminal acceptor by oxygen in the medium. The addition of increasing concentration of reduced spinach Pc resulted in slowing of the $\Delta\psi$ decay (Fig. 1A, trace 2) due to competition of Pc with (F_A/F_B)⁻ for reduction of P700⁺.

Fig. 1B shows the dependence of the relative contribution of the passive discharge slow phase on Pc concentration. At Pc concentrations >100 μM, the relative contribution of this $\Delta\psi$ decay phase exceeded 90% of the overall decay. At Pc concentrations exceeding 50 μM, an additional $\Delta\psi$ rise phase is observed in the kinetics of the photoelectric response (Fig. 1A, trace 3). The kinetics of this phase at a Pc concentration of 400 μM is presented in a faster time scale in Fig. 2A (trace 3). Fig. 2B shows the dependence of relative amplitude of this phase on Pc concentration. The maximal amplitude comprises ~20% of that of the fast phase attributed to the P700⁺(F_A/F_B)⁻ charge separation.

As shown in Fig. 2A (trace 1), the fast unresolved rise in the kinetics of the flash-induced $\Delta\psi$ generation in the absence of added Pc is followed by small decay phases with lifetimes (τ) of ~10 μs and 100–200 μs. According to our previous considerations [20], these lifetimes correspond to the lifetimes of A₁⁻ back reactions derived from optical data [19], however we cannot rigorously exclude that the 5–10 μs decay phase is related to some electrical artifact. It should be noted that increasing concentration of Pc resulted in a proportional decrease in the contribution of these decay phases. At the highest Pc concentration used (Fig. 2A, trace 3) no decay phases in the submillisecond time range were observed. In order to

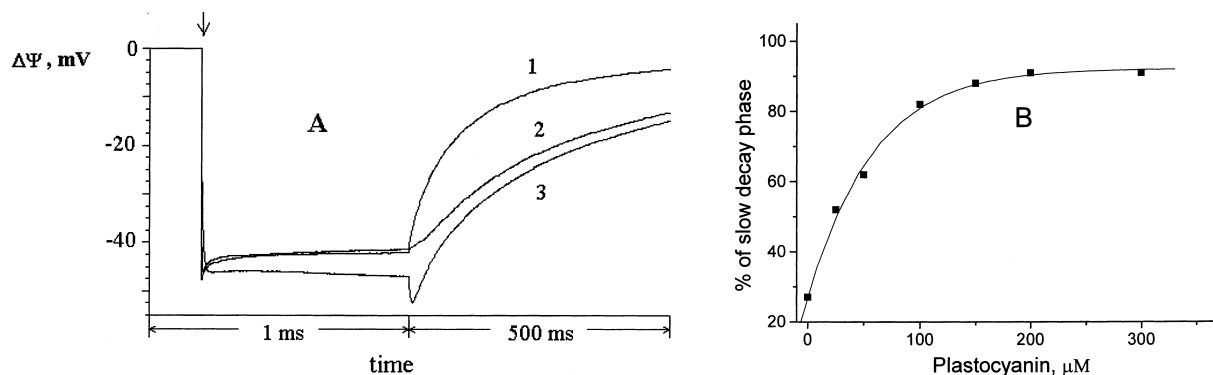


Fig. 1. Effect of Pc on the kinetics of the flash-induced $\Delta\psi$ decay in proteoliposomes containing PS I complexes. A: Representative flash-induced photoelectric response. Trace 1: in the absence of Pc; traces 2 and 3: in the presence of 50 μM and 400 μM of Pc, respectively. B: Dependence of the contribution of the slow decay phase on Pc concentration. The incubation medium contains 25 mM Tris-HCl (pH 8.1), 10 mM sodium ascorbate and 4 μM 2,6-dichlorophenolindophenol. Slow decay phases with lifetimes >500 ms were ascribed to the passive discharge across the membrane.

