PsaC Subunit of Photosystem I Is Oriented with Iron-Sulfur Cluster F_B as the Immediate Electron Donor to Ferredoxin and Flavodoxin

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ABSTRACT The PsaC subunit of photosystem I (PS I) binds two [4Fe-4S] clusters, F_A and F_B, functioning as electron carriers between F_X and soluble ferredoxin. To resolve the issue whether F_A or F_B is proximal to F_X, we used single-turnover flashes to promote step-by-step electron transfer between electron carriers in control (both F_A and F_B present) and $HgCl_2$-treated (F_B-less) PS I complexes from *Synechococcus* sp. PCC 6301 and analyzed the kinetics of $P700^+$ reduction by monitoring the absorbance changes at 832 nm in the presence of a fast electron donor (phenazine methosulfate (PMS)). In control PS I complexes exogenously added ferredoxin, or flavodoxin could be photoreduced on each flash, thus allowing $P700^+$ to be reduced from PMS. In F_B-less complexes, both in the presence and in the absence of ferredoxin or flavodoxin, $P700^+$ was reduced from PMS only on the first flash and was reduced from F_X^- on the following flashes, indicating lack of electron transfer to ferredoxin or flavodoxin. In the F_B-less complexes, a normal level of P700 photooxidation was detected accompanied by a high yield of charge recombination between $P700^+$ and F_A^- in the presence of a slow donor, 2,6-dichlorophenol-indophenol. This recombination remained the only pathway of F_A^- reoxidation in the presence of added ferredoxin, consistent with the lack of forward electron transfer. F_A^- could be reoxidized by methyl viologen in F_B-less PS I complexes, although at a concentration two orders of magnitude higher than is required in wild-type PS I complexes, thus implying the presence of a diffusion barrier. The inhibition of electron transfer to ferredoxin and flavodoxin was completely reversed after reconstituting the F_B cluster. Using rate versus distance estimates for electron transfer rates from F_X to ferredoxin for two possible orientations of PsaC, we conclude that the kinetic data are best compatible with PsaC being oriented with F_A as the cluster proximal to F_X and F_B as the distal cluster that donates electrons to ferredoxin.

INTRODUCTION

PsaC is a photosystem I-bound, 8.9-kDa polypeptide that contains two [4Fe-4S] clusters termed F_A and F_B (Hayashida et al., 1987). Although its three-dimensional structure has not yet been solved, the main-chain folding pattern of PsaC is presumed to be similar to the small bacterial dicluster ferredoxins from *Peptococcus asaccharolyticus* (Adman et al., 1976) (formerly *P. aerogenes)* and *Clostridium acidi-urici* (Duee et al., 1994), which contain two α-helices near the iron-sulfur clusters and two regions of two-stranded antiparallel β-sheet. In common with these proteins, PsaC is likely to possess a pseudo-C_2 symmetry axis that is oriented perpendicular to a distance vector connecting the two iron-sulfur clusters, F_A and F_B. The amino acid sequence of 2[4Fe-4S] ferredoxins usually contains two CxxCxxCxxxCP iron-sulfur binding motifs (Dunn and Gray, 1988; Oh-oka et al., 1988) in which the first three cysteines in one motif cooperate with the fourth cysteine in the other motif to bind one cubane cluster. The location of F_A and F_B relative to the cysteine ligands was first deduced by in vitro mutagenesis studies (Zhao et al., 1992) and has been confirmed using in vivo mutagenesis (Yu et al., 1997; Jung et al., 1997; Mannan et al., 1996). F_A with principal *g*-values of 1.86, 1.94, and 2.05 and a midpoint potential of -540 mV is identified as the cluster ligated by cysteines 21, 48, 51, and 54. F_B with principal *g*-values of 1.89, 1.92, and 2.07 and a midpoint potential of -590 mV is identified as the cluster ligated by cysteines 11, 14, 17, and 58 (for review see Brettel, 1997).

An electron paramagnetic resonance (EPR) study of membrane-oriented photosystem I (PS I) complexes (Guigliarelli et al., 1993) provided the first indication that the F_A-F_B axis was tilted from the membrane plane, implying that electron transfer from F_X to ferredoxin occurs sequentially through the two iron-sulfur clusters. X-ray crystallographic studies showed that the center-to-center distances between F_X and the two PsaC-bound [4Fe-4S] clusters are 15 Å and 22 Å, respectively; the distance between F_A and F_B is 12 Å ± 0.5 Å; and the distance vector connecting F_A and F_B is tilted 54° (± 5°) from the membrane normal (Krauss et al., 1993; 1996). The full *g*-tensor orientation of F_A and F_B in single crystals of PS I further fixes the orientation of PsaC along a rotation axis that passes through F_A and F_B (Kamlowski et al., 1997a; 1997b). However, because of its pseudo-C_2 symmetric axis, there remains a twofold ambiguity in the orientation of PsaC, leaving the issue of whether F_A or F_B is proximal to F_X unresolved.

