Control of Electron Transport in Photosystem I by the Iron-Sulfur Cluster F_X in Response to Intra- and Intersubunit Interactions*

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Photosystem I (PS I) is a transmembraneal multisubunit complex that mediates light-induced electron transfer from plastoeycanine to ferredoxin. The electron transfer proceeds from an excited chlorophyll a dimer (P700) through a chlorophyll a (A0), a phylloquinone (A1), and a [4Fe-4S] iron-sulfur cluster F_X, all located on the core subunits PsaA and PsaB, to iron-sulfur clusters F_A and F_B, located on subunit PsaC. Earlier, it was attempted to determine the function of F_X in the absence of F_A/B mainly by chemical dissociation of subunit PsaC. However, not all PsaC subunits could be removed from the PS I preparations by this procedure without partially damaging F_X. We therefore removed subunit PsaC by interruption of the psaC2 gene of PS I in the cyanobacterium Synechocystis sp. PCC 6803. Cells could not grow under photosynthetic conditions when subunit PsaC was deleted, yet the PsaC-deficient mutant cells grew under heterotrophic conditions and assembled the core subunits of PS I in which light-induced electron transfer from P700 to A1 occurred. The photoreduction of F_X was largely inhibited, as seen from direct measurement of the extent of electron transfer from A1 to F_X. From the crystal structure it can be seen that the removal of subunits PsaC, PsaD, and PsaE in the PsaC-deficient mutant resulted in the braking of salt bridges between these subunits and PsaB and PsaA and the formation of a net of two negative surface charges on PsaA/B. The potential induced on F_X by these surface charges is proposed to inhibit electron transport from the quinone. In the complete PS I complex, replacement of a cysteine ligand of FX by serine in site-directed mutagenesis C565S/D566E in subunit PsaB caused an ~10-fold slow down of electron transfer from the quinone to F_X without much affecting the extent of this electron transfer compared with wild type. Based on these and other results, we propose that F_X might have a major role in controlling electron transport through PS I.

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§§ The abbreviations used are: PS I, photosystem I; Chl, chlorophyll; DCMU, 2,6-dichlorophenolphosphol; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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inefficient or inhibited will charge recombination between intermediate carriers and the primary donor occur. The rate of recombination increases with decreasing distance of the electron carrier from the primary donor and can be used to determine the efficiency of the reduction of a given carrier. The rate of recombination therefore is expected to increase if the electron transfer to $F_X$ become less efficient in response to the redox state of $F_{A/B}$, to the absence of these iron-sulfur clusters, to temperature, and possibly to the subunit interaction in PS I. Thus, at low temperature, the single turnover flash reduction of $F_X$ was either partially or totally inhibited (12, 18, 19). In the absence of an extrinsic acceptor, following a single turnover flash the $t_{1/2}$ of 45 ms for the reduction of $P700^-$ was assigned to the recombination of $P700^- F_{A/B}^-$. When $F_{A/B}$ were pre-reduced by dithionite, a one turnover flash $t_{1/2}$ of 250 $\mu$s was determined (21). Although it was expected that when $F_{A/B}$ were pre-reduced the recombination would occur between $P700^-$ and $F_X^-$, spectral analysis of this reaction in cyanobacterial PS I indicated that $A_1^-$ was the dominant electron donor in this recombination; thus electron transfer from $A_1$ to $F_X$ appeared to be largely inhibited under such conditions (19). Removal of $F_{A/B}$ by dissociation of subunit PsaC with urea treatment resulted in a $t_{1/2}$ of 1.2 ms for most of $P700^-$ reduction. In the absence of final iron-sulfur clusters of PS I it was reasonable to attribute the reduction of $P700^-$ to recombination with $F_X^-$ (22, 23). Indeed under such conditions the rate of electron transfer from $A_1$ to $F_X$ was reported to be the same as in untreated PS I (9). Yet in PS I lacking PsaC by genetic deletion of the subunit (24) or as a result of mutation in the ligands of $F_{A/B}$ (25), only partial reduction of $F_X$ was observed. Although electron transfer to $F_X$ was not directly measured in these works, a significant part of the total reduction of $P700^-$ proceeded at a rate that was faster than the rate of $P700^-/F_X^-$ recombination. This indicated that complete removal of subunit PsaC, and the iron-sulfur clusters $F_A$ and $F_B$ partially inhibited forward electron transport from $A_1$ to $F_X$. In this work we report the effect of the interruption of the psaC gene, in the cyanobacterium Synechocystis sp. PCC 6803, on electron transport in PS I. The intra- and intersubunit interactions were studied in PS I mutated in a cysteine ligand of FX in PsaB and in psaC mutants, respectively. PS I was assembled in the absence of subunit PsaC and the associated iron-sulfur clusters $F_A$ and $F_B$. Single turnover flash experiments showed that a 10-fold slow down and an inhibition of the forward electron transfer to $F_X$ occurred in PS I prepared from the cysteine to serine mutant in psaB and the psaC- mutant, respectively.