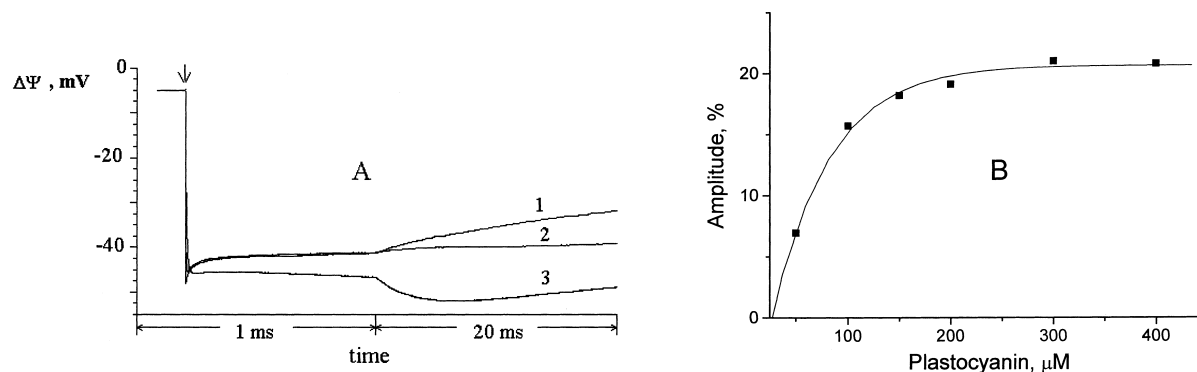


Fig. 2. Effect of Pc on the kinetics and amplitude of the flash-induced $\Delta\psi$ generation in PS I containing proteoliposomes. A: Flash-induced photoelectric responses in the absence (trace 1), and in the presence of 50 μ M (trace 2) and 400 μ M (trace 3) of Pc. B: Dependence of relative amplitude of the millisecond phase of $\Delta\psi$ generation on Pc concentration. Experimental conditions as in Fig. 1.

reveal the kinetic components that appeared in the presence of Pc in the submillisecond time range, the voltage signal obtained in the absence of Pc was point-by-point subtracted from those obtained in the presence of a certain concentration of Pc. The resulting difference between traces 3 and 1 of Fig. 2A is presented in Fig. 3A.

The kinetics is clearly biphasic: the small fast component is characterized by τ of ~ 30 μ s and the slow one by τ of ~ 3 ms. The latter lifetime is similar to the monophasic kinetics presented in Fig. 2A (trace 3). We believe that because both back reactions from A_1^- and a possible electrical artifact are not affected by the addition of Pc, the $\Delta\psi$ kinetics obtained by the subtraction procedure would reflect the true kinetics of $\Delta\psi$ generation in the presence of Pc. The lifetime of the fast kinetic component did not depend on the concentration of Pc, indicating a first-order reaction, while its amplitude increased with increasing Pc concentration. Fig. 3B shows the dependence of the relative contribution of the 30 μ s kinetic component on the Pc concentration. The dependence is virtually linear and contributes $\sim 10\%$ to the overall electrogenic step attributed to the $Pc \rightarrow P700^+$ electron transfer at the highest Pc concentration used (400 μ M).

4. Discussion

Comparative kinetic analysis of electron transfer between Pc and photooxidized P700 in a variety of evolutionarily differentiated organisms made it possible to propose different

reaction mechanisms for PS I reduction [13]. In most primitive cyanobacteria, like *Synechocystis* and *Synechococcus*, a linear Pc concentration dependence of the $P700^+$ reduction rate constant was observed [11]. This kind of behavior corresponds to a simple bimolecular collisional mechanism (type I). In other cyanobacteria, like *Anabaena* and *Pseudanabaena*, and in hybrid systems composed of eukaryotic PS I and cyanobacterial Pc, the pseudo-first-order rate constant of $P700^+$ reduction revealed a saturation profile in the Pc concentration dependence. This profile was attributed to a two-step mechanism (type II), which involved complex formation prior to the electron transfer. The kinetic profiles following type I and type II mechanisms were monophasic, with no detectable fast phases. The electron transfer kinetics between Pc and PS I in higher plants and eukaryotic algae was shown to be two-exponential with a fast first-order rate constant ($t_{1/2} = 10\text{--}20$ μ s) and a slower second-order rate constant. A three-step kinetic mechanism (type III), involving a rate-limiting conformational change within the Pc–PS I complex before intracomplex electron transfer, was suggested to explain the experimental data at saturating Pc concentrations.

The data presented in this work seem to be consistent with a type III mechanism. Indeed, in the presence of Pc, the kinetics of membrane potential generation contains two phases associated with the vectorial electron transfer from Pc to $P700^+$. The saturation behavior of the concentration curves of the kinetic components, for which the reaction is second-order at low Pc concentrations but becomes first-order at high