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There are thus two possibilities for arranging F_A and F_B in the sequence of electron transfer from F_X to ferredoxin or flavodoxin. 1) The sequence $F_X \rightarrow F_B \rightarrow F_A$ was invoked from the preferential F_B photoreduction in the presence of chemically reduced F_A (Heathcote et al., 1978) and the lack of F_A photoreduction upon F_B destruction by diazonuim benzene sulfonate (Malkin, 1984). This arrangement also complies with the fact that F_A is more electropositive than F_B. Additional evidence for this arrangement follows from studies of rebinding of a mutant PsaC with specific amino acid substitutions around the two iron-sulfur cluster binding sites. Based on the premise of electrostatic interaction between D9 in PsaC (which is in close proximity of F_B) and an arginine of one of the external loops of either PsaA or PsaB, Biggins et al. (1995; Rodday et al., 1996) suggested the most favorable orientation of PsaC has F_B as the proximal cluster to F_X. The lack of efficient reconstruction of a PS I core with a loop-deleted PsaC (Naver et al., 1996) also favors the $F_X \rightarrow F_B \rightarrow F_A$ sequence. 2) The sequence $F_X \rightarrow F_A \rightarrow F_B$ was invoked from the EPR data on efficient photoreduction of F_A in the presence of chemically reduced F_B (Cammack et al., 1979; Nugent et al., 1981) and negligible F_B photoreduction in the presence of chemically reduced F_A (Bearden and Malkin, 1976). Studies on Hg-treated PS I complexes provide the strongest arguments for tk;2F_A as the F_X-proximal cluster. Sakurai et al. (1991) reported that F_B was more easily extracted by $HgCl_2$ treatment, whereas F_A was left almost intact, consistent with lower steric hindrance of F_B. The steady-state rates of electron transfer from plastocyanin to $NADP^+$ or to ferredoxin in spinach PS I (He and Malkin, 1994) and from cytochrome c_6 to $NADP^+$ or to flavodoxin in *Synechococcus* sp. PCC 6301 PS I (Jung et al., 1995) were inhibited by ~70% upon $HgCl_2$-treatment. In the latter study, $NADP^+$ reduction was completely restored upon rebuilding of F_B cluster. This implies that F_B functions as the terminal electron acceptor bound to PS I complex. However, these results are not unambiguous because a lower quantum efficiency of electron transfer might not be apparent in a steady-state measurement and therefore cannot be excluded as an alternative interpretation of low $NADP^+$ reduction rates seen in $HgCl_2$-treated samples.

In this study, we used single-turnover saturating flashes at room temperature to promote a step-by-step electron transfer to the terminal electron acceptor in control (containing both F_A and F_B) and F_B-less PS I complexes in the absence and presence of electron acceptors. By analyzing the kinetics of $P700^+$ reduction kinetics from external donors (phenazine methosulfate (PMS) and 2,6-dichlorophenol-indophenol (DCPIP)), we find that F_B is required for forward electron transfer to ferredoxin or flavodoxin. This result addresses the structural twofold uncertainty in the orientation of PsaC on the PS I complex by supporting the following sequence of electron transfer: $F_X \rightarrow F_A \rightarrow F_B$.

MATERIALS AND METHODS

Trimeric PS I complexes from *Synechococcus* sp. PCC 6301 were isolated using Triton X-100 and sucrose gradient ultracentrifugation (Golbeck, 1995). Preparation of F_B-less TX-PS I complexes by treatment with $HgCl_2$ and reinsertion of the F_B iron-sulfur cluster using $FeCl_3$, Na_2S, and β-mercaptoethanol were performed as described previously (Jung et al., 1995). Recombinant ferredoxin from *Synechocystis* sp. PCC 7002 was overproduced in *Escherichia coli* strain BL21 cells harboring *petF* in the expression plasmid pSE280 (Mühlenhoff et al., 1996). Recombinant flavodoxin from *Synechocystis* sp. PCC 7002 was overproduced in *E. coli* strain BL21 cells harboring *isiB* in the expression plasmid pSE280 (Mühlenhoff et al., 1996). Ferredoxin and flavodoxin were purified as described elsewhere (Bottin and Lagoutte, 1992).