Based on these and other results, we propose that $F_X$ has a major role in controlling electron transfer through PS I. The redox potential of $F_X$ is influenced by electronic interaction with $F_A$ and $F_B$. Reduction or removal of $F_A$ and $F_B$ may lower the redox potential of $F_X$ and thus block electron transfer from $A_1$ to $F_X$. The inhibition of $F_X$ reduction resulted in charge recombination of $A_1^-$ with $P700^-$. We suggest that this control switch has physiological importance in prevention of photodamage that could result from reduction of oxygen by reduced $F_X$ and $F_{A/B}$. Photodestruction damage to $F_{A/B}$ was shown to happen during selective activation of PS I under high light intensity (26).

**EXPERIMENTAL PROCEDURES**

_In Vitro Insertional Inactivation of the psaC2 Gene.—_Plasmid p61–2.4 containing a 2.26-kb fragment of the psaC2 gene from _Synechocystis_ (GenBank™ accession number D63999) (27) was used to construct a vector for inactivation of the psaC2 gene. Plasmid p61–2.4 was a kind gift from Dr. Klaus Steinmüller (University Dusseldorf, Dusseldorf, Germany). A 2-kb $\Omega$ fragment from the pH45t1 plasmid, containing the streptomycin and spectinomycin resistance-conferring gene (28), was digested with Smal and subcloned in the filled-in Xbal site of $psaC2$ gene in p61–2.4 plasmid resulting in plasmid pCA2 (Fig. 1). Synechocystis cells from wild type and mutant C5656S/D566E in PsaB (29) were transformed with the pCA2 plasmid for inactivation of the $psaC2$ gene, essentially as described (30). After transformation, selections of spectinomycin colonies were carried out under light-adapted heterotrophic growth conditions (5) for $psaC$ mutants. Single spectinomycin colonies were plated and sequentially transferred in increasing spectinomycin concentrations from 5 to 20 $\mu$g/ml, respectively, until full segregation was obtained.

**Cyanobacterial Strains and Growth Conditions—_Synechocystis_ sp. PCC 6803 cells, a glucose-tolerant strain, were grown in BG-11 medium supplemented with 5 mM TES, pH 8.0, and 5 mM glucose at 30°C (31). Mixotrophic cultures were grown in continuous light at photon flux density of 40 $\mu$mol m$^{-2}$ s$^{-1}$. Heterotrophic cultures were grown in dim light (photon flux density of 2 $\mu$mol m$^{-2}$ s$^{-1}$) or under light-adapted heterotrophic growth conditions: in complete darkness, except for 10 min of light (photon flux density of 40 $\mu$mol m$^{-2}$ s$^{-1}$) every 24 h (5). For photoautotrophic growth, cells were grown in the medium without addition of glucose in continuous light (photon flux density of 40 $\mu$mol m$^{-2}$ s$^{-1}$) every 24 h (5). For growth on plates, BG-11 medium was supplemented with 1.5% (w/v) Bacto-agar and 0.3% sodium thiosulfate (32). Growth of cells was monitored by measurement of absorption changes at 750 nm.

**Manipulation of DNA—_Most of the techniques for DNA analysis and manipulation used in this study were according to standard procedures (33). Synechocystis genomic DNA, used as a template for psaB and psaC2 genes, was extracted essentially as described (30) and purified by using a GENECLEAN Kit (BIO 101). DNA sequencing was performed on double-stranded plasmid or was PCR amplified DNA by the dideoxy sequencing method (34) with a Sequenase Version 2.0 kit (USB). A GCG program (Wisconsin Package Version 9.1; Genetics Computer Group) was used to design oligonucleotides as primers and analyze DNA sequences.