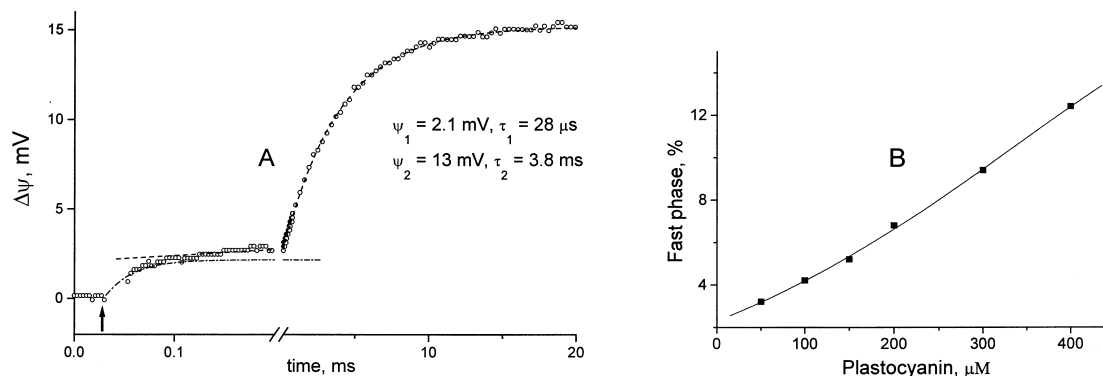


Fig. 3. Kinetics and relative contribution of the fast kinetic component of the $\Delta\psi$ generation. A: Difference between the flash-induced photoelectric responses in the presence of 400 μ M of Pc (Fig. 2A, trace 3) and in the absence of Pc (Fig. 2A, trace 1). B: Dependence of relative amplitude of the 30 μ s kinetic component on Pc concentration. Conditions as in Fig. 1.

concentrations, is similar to what has been observed for bacterial RCs with cyt c_2 [21,22]. A similar kinetic pattern was also shown to be typical of the photoinduced reaction of electron transfer from high-potential iron–sulfur protein to the photosynthetic RC of *Rhodospirillum rubrum* [23]. This behavior was interpreted assuming two mechanisms: (1) RC aggregation at a high concentration of soluble donor in the dark [24] and (2) formation of an equilibrium complex between RC and soluble donor after the excitation flash (see [23] and references therein). Based on the Pc concentration dependence of amplitudes of fast and slow phases of membrane potential generation (Figs. 2 and 3), the dissociation constants for these complexes can be estimated as $K_{d1} > 400$ μM and $K_{d2} \approx 80$ μM , respectively. These values of K_d are larger than the dissociation constants of complexes between Pc and PS I described in the literature [25,26]. This particularly concerns the K_{d1} value. Such an apparent discrepancy is not surprising, because the data given in [25,26] were obtained with spinach Pc and spinach PS I, whereas we studied a hybrid system. Obviously the affinity of spinach Pc for spinach PS I should be higher than the affinity of spinach Pc for cyanobacterial PS I.

Based on these mechanisms, the concentration dependence of the rate of electron transfer from Pc to P700^+ can be calculated using Eq. 1 derived in [27]:

$$k_{\text{obs}} = k_{\text{sm}}[\text{Pc}]_0 / (K + [\text{Pc}]_0) \quad (1)$$

where k_{obs} is the experimentally observed value of the rate constant of the slow phase; $[\text{Pc}]_0$ is the concentration of reduced free Pc in the solution immediately after flash; k_{sm} is the maximum limiting value of the rate constant of the slow phase.

Because the concentration curve of the fast kinetic component was not saturated in our experiments ($K_{d1} > 400$ μM), the value of $[\text{Pc}]_0$ could not be calculated analytically. However, we used the experimental curve shown in Fig. 3 to estimate the fraction of Pc–P700 complexes formed prior to photoexcitation. A fit of the experimental data shown in Fig. 2 to Eq. 1 using the estimated values of $[\text{Pc}]_0$ (Fig. 4) gives $k_{\text{sm}} = 850$ s^{-1} and $K = 420$ μM . This limiting value of k_{sm} is significantly smaller than the rate constant of the fast electrogenic phase (4.89×10^4 s^{-1}), implying the formation of two

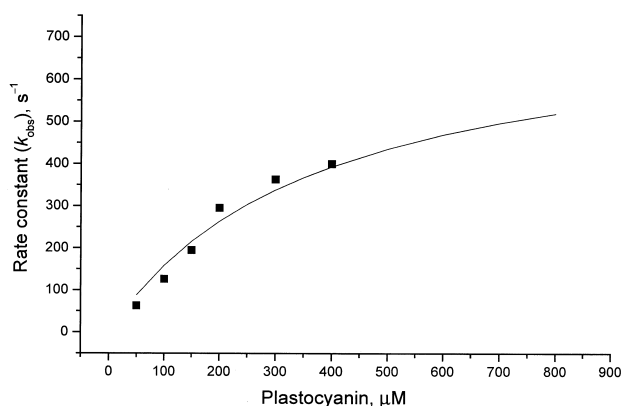


Fig. 4. Experimental points and theoretical curve of the dependence of the millisecond phase rate constant on Pc concentration. The theoretical curve was plotted from Eq. 1 at $k_{\text{sm}} = 850$ s^{-1} and $K = 420$ μM . Conditions as in Fig. 1.