PS I complexes were suspended to a Chl *a* concentration of 50 μg/ml in a 10-mm × 4-mm quartz cuvette. Kinetics of absorbance changes at 832 nm (ΔA_{832}) were measured with a spectrophotometer described previously (Vassiliev et al., 1997), except that the detection beam (power, 30 mW; λ, 832 nm) was provided by a PMT-25 laser diode assembly (Power Technology Inc., Little Rock, AR). Single turnover flashes were provided by a frequency-doubled (λ, 532 nm), Q-switched (FWHM, 10 ns) Nd-YAG laser model DCR-11 (Spectra-Physics, Mountain View, CA) at a flash energy of 10 mJ. Multiple flash excitation at 15-ms intervals was provided by a Xenon flash lamp (FWHM, 10 μs, flash energy ~5 mJ) Model 6100E-72 (Photochemical Research Associates, London, ON, Canada). The multiexponential fits of ΔA_{832} kinetics were performed by the Marquardt algorithm in Igor Pro v. 3.03 (Wavemetrics, Lake Oswego, OR) on a Power Macintosh 7100/88 computer. In multiple flash experiments (Fig. 1), the four kinetics were analyzed by a global two-exponential fit to an equation $A(t) = A_1 \times \exp(-t/\tau_1) + (A_0 - A_1 - A_3) \times \exp(-t/\tau_2) + A_3$, in which τ_1 and τ_2 are the global lifetimes of the components, A_1 is the free running amplitude of the first component, A_3 is the free running baseline amplitude, and A_0 is the global sum of all amplitudes; the amplitude of the second component A_2 is represented by $A_0 - A_1 - A_3$.

Treatment of cyanobacterial PS I complexes with $HgCl_2$ under the conditions specified in Jung et al. (1995) resulted in 90% destruction of the F_B iron-sulfur cluster and in the retention of 80% of the F_A iron-sulfur cluster when assayed by low temperature EPR spectroscopy (data not shown). To obtain kinetic confirmation for the removal of a single iron-sulfur cluster, we used ΔA_{832} measurements and a multiple flash excitation protocol (Sauer et al., 1978) that enables quantitation of the number of photoactive electron acceptors at room temperature in PS I. This approach is based on a selected combination of two experimental conditions. The first condition is the use of a fast electron donor to $P700^+$ (reduced PMS) provided at appropriate concentrations so that it reduces $P700^+$ faster than the back-reaction of $(F_A/F_B)^-$ but slower than the back-reaction of F_X^-. Given that the lifetimes of the back-reaction from $(F_A/F_B)^-$ are ~10 and 80 ms (1:4 amplitude ratio) and the lifetimes of the F_X^- back-reaction (in the presence of F_A^- and F_B^-) are 450 μs and 1.5 ms (5:1 amplitude ratio) (Vassiliev et al., 1997), the 6-ms electron donation from 50 μM PMS provides a favorable forward electron transfer time. The second condition is the use of excitation flashes fired at time intervals (15 ms) that are shorter than the lifetime of $P700^+$ $(F_A/F_B)^-$ but longer than the forward transfer of electron donation from the exogenous donor. When these conditions are satisfied, the first two flashes lead to sequential reduction of F_A and F_B that remain reduced during the time interval before the third excitation flash. Hence, after the first two flashes, $P700^+$ is reduced primarily from PMS, whereas on subsequent flashes $P700^+$ is reduced primarily from F_X^-. The presence of the 6-ms component can therefore be used as an indicator of active forward electron transfer from F_X to one (or both) of the terminal clusters. If a soluble electron acceptor is present in the media, the efficiency of forward electron transfer from F_X^- will depend on the ability of F_A^- and F_B^- to be oxidized by this component.

RESULTS

The global multiexponential fit of the kinetics of control PS I complexes (Fig. 1 *A*) yields two major components, which we denote as the F_X-component (τ, 468 μs) and the PMS-component (τ, 6.1 ms). Ideally, the multiple flash protocol