**Isolation of Thylakoid Membranes and PS I Complexes—_Harvested cells were washed and broken in a French pressure cell at 500 p.s.i., and the thylakoids were isolated. PS I was solubilized by the detergent n-dodecyl $\beta$-D-maltoside and purified on DEAE-cellulose columns and on a sucrose gradient as described (35). SDS-polyacrylamide gel electrophoresis and Western blotting were done as described previously (36, 37). Protein in the membranes was determined after solubilization in 1% SDS as described (38). Chlorophyll concentration and P700 chemical oxidation and photooxidation were determined according to published methods (39, 40).

**Determination of Acid-labile Sulfide and Iron in the Photosystem I Complexes—_The iron content in iron-sulfur clusters in PS I was determined by an adaptation of an earlier reported method (41, 42). Acid-labile sulfide was determined by modification of published methods (43, 44). In the modified procedure the amount of acid-labile sulfide was determined after the extraction of chlorophyll and measurement of absorption of the dye at 750 nm in the presence of 4 M KCl. It was found that there is less interference from chlorophyll breakdown products left after extraction, when determination was carried out by measurement
of the absorption at 750 nm rather than at 640 nm used in the standard assay. Ferredoxin (molecular mass 6.2 kDa) from Clostridium pasteurianum (45, 46) was used as standard. For each preparation, the mean values of three independent experiments, each in triplicate, were calculated to obtain 95% statistical confidence. The values were normalized on the basis of the chlorophyll content in the samples.

Isolation and Reconstitution of the PS I Core Complexes and the Subunits PsaC, PsaD, and PsaE—Preparation of PS I core and isolation of subunits PsaC, PsaD, and PsaE were carried out essentially according to the methods as described (47, 48). PS I was dissociated by treatment with 6.8 x 10^-3 urea for 10–30 min until the P700+ reduction reached τ, of 1 ms. Following removal of the urea by dialysis, the dissociated peripheral low molecular mass polypeptides PsaC, PsaD, and PsaE were separated from the core by ultrafiltration and concentrated by the same method.

Reconstitution of iron-sulfur clusters in PS I core preparation and in the PS I complexes from the PsaC-deficient mutant strains was adapted from the previously reported studies (47, 48). Essentially PS I core preparations in the absence or the presence of the isolated small subunits obtained from urea dissociation were suspended in N2-purged solutions 0.5% β-mercaptoethanol to which FeCl3 and Na2S3 solutions were added and incubated for 12–18 h. The solution was concentrated near dryness by ultrafiltration, washed twice with a solution containing 0.05% β-mercaptoethanol. The reconstituted PS I complexes were stored at −80 °C in the presence of 15% glycerol.

Spectroscopic Measurements—The kinetics of P700+ reduction were measured by monitoring flash-induced transient absorption changes at 700 and 820 nm. Measurements at 820 nm were performed on the set-up as described earlier (49). In brief, the sample was excited by 352-nm laser flashes of 300-ps duration and an energy of ~0.7 mJ/cm2. Continuous measuring light from an 820-nm laser diode passed through the sample (path length, 10 mm) and was monitored by a silicon photodiode, amplified, and digitized. The electronic bandwidth of the detection system was usually limited to 20 MHz. Typically, 16 transients were averaged at a repetition rate of 1 Hz. Measurements at 700 nm and measurements at 820 nm on reconstituted samples used a modified flash photolysis setup as described earlier (50), which included a 10-ps xenon flash lamp (Lambda Physik, EBL 100Q). A measuring light beam was provided by a tungsten-iodide source passed through a monochromator. The absorption changes at 820 and 700 nm were monitored by a photomultiplier, interfaced by Tektronix TDS 520A digitizing oscilloscope, and recorded on a personal computer.