different complexes between Pc and P700. Therefore, the results of this study are consistent with a type III mechanism. However, this conclusion is apparently in conflict with the previous attribution of the interaction observed in hybrid systems to a type II mechanism. The apparent discrepancy is possibly due to the fact that the signal-to-noise ratio achieved in electrometric measurements is much higher than in the conventional flash-spectrometric technique. Indeed, the contribution of the fast phase, even at a very high concentration of Pc, was much smaller ($\sim 10\%$ of the overall process) than in case of higher plants and eukaryotic algae.

This raises the problem of the existence of a pure type II mechanism suggested earlier to explain the monophasic saturation profile in Pc concentration dependence of the kinetics of P700^+ reduction, observed in hybrid systems and some cyanobacteria (see above). It seems more probable that the evolution selected only two mechanisms – a simple collisional mechanism (type I) for primitive cyanobacteria and a more complex three-step mechanism (type III) for the other photosynthetic oxygen-evolving organisms. Generally, this statement is supported by critical analysis of different kinetic mechanisms recently made by Hope [26]. Indeed, a type II mechanism would behave like type I if the internal electron transfer rate were much greater than the association rates. It was also concluded that very likely a type III mechanism may function in all cases, whereas the kinetics may appear to be describable by the other mechanisms because of the relative speeds of the electron transfer or rearrangement steps.

The presently accepted model of PS I indicates that the subunits PsaA and PsaB are almost exclusively responsible for binding the soluble electron donor proteins. The most probable binding site for Pc on the surface of the heterodimer was probed with the aid of computer modeling, and the distance between the Cu^{2+} atom and P700 was estimated to be about 20 Å [28]. As was mentioned above, the surface-exposed imidazole ring of His 87 seems to be crucial for the electron transfer pathway between the copper ion and PS I [4,5]. The distance between the nitrogen atom of His 87 and the copper atom for the reduced Pc is 2.4 Å [29]. Therefore the electron transfer within Pc does not significantly contribute to electrogenicity due to electron transfer from Pc to P700^+ in PS I complexes.

It may be suggested from the X-ray structure of the PS I complex [6,7] that the sum of projections of the center-to-center vectors of electron transfer $\text{P700} \rightarrow \text{F}_B$ onto the membrane normal (D_{PB}) is equal to 53 Å, whereas the projection of the $\text{Pc} \rightarrow \text{P700}$ vector (D_{DP}) is ~ 20 Å. The photovoltage amplitudes, as normalized to the amplitude of the $\text{P700} \rightarrow \text{F}_B$, yield values of 1 and 0.2 relative units for the $\text{P700} \rightarrow \text{F}_B$ and $\text{Pc} \rightarrow \text{P700}$ components of $\Delta\psi$, respectively. Considering that the corresponding electron transfer reactions are essentially irreversible, the ratio between the effective dielectric constant values (ϵ) corresponding to electron transfer from the donor protein (Pc) to P700 and from P700 to F_B ($\epsilon_{\text{DP}}/\epsilon_{\text{PB}}$) is related to the photovoltage drop between Pc and P700 (ψ_{DP}) and between P700 and F_B (ψ_{PB}) by the approximate equation:

$$\epsilon_{\text{DP}}/\epsilon_{\text{PB}} = (D_{\text{DP}}/D_{\text{PB}}) : (\psi_{\text{DP}}/\psi_{\text{PB}}) = 0.38 : 0.2 = 1.9 \quad (2)$$

where D_{DP} and D_{PB} are the projections for the $\text{Pc} \rightarrow \text{P700}$ and $\text{P700} \rightarrow \text{F}_B$ distance vectors to the membrane normal, respectively.

It should be noted that both the location of the Pc binding site and the distance between the copper atom of Pc and Mg-porphyrin rings of P700 are only an estimate, as X-ray structural information on the Pc-PS I complex is lacking. However, the fact that the average dielectric constant value in the Pc→P700 region is about two times higher than in the P700→F_B region is consistent with the three-phase model for the membrane proteins [30]. According to this model, the redox centers that are more remote from the hydrophobic core are usually located in the protein region with the higher dielectric constants.

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