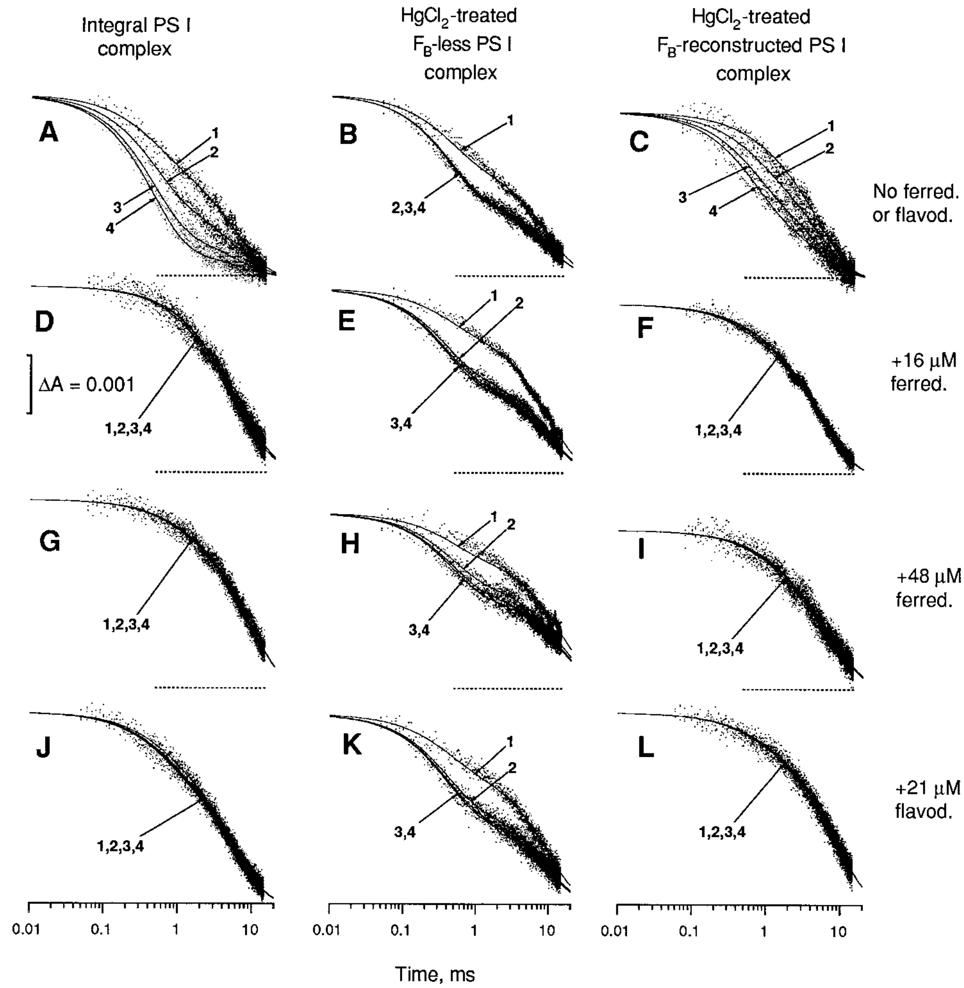

FIGURE 1 Kinetics of absorbance change at 832 nm (ΔA_{832}) of integral (*left column*), $HgCl_2$-treated (*middle column*), and F_B-reconstructed (*right column*) PS I complexes upon excitation with trains of four consecutive flashes at 15 ms intervals; flash numbers are indicated near the traces. The samples were suspended in 25 mM 2-[*N*-morpholino]ethanesulfonic acid buffer, pH 6.3, with 0.02% Triton X-100, 50 μM PMS, and 2 mM sodium dithionite and incubated in the dark for 1 min before excitation. Three sets of four-flash trains separated by 1-min intervals were applied to each sample; then ferredoxin (*2nd* and *3rd rows*) was added to the media at concentrations indicated in the figure, and four-flash trains were again applied at 1-min dark intervals. Flavodoxin (*4th row*) was added to a new sample instead of ferredoxin in identical experimental conditions. The kinetics in response to the flash trains were acquired as single traces, which were then averaged and cut into individual kinetics. The results of the global two-exponential fit are shown as *solid lines*.

calls for the appearance of the PMS-component only after the first two flashes and the appearance of the F_X-component on all subsequent flashes. In practice, mixed kinetics occur in the experiment, which are due, in part, to the relatively close lifetime of the F_X^- back-reaction and the forward electron donation time from PMS to $P700^+$. The presence of the F_X-component on the first two flashes is also a consequence of the unavoidable chemical reduction of a fraction of F_A and/or F_B, and the presence of the PMS-component on subsequent flashes indicates that PMS overrides the reduction of $P700^+$ from F_X^- also in a small percentage of reaction centers. Finally, the small fraction of reaction centers with both clusters reduced is increased on the second flash due to photochemical reduction of the second PsaC-bound cluster in those centers where one of the clusters was chemically prereduced. This leads to an even higher contribution of the F_X-component on the second flash. The net result is that the contribution of the PMS-component is 67% on the first flash and 46% on the second flash, whereas on the third and the fourth flashes it drops to 26 and 16%, respectively.

Consistent with the results of Sakurai et al. (1991), the kinetics of the $HgCl_2$-treated PS I complex on the first flash differs dramatically from those on the second and all subsequent flashes, which are nearly identical (Fig. 1 *B*). The contribution of the PMS-component is 64% on the first flash, whereas on the next three flashes it has lower values of 46, 45, and 46%, respectively. This indicates that unlike the control sample that has different contributions of the F_X^- back-reaction on the second and subsequent flashes, the Hg-treated PS I complex has identical contributions of the F_X^- back reaction on the second and subsequent flashes. Therefore, the F_A cluster acts as an efficient electron acceptor from F_X at room temperature as well as cryogenic temperatures (Jung et al., 1995). A higher contribution of the PMS-component in $HgCl_2$-treated is due to a slight acceleration of the back-reaction between $P700^+$ and F_A^-, which was uncovered in single-flash excitation experiments in the presence of a slow external donor to $P700^+$ (see below; Vassiliev et al., 1997).