The kinetics of A700 oxidation in time scales shorter than 5 μs were measured by monitoring flash-induced transient absorption changes at 380 nm on a set-up similar to the one described earlier (51). The sample was excited by 532-nm laser flashes of 300-ps duration and an energy of ~0.5 mJ/cm2. The measuring light was provided by the relatively flat top of a 50-μs xenon flash and was filtered by a combination of interference and colorless glass filters placed in the measuring beam before and in front of the detector silicon photodiode FND 100Q from EG&G, yielding a spectrum of 8-nm width centered at 380 nm. The detector output to 50 ohms was amplified (30 dB, 500 Hz–1.7 GHz) and recorded by a digitizing oscilloscope (DSA 602A with amplifier plug-in 502A from Tektronix). The time resolution of the detection system was 2 ns. The baseline was recorded separately with mechanically blocked excitation flash and subtracted from the transient recorded with excitation. Deviating from previous methodology (51, 52), the sample was contained in a quartz cell of 20-mm width, 30-mm height, and 2-mm path length for the measuring light. This cell was allowed to spread the measuring light on a larger cross-section than previously and to diminish a potential actinic effect of the measuring light. Typically, 2048 transients were averaged at a repetition rate of 1 Hz. The same set-up was used to monitor absorption changes at 430 nm (essentially because of oxidation and re-reduction of P700) under identical conditions of excitation and time resolution. The spectral bandwidth of the measuring light was 3 nm, and only 256 transients were averaged.

Absorption changes at 380 nm in a time scale up to 500 μs were recorded essentially as described previously (53). The excitation energy was ~0.3-mJ/cm2 in a 300-ps flash at 532 nm. The measuring light from a continuous 100-W tungsten halogen lamp was filtered as above. A shutter placed in the measuring light beam in front of the sample was opened 3 ms before the excitation flash and closed 5 ms after the flash to prevent exposure of the sample to the measuring light. The time resolution of the detection system was ~1 μs. The sample was contained in a 10 x 10-mm standard cuvette. 256 transients were averaged at a repetition rate of 2 Hz. For measurements at 380 nm in a 10-ms time scale, the measuring light was continuous. The sample was contained in a quartz cell of 20-mm width, 30-mm height, and 2-mm path length, allowing to spread the measuring light to a larger cross-section and to diminish its actinic effect. The time resolution of the detection system was limited to 5 μs. 512 transients were averaged at a repetition rate of 1 Hz.

Unless specified otherwise, samples contained 25 mM Tris, pH 8.3, 10 mM sodium ascorbate, 500 μM DCIP, and PS I complexes as indicated in the figure legends. All spectroscopic measurements were performed at room temperature. Absorption change transients were analyzed by fitting with a multiexponential decay using Marquardt least-squares algorithm programs (KaleidaGraph 3.5 from Synergy Software, Reading, PA, and DEC60, kindly provided by P. Seti, Commissariat à l’Energie Atomique, Saclay, France).

**RESULTS**

Assembly of PS I in the PsaC-deficient Mutants—The segregated cells of the PsaC-deficient mutants, pscA– and pscC– C565S/D566E, failed to grow either mixotrophically or autotrophically under light intensity of 40 μmol/m2·s−1, but could grow heterotrophically at wild type growth rate under a low light intensity of 2 μmol/m2·s−1. The absorption of the chlorophyll/cell at 440 and 680 nm in the pscA– cells was smaller than in the wild type cells suggesting that photosynthetic complexes were assembled at a reduced level in the mutants. A semi-quantitative evaluation of the assemblies of the PS I complexes in the PsaC-deficient mutants was done by Western blot analysis of the thylakoid membranes and in isolated PS I with the antibodies against the PsaA/PsaB subunits. A reduced amount of the PsaA/PsaB subunits was detected in the thylakoids from the mutants pscA– C565S/D566E and in isolated...
PS I of psaC− and psaC− C565S/D566E when compared with the wild type and the C565S/D566E mutant (Fig. 2a). Western blot analysis of the isolated PS I using the antibody against the PsaC subunit indicated that the mutants psaC− and psaC− C565S/D566E did not contain detectable amounts of the PsaC subunit (Fig. 2b). These results indicated that inactivation of the PsaC-encoding gene psaC2 prevented the assembly of the PsaC subunit but did not significantly affect biogenesis and stability of the PS I core complex.

A Chl/P700 ratio was determined spectroscopically by photooxidation and by chemical oxidation of P700 with ferricyanide. In PS I complexes from the wild type, C565S/D566E, psaC−, and psaC− C565S/D566E mutants, a Chl/P700 ratio of 100, 110, 120, and 150, respectively, was determined. The ratio of Chl/P700 in the mutants psaC− and psaC− C565S/D566E was slightly higher than in the wild type and in the mutant C565S/D566E, respectively. These results complement the immunoassay data, which indicated the assemblies of the PS I complexes in the mutants psaC− and psaC− C565S/D566E at reduced levels.

Forward Electron Transfer from the Phylloquinone A1 to Iron-Sulfur Cluster FX and Charge Recombination Reactions—In isolated PS I, photooxidized P700 is reduced by either the final electron acceptor or by the intermediate electron transport carriers. The rate of reduction depended on proximity of the reducing carrier to P700− and the redox potential differences. On removal of PsaC subunit containing iron-sulfur clusters FX and FB from PS I, it was expected that P700− will be reduced by FX−. Yet the rate of reduction of P700− was much faster in the psaC− PS I (τs ≈ 10 μs for the main component; see Table I and Fig. 3C) compared with the urea-dissociated PS I core complex, where the PsaC subunit was chemically dissociated (τs ≈ 0.4 and 1.2 ms for the main components; see Table I and Fig. 3D). The faster P700− reduction could be a result of either an inhibition of electron transfer to FX that causes A1−/P700− recombination or a faster rate of reduction by FX− because of the subunit modification. Such alternatives could be resolved by measurement of the rate of forward electron transfer between A1 and FX. Measurement of the forward electron transfer could also verify our suggestion that a slower forward rate resulted in slower growth rate of the mutant C565S/D566E (50).

Wild type PS I—The rate of forward electron transfer from A1− was determined from measurements of light-induced absorption changes at 380 nm. At this wavelength, there is an increase in absorption because of the reduction of the phylloquinone, A1, that is followed by bleaching caused by A1− reoxidation. Oxidation of A1− can either be because of forward electron transfer to FX or because of A1−/P700− recombination. It is possible however, to distinguish between the two pathways of oxidation if the rate of oxidation of A1− is compared with the rate of reduction of P700−. The oxidation of A1− is a result of A1−/P700− recombination when the rate of oxidation of A1− agrees with the rate of reduction of P700−. To this end both rates were determined in the various preparations under similar experimental conditions. In the wild type PS I, flash-induced reduction of A1−, which is recorded as unresolved fast increase in absorption, is followed by biexponential decay that could be fitted with τs ≈ 8.3 ns (19% of the total absorption change) and 180 ns (81%) (Fig. 4A). These results are in harmony with earlier measurements (12). The kinetics of P700− was obtained in the same sample under identical excitation conditions and time resolution by monitoring absorbance changes at 430 nm where oxidation of P700 is accompanied by a strong bleaching (40). As shown in Fig. 4B, the flash-induced bleaching at 430 nm did not recover over at least 3 μs, in line with previous reports (12, 52, 54). These results demonstrate that there was no reduction of P700− during the full oxidation of A1− in the wild type PS I. Hence, A1− was oxidized by electron transfer to FX and not by charge recombination with P700−. Indeed the decay rate of P700− measured at 820 nm
where singlet-excited Chl uncoupled antenna chlorophylls, because an absorbance decay (inset) that might reflect the decay of the singlet excited state of subsequent absorption decrease to reoxidation of A_1. The inset shows an expansion of the same signal. B, flash-induced absorption changes at 430 nm. The fast decrease in absorption following a flash is attributed essentially to oxidation of P700. Signals in A and B were measured on the same sample. Time resolution, 2 ns. Optical density in the red absorption maximum of the sample, OD_{red} = 1.02.

was slow, with t values of 18 and 68 ms (see Fig. 3A and Table 1). These were ascribed to charge recombinations of P700^+/FA^- and reduction of P700^- by the artificial donor DCIP in those PS I complexes where the electron on FA^- had escaped to an exogenous acceptor (23).