The addition of 16 μM ferredoxin affects the kinetic pattern of the ΔA_{832} kinetics in the control but not in the $HgCl_2$-treated PS I complexes (Fig. 1, *D* and *E*). An increase in ferredoxin concentration up to 48 μM does not affect the kinetics in the $HgCl_2$-treated complexes (Fig. 1, *G* and *H*). Addition of 21 μM flavodoxin as an alternative electron acceptor also leads to a complete elimination of the flash-number-dependency in the control but not in the $HgCl_2$-

treated PS I complexes (Fig. 1, *J* and *K*). A large increase in flavodoxin concentration up to 83 μM has no additional effect on the $HgCl_2$-treated complexes (not shown). To show that the lack of effect of ferredoxin and flavodoxin on $HgCl_2$-treated complexes is due to the loss of the F_B cluster and not to damage to the ferredoxin/flavodoxin docking site, we reconstructed the F_B cluster in the Hg-treated PS I complexes and repeated the ΔA_{832} kinetic measurements. As shown in Fig. 1 *C*, the normal flash number dependency is almost completely restored, and the addition of either ferredoxin (Fig. 1, *F* and *I*) or flavodoxin (Fig. 1, *L*) leads to the complete loss of the flash number dependency.

Additional evidence for the lack of electron transfer to ferredoxin and flavodoxin in $HgCl_2$-treated PS I complexes is provided by single flash experiments in the presence of reduced DCPIP, a slow electron donor to $P700^+$. Unlike our previous study (Vassiliev et al., 1997), we used aerobic rather than anaerobic conditions so as to provide oxygen as an electron trap when using methyl viologen as the immediate electron acceptor. Under aerobic conditions the terminal iron-sulfur cluster of PS I is reoxidized by oxygen present in solution, and the contribution of the component arising because of direct reduction of $P700^+$ from DCPIP (4- to 10-s lifetime) is 35 to 45% (Fig. 2), which is about two times higher than under anaerobic conditions (Vassiliev et al., 1997). Addition of 30–100 μM ferredoxin leads to a significant increase (up to 75%) of the contribution of the DCPIP-mediated component in the control PS I complex (Figs. 2 *A* and 3 *A*). In the $HgCl_2$-treated PS I complex, most of the back-reaction is derived from F_A^- with life times of 17 and 91 ms (47 and 27% amplitude, respectively) and with a negligible contribution of the slower DCPIP-component that can be resolved in most of these experiments only as a baseline (5 to 8% amplitude). The remainder of the ΔA_{832} decay is brought about by components with lifetimes of 61 μs (11%) and 1.9 ms (10%), which arise because of back-reactions of A_1^- and F_X^-. The contribution of the latter two components is lower than we found in the $HgCl_2$-treated sample in our previous work (Vassiliev et al., 1997) and indicates an even greater retention of F_A in these samples. Unlike the control PS I complex, addition of ferredoxin up to 430 μM has no effect on the kinetics of the $HgCl_2$-treated PS I complex (Figs. 2 *B* and 3 *B*).

Methyl viologen is an efficient electron transport mediator between the acceptor side of PS I and molecular oxygen (Hiyama and Ke, 1971). As the photo-reduced terminal acceptor of PS I cannot participate in the recombination reaction because of its efficient oxidation by methyl viologen (Figs. 2 *A* and 3 *B*), greater than 90% of $P700^+$ is reduced by DCPIP in the presence of 50 μM methyl viologen. This concentration of methyl viologen has no effect on $HgCl_2$-treated complex, but an additional increase of its concentration to the millimolar range results in more than 80% of $P700^+$ reduction occurring from DCPIP (Fig. 3 *B*; also see Fujii et al., 1990). Hence, unlike ferredoxin or flavodoxin, methyl viologen has the ability to accept electrons from F_A, but only at a concentration nearly two orders of magnitude greater than is required in control PS I complexes.

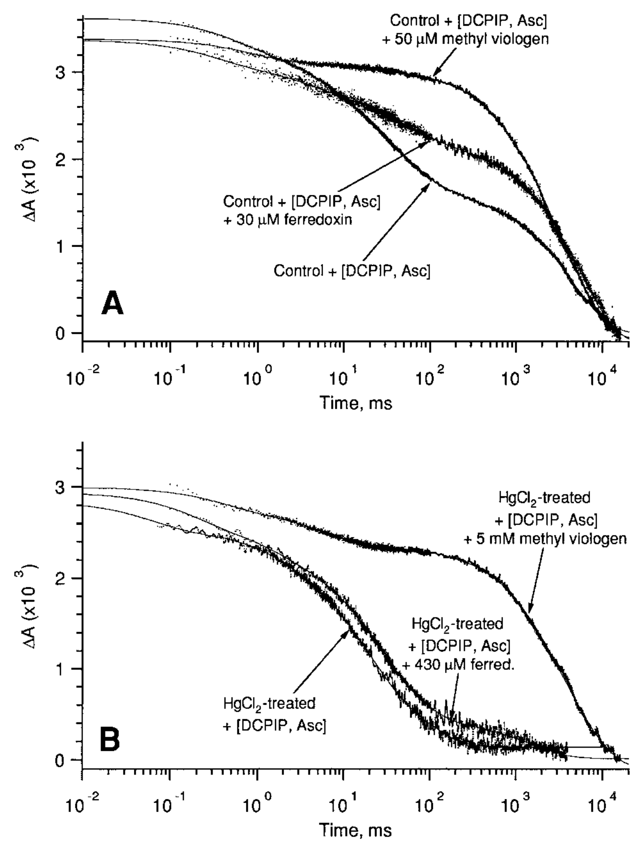

FIGURE 2 Kinetics of ΔA_{832} decay in control (*A*) and $HgCl_2$-treated (*B*) PS I complexes in 25 mM Tris buffer, pH 8.3, with 0.02% Triton X-100, 10 mM sodium ascorbate, and 4 μM DCPIP in the absence and in the presence of ferredoxin or methyl viologen upon laser flash excitation, average of 12 traces acquired at 150-s intervals. Multiexponential fits are shown as *solid lines*.

DISCUSSION

Even though F_A and F_B were among the first bound electron transfer cofactors discovered in PS I, the issue of whether electron transfer proceeds from $F_X \rightarrow F_A \rightarrow F_B$ or from $F_X \rightarrow F_B \rightarrow F_A$ remained unclear. The reason for this uncertainty lies mostly in the identical optical signatures of F_A and F_B, which disallows the use of time-resolved optical spectroscopy to distinguish one acceptor from another at room temperature. Consequently, most of the functional data on the photoreduction of the iron-sulfur clusters has been provided by EPR measurements performed at cryogenic temperatures. However, even though F_A and F_B have distinguishable *g*-tensors, the time resolution of EPR is insufficient to perform the requisite kinetic measurements.

In attempting to resolve this issue, we examine our kinetic data in the context of previous work and in light of the two possible orientations of PsaC.

A possible orientation of PsaC would be that first F_B is proximal to F_X. In the absence of F_B the electron transfer between F_X and F_A would need to span a center-to-center distance of 22 Å. The distance between F_A and the iron-sulfur cluster on ferredoxin will likely be unchanged. Given

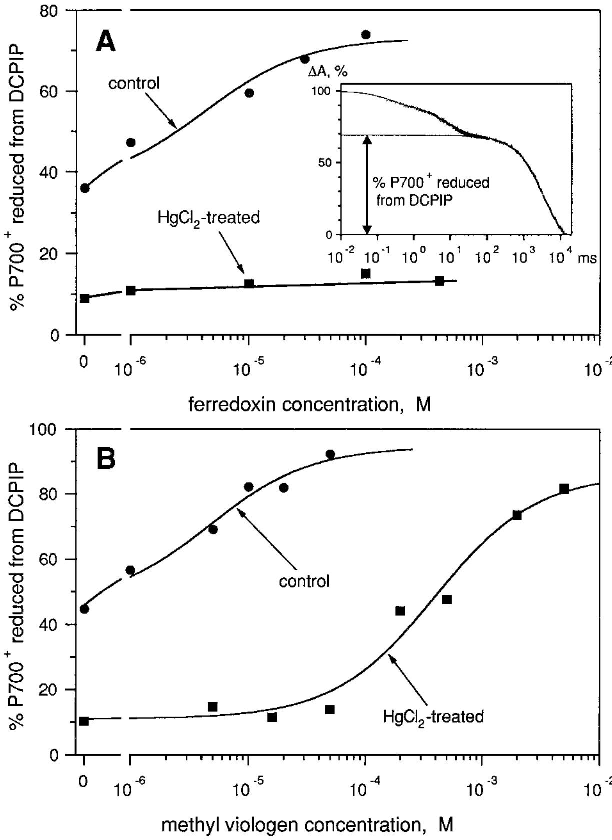

FIGURE 3 The dependency of the amplitude of the slow (4 to 10 s) component arising caused by direct reduction of $P700^+$ from DCPIP on the concentration of ferredoxin (*A*) and methyl viologen (*B*) in control (*circles*) and $HgCl_2$-treated (*squares*) PS I complexes. The basic media consisted of 25 mM Tris buffer, pH 8.3, with 0.02% Triton X-100, 10 mM sodium ascorbate, and 4 μM DCPIP. The amplitudes are normalized to the initial amplitude of the absorbance change derived from the multiexponential fit as shown in the *inset*.