**PS I from C565S/D566E Mutant**—The decay of flash-induced P700^- in PS I from C565S/D566E mutant monitored at 820 nm could be analyzed into four exponential components (see Fig. 3B and Table 1). 85% of the total amplitude decayed with virtually the same kinetics in the 20- to 80-ns range as in wild type PS I (see above), indicating that forward electron transfer to FA^- occurred in the large majority of C565S/D566E mutant PS I. 15% of the 820-nm absorbance change decayed in 3 to 20 µs, a time range typical for charge recombination between A_1^- and P700^- (55); decay of the triplet state of uncoupled antenna chlorophylls may have contributed to these µs kinetics, as well. The oxidation of A_1^- measured at 380 nm occurred essentially with t \approx 3 µs, i.e. more than one order of magnitude slower than in wild type PS I (Fig. 5A). There appears to be a small (~10%) additional phase with t \approx 80 ns, but in view of the signal-to-noise ratio, the significance of this phase is uncertain. At early times, there is a rising phase with t \approx 5 ns (Fig. 5A, inset) that might reflect the decay of the singlet excited state of uncoupled antenna chlorophylls, because an absorbance decay with similar kinetics was observed at 820 nm (data not shown), where singlet-excited Chl α has some absorption. We cannot exclude that a fraction of A_1^- was oxidized in less than 10 ns and that the corresponding absorbance decrease at 380 nm was overcompensated by the 5-ns rise. The 3-µs phase at 380 nm can be attributed essentially to forward electron transfer, as virtually no reduction of P700^- was observed in the same time scale at 430 nm (Fig. 5B). A small phase with t \approx 30 ns at 430 nm presumably reflects charge recombination in the primary pair P700^-A_0^- in a small fraction of these mutant PS I complexes. To our knowledge, this is the first reported case in which alteration in the ligands of FX caused a slow down in the rate of forward electron transfer from A_1^- . This slow down may contribute to the decreased growth rate of the C565S/D566E mutant (29).

**PS I from psaC^- Mutant**—The decay of flash-induced P700^- in PS I from psaC^- mutant monitored at 820 nm (Fig. 3C) is much faster and kinetically more complex than in the wild type and in the C565S/D566E mutant. A fully satisfying fit by a sum of exponential decays required five components (see Table 1). 86% of P700^- decayed with half-times in the range from ~5 to 200 µs that are typical for charge recombination between P700^- and A_1^- in PS I lacking FX (53, 55). Only 9% of P700^- decayed with a half-time in the order of 1 ms expected for P700^-FX^- recombination. The remaining 5% decaying in ~100 ms may reflect PS I complexes where an electron escaped from the acceptor side (to O_2 or another soluble acceptor), and P700^- was reduced by reduced DCIP. In line with these assignments, very little oxidation of A_1^- measured at 380 nm (Fig. 6A; the rising phase with t \approx 5 ns seen in the inset is presumably because of some singlet excited chlorophyll, as discussed above for the C565S/D566E mutant) took place in the tens to hundreds nanosecond range, i.e. the time scale in which electron transfer from A_1^- to FX occurs in intact wild type PS I. Following the A_1^- absorption at 380 nm for several hundred microseconds (Fig. 7A), A_1^- was found to decay with similar kinetics.
in the 5- to 200 ms range as most of P700\(^+\), i.e. A\(_1\)\(^-\) decayed essentially by charge recombination with P700\(^+\). It therefore seems that in psaC\(^-\) the removal of the small subunits and the iron-sulfur clusters FA/B inhibited most of the forward electron transfer from A\(_1\)\(^-\) to FX. As an alternative interpretation of the data, it can be argued that genetic deletion of the PsaC subunit caused disintegration of the iron-sulfur cluster FX in more than 80% of the reaction centers. However, the quantification of acid-labile sulfide and iron, and the results of reconstitution experiments presented below in this work, as well as EXAFS studies presented elsewhere (56), do not support such an interpretation.

**Urea Dissociated PS I Core Preparation**—The wild-type PS I complexes were incubated with 6.8 M urea to remove the FA/FB-containing subunit PsaC. This procedure, which resulted in the dissociation of the PsaC subunit from the core subunits PsaA/PsaB, was also accompanied by oxidative degradation of the iron-sulfur clusters FA/FB (22, 57). The treatment with urea proceeded until the rate of the decay kinetics of P700\(^+\) indicated a lack of reduction of FA/FB. However, it can be seen from the immunoblot of this preparation that a detectable amount of PsaC subunit was still attached to the core after the treatment of PS I (Fig. 2). The decay of flash-induced P700\(^+\) in urea-treated PS I monitored at 820 nm (Fig. 3D) was again much faster than in untreated wild-type PS I, yet not as fast as in PS I from the psaC\(^-\) mutant. According to the best fit with five exponential decay phases (see Table I), most of P700\(^+\) decayed with \(t_{1/2}\) of 1.22 ms (41%) and 442 ms (22%), similar to kinetics reported previously for such preparations and attributed to charge recombination between P700\(^+\) and FX\(^-\) (23, 55) (see below). The small 41-ms component (8%) may be because of PS I complexes where FA and FB were still present and/or where the electron had escaped from FX. The phases with \(t_{1/2}\) of 7.2 ns (11%) and 74 ns (18%) are typical for charge recombination between P700\(^+\) and A\(_1\)\(^-\), indicating loss or inactivation of FX in about 30% of the sample.