that the photoreduction of F_A is highly efficient at both cryogenic (Jung et al., 1995) and room temperatures (this study), it is difficult in this model to rationalize the failure of ferredoxin and flavodoxin to become reduced. Although it might be argued that the binding site is destroyed by the Hg-treatment, it would need to be a reversible loss as steady-state rates of $NADP^+$ photoreduction (Jung et al., 1995) and flash-induced ferredoxin and flavodoxin reduction (this study) are restored when F_B is rebuilt with β-mercaptoethanol, inorganic iron, and sulfide. Additionally, the binding site for ferredoxin and flavodoxin is likely to involve PsaD (and possibly PsaE) rather than PsaC (Chitnis et al., 1995). In principle, conformational changes in PsaC induced by the loss of F_B could be transmitted to PsaD (and perhaps PsaE). However, the absence of PsaD leads to large changes in the EPR spectrum of F_A and F_B, implying that the g-tensor is sensitive to protein conformation (Li et al., 1991; Chitnis et al., 1996). Therefore, conformational changes in PsaC should have resulted in significant changes in the EPR spectrum of F_A. Yet, with the exception of a slight upfield shift of the g_x resonance, the EPR spectrum of F_A is identical to that of the control (Jung et al., 1995). Any changes in the binding site for ferredoxin and flavodoxin also should be irrelevant to the rates of reduction of methyl viologen. Given that this is likely to be a diffusion-mediated process that does not require a docking site, it is difficult to rationalize the need for a two-order of magnitude increase in the concentration required for electron acceptance from F_A^-. This orientation of PsaC is also incompatible with distance versus rate considerations. Although no direct data on the rate of F_A photoreduction exist, analysis of various spectroscopic and electrometric data yields lifetime values of forward electron transfer from F_X^- ranging between 50 and 800 ns (Brettel, 1997). Assuming λ (a coefficient that depends on the intervening medium in propagating the wave function) of 1.4 $Å^{-1}$ for electron transfer in proteins and given an electron transfer rate constant of 10^{13} at van der Waals contact (Moser et al., 1992), a 7-Å increase in distance leads to a prediction of an about 18,000-fold increase in the electron transfer time up to a value ranging from 900 μs to 14 ms (Fig. 4 *A* for illustration). The $P700^+$ F_X^- recombination kinetics in the $HgCl_2$-treated PS I complex (with 100 mM dithionite) is mainly composed of two components with lifetimes of 270 μs and 842 μs at approximately 1:1 ratio (not shown). Such kinetics are consistent with those found in integral PS I complexes with prereduced F_A and F_B and in PsaC-devoid core preparations (Vassiliev et al., 1997). Therefore, if the F_B cluster is located between F_X and F_A, the large increase of the forward electron transfer rate from F_X^- should have led to a measurable increase in the contribution of the F_X^- back-reaction.

A second possible orientation of PsaC would be that F_A is proximal to F_X. EPR studies in urea-ferricyanide-treated

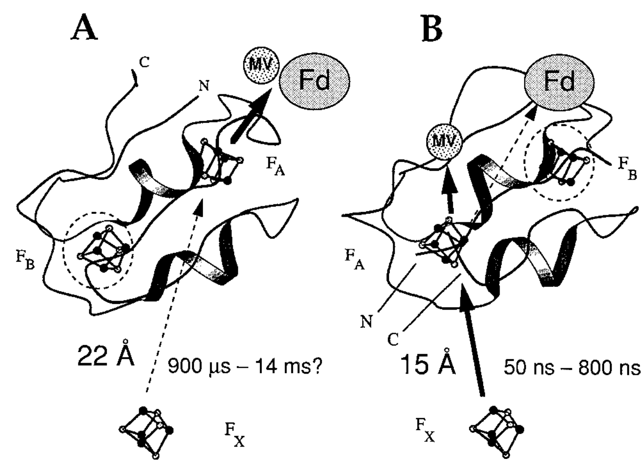

FIGURE 4 Schematic representation of possible electron transport pathways from F_X to exogenously added ferredoxin (Fd) and methyl viologen (MV) for two possible orientations of PsaC assuming that the cluster proximal to F_X is F_B (*A*) or F_A (*B*). The structural diagrams of PsaC are reprinted from Kamlowski, A., A. Van der Est, P. Fromme, N. Krauss, W. D. Schubert, O. Klukas, and D. Stehlik. 1997. The structural organization of the PsaC protein in Photosystem I from single crystal EPR and x-ray crystallographic studies, *Biochim. Biophys. Acta* 1319:185–198, with kind permission of Elsevier Science—NL, Sara Burgerhastraat 25, 1055, KV Amsterdam, The Netherlands.