Direct measurements of A\(_1\)\(^-\) at 380 nm, showed that about half of A\(_1\)\(^-\) was oxidized within less than 1 µs (Fig. 8A). This kinetics was best fit with two exponential phases of \(t_{1/2} = 15.6\) ns (38%) and 126 ns (62%). At the same time scale, hardly any reduction of P700\(^+\) as measured by the absorption changes at 430 nm is
The fate of the remaining $A_1^-$ was studied at 380 nm on micro- and millisecond time scales (Fig. 6B). About two-thirds decayed with kinetics in the 10- to 150-$\mu$s range. We attribute this decay to recombination of $A_1^-$ with $P700^-$ in a fraction of the PS I complexes where $F_X$ was lost or inactivated, in line with the corresponding kinetics of $P700^-$ observed at 820 nm. Interestingly, a significant amount of the flash-induced absorption change at 380 nm decayed with $t_c \sim 1$ ms (inset of Fig. 6B), i.e. the kinetics that is usually attributed to recombination between $P700^-$ and $F_X$ (2). This observation supports a previous suggestion (16) that the $t_c \sim 1$ ms kinetics in urea-treated PS I represents charge recombination between $P700^-$ and a quasi-equilibrium between $A_1^- \sim F_X^-$, where the equilibrium constant, $K$, is in the order of three. Quantification of our present results shows that about 15% of total $A_1^-$ decayed with $t_c \sim 1$ ms, whereas about 60% of total $P700^-$ decayed with similar kinetics. These numbers are consistent with $K \sim 3$ (three-fourths of $F_X^-$ and one-fourth of $A_1^-$ present in the quasi-equilibrium that recombines with $P700^-$).

Comparing these results with those on the psaC$^-$ mutant, it is puzzling that destruction of $F_A$ and $F_B$ by urea treatment inactivated electron transfer from $A_1^-$ to $F_X$ less than genetic depletion of psaC. Possible explanations will be considered under “Discussion.”

The Composition of Acid-labile Sulfide and Iron in the psaC$^-$ Mutants and the Core Complexes—The content of acid-labile sulfide and iron in PS I was determined to verify whether the iron-sulfur cluster $F_X$ was depleted as a result of the genetic deletion of the Psac subunit. A disintegration of the iron-sulfur cluster $F_X$ in 80% of the reaction centers could have caused the observed decrease in reduction of $F_X$ in these mutants. The various preparations were analyzed and normalized on the basis of chlorophyll content in the samples. The calculated molar ratios of $P700$ per acid-labile sulfide were $1:2.3 \pm 1.2$, $1:4 \pm 0.5$, $1:4 \pm 0.5$, and $1:4 \pm 0.5$ in PS I from the wild type, the core preparation, mutant psaC$^-$, and mutant psaC$^-$/psaC$^-$C565S/D566E, respectively. The molar ratios of $P700$ to iron were $1:12.1 \pm 2.2$, $1:4 \pm 0.8$, $1:4 \pm 0.5$, and $1:4 \pm 0.5$ for PS I from the wild type, the core preparation, mutant psaC$^-$, and mutant psaC$^-$C565S/D566E, respectively. The data confirm a content of three [4Fe4S] clusters in the wild type and one iron-sulfur cluster in the psaC$^-$ mutants and the core complexes as anticipated.

Reconstitution of the Iron-Sulfur Cluster $F_X$—Chemical analysis indicated that the contents of acid-labile sulfide and iron were adequate to form an iron-sulfur cluster in PS I of the Psac-deficient mutants. It was still possible that only less than 20% of the clusters are functionally competent. To test this possibility, $F_X$ was reconstituted in the PS I complexes from the Psac-deficient mutants and in the urea-dissociated core complexes. The reconstitution was carried out in the presence of $\beta$-mercaptoethanol, $Fe^{3+}$, and $S^{2-}$ or in the presence of $\beta$-mercaptoethanol alone. It was only in the PS I of the mutant psaC$^-$C565S/D566E that the reconstitution increased the extent of the $P700^-$ reduction phase with a half-time of $\sim 700 \mu$s, up to 15% of the total absorption change. Treatment of the core complex and of PS I of the mutant psaC$^-$ did not change the kinetics of $P700^-$ reduction. These results suggest that only in the PS I from the mutant psaC$^-$C565S/D566E was there some degradation of $F_X$. X-ray absorption fine structure analysis of the iron in Psac$^-$ PS I also indicated the presence of [4Fe4S] cluster with average iron-sulfur bond distance of 2.27 Å and an average iron-iron bond distance of 2.69 Å (56).