PS I complexes that preferentially retain F_A rather than F_B (Golbeck and Warden, 1982), kinetic measurements in Hg-treated PS I complexes, which totally lack F_B (Sakurai et al., 1991), and the restoration of NADP+ reduction after reconstitution of F_B in Hg-treated PS I complexes (Jung et al., 1995) provide the strongest arguments for F_A as the F_X proximal cluster. The one lingering issue is that a lower quantum efficiency of electron transfer caused by the irreversible loss of the ferredoxin and flavodoxin docking site cannot be excluded as an alternative interpretation of the results of He and Malkin (1994) and Jung et al. (1995). In this study, we eliminated this contingency by showing in a F_B-less PS I complex: 1) a normal level of photochemical activity of P700, based on the amplitudes of the photoinduced absorbance change at 832 nm; 2) an unimpaired functioning of F_A at room temperature based on a high yield of charge recombination on the tens-of-ms time scale between $P700^+$ and F_A^-; 3) lack of direct electron transfer from F_A^- to either ferredoxin or flavodoxin; and 4) a decreased efficiency of methyl viologen reduction in the absence of F_B, which implies a diffusion barrier but a normally functioning F_A cluster. In the absence of F_B, the electron transfer between F_X and F_A would remain at a center-to-center distance of 15 Å. This is compatible with the efficient reduction of F_A at both cryogenic (Jung et al., 1995) and room temperatures (this study). The distance between F_A and ferredoxin is difficult to judge given that the three-dimensional structure of PsaC as well as the binding site and orientation of ferredoxin on the PS I complex are only known to approximation. Assuming for the purpose of argument that the distance vector between F_A and the [2Fe-2S] cluster in ferredoxin passes through F_B, an additional distance of 12 Å would need to be spanned without participation of a cofactor if F_B is absent (Fig. 4 *B*). Reduction of both ferredoxin and flavodoxin involves complex formation preceding electron transfer, which follows multicomponent kinetics (Hervas et al., 1992; Medina et al., 1992). Three phases with halftimes of 500 ns, 20 μs, and 100 μs have been attributed to reduction of ferredoxin on a single flash (Sétif and Bottin, 1994). The increased distance (without mediation by a redox-active cofactor) leads to the prediction of a 2.1×10^7-fold increase in the electron transfer time to $\sim$10 s for the fastest phase of ferredoxin reduction. This is about two orders of magnitude slower than the $P700^+$ F_A^- back-reaction and would result in negligible quantum yield of ferredoxin reduction, which agrees with the experiment. The recovery of photoreduction of ferredoxin and flavodoxin on a single turnover flash (this study), as well as the recovery of steady-state $NADP^+$ photoreduction mediated by ferredoxin and flavodoxin (Jung et al., 1995), correlates with the restoration of a functional F_B cluster as an essential intermediate electron carrier. A small molecule such as methyl viologen with a mass of 186 is capable of accepting the electron from F_A^- but at a concentration of two orders of magnitude greater than is required in the control. We attribute this to steric hindrance in which a higher concentration of methyl viologen is required to overcome the diffusion barrier to the buried F_A cluster.

The finding that F_X fails to donate electrons to ferredoxin and flavodoxin when F_B is missing and that it succeeds in donating to ferredoxin and flavodoxin when F_B is restored is best compatible with an orientation of PsaC with F_A as the F_X proximal iron-sulfur cluster. The implied electron transfer sequence is therefore $F_X \rightarrow F_A \rightarrow F_B \rightarrow$ ferredoxin. Note that this orientation of PsaC involves an electron transfer from F_A (E_m, −540 mV) to a more electronegative acceptor, F_B (E_m, −590 mV), which results in the presence of a positive Gibbs free energy step in PS I (given that the known midpoint potential values for the [4Fe-4S] clusters determined for PS I at cryogenic temperatures apply at room temperature and that reduced F_A^- does not influence the determination of midpoint potential of F_B). It is noteworthy that ferredoxin reduction is not affected in the K52S/R(53)A mutant of PsaC in which the preferential photoreduction of F_B is attributed to a more negative redox potential of F_A (Fischer et al., 1997). Recent studies indicate that small positive Gibbs free energy changes may be common in multicofactor enzymes; examples are found in two segments of mitochondrial respiratory chain (Ohnishi and Salerno, 1982), [NiFe] hydrogenase (Fontecilla-Camps, 1996), and the tetraheme cytochromes of bacterial reaction centers (Nitschke et al., 1993). The relevance, if any, of this uphill electron transfer step to PS I function is unknown. The important issue for an efficient electron transfer from the primary donor of PS I to ferredoxin is a net negative change in Gibbs free energy from A_1 to ferredoxin, which still occurs in the $A_1 \rightarrow F_X \rightarrow F_A \rightarrow F_B \rightarrow$ ferredoxin sequence.

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