Reconstitution of the Iron-Sulfur Clusters $F_A/F_B$—The iron-sulfur cluster $F_X$ seemed to be intact in PS I from the psaC$^-$ mutant, as seen from the reconstitution studies of this cluster. Yet only 9% of the reduction of $P700^-$ was because of recombination of $P700^-/F_X^-$. It is possible that, although $F_X$ was intact, it could not be fully reduced in the absence of Psac and $F_A/F_B$. To find out whether $F_X$ was functionally functional, the clusters $F_A/F_B$ were reconstituted in the PS I complexes from the Psac-deficient mutants and in the core preparation. For reconstitution of $F_A/F_B$, the $F_A/F_B$-binding subunit Psac and subunits Psad and PsaE were mixed with the $P700$-$F_X$ containing complexes in the presence of $\beta$-mercaptoethanol, $Fe^{3+}$, and $S^{2-}$ as described previously (47, 48, 58). The kinetics of $P700^-$ reduction at 820 or 700 nm were measured to evaluate the function of $F_X$ in the reconstituted PS I.

The reconstitution caused an increase in the extent of the decay component with a half-time $> 5$ ms, corresponding to charge recombination between $P700^-/F_A/F_B^-$ (see Fig. 9 for PS I from the psaC$^-$ mutant). These results indicated that $F_X$ in the core preparation and in the PS I complexes from the Psac-deficient mutants psaC$^-$ and psaC$^-$C565S/D566E had the capacity to mediate electrons from $A_1$ to $F_A/F_B$ once the full electron-transfer chain $P700^-/F_A/F_B^-/F_X^-/F_A/F_B^-$ was restored by reconstitution. Therefore, it can be suggested that $F_X$ in the core preparation and in the PS I complexes of these Psac-deficient mutants is potentially functional. The extent of the $P700^-$ reduction due to $P700^-/F_A/F_B^-/F_X^-/F_A/F_B^-$ recombination was 58, 32, and 32% in the reconstituted core preparation and in the reconstituted PS I from the mutants psaC$^-$ and psaC$^-$C565S/D566E.
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DISCUSSION

The Location of F<sub>X</sub> at the Subunit Interphase—The iron-sulfur cluster F<sub>X</sub> is located on the core subunits PsaA/B at the interphase with subunit PsaC in PS I. It is the last carrier of the primary section of the electron transfer chain. F<sub>X</sub> mediates electrons across the subunit interphase to the terminal carriers F<sub>AB</sub> on subunit PsaC. It is suggested that this strategic location F<sub>X</sub> facilitates a potential role of the carrier as a control switch of electron transfer in PS I. For such a role, the carrier should be able to affect the electron transport in response to changes in the environment. Indeed, there are numerous findings that indicate that the electron transfer to F<sub>X</sub> is changed in response to the state of the surroundings. The rate is sensitive to the redox state of F<sub>AB</sub> to the presence of these iron-sulfur clusters, to temperature, and to inter- and intrasubunit interactions. All these responses are indicative of a potential role of controlling the electron flow in PS I. In this work, we evaluated the inter-subunit interactions by deletion of subunit PsaC. Intrasubunit interactions were studied by site-directed mutations.

Simultaneous Measurements of A<sub>1</sub><sup>−</sup> Oxidation and P700<sup>+</sup> Reduction—Earlier attempts were made to determine the function of F<sub>X</sub> in the absence of F<sub>AB</sub> by chemical dissociation of subunit PsaC and by genetic modifications. In these experiments, the effect of the modifications on the intermediate electron transport were mostly evaluated by monitoring the rate of reduction of P700<sup>+</sup>. The rate of reduction of part of P700<sup>+</sup> in the PsaC-depleted PS I was found to be faster than that expected for P700<sup>+</sup>/F<sub>X</sub> recombination (24, 25). The faster P700<sup>+</sup> reduction could be a result of either an inhibition of electron transfer to F<sub>X</sub>, which caused A<sub>1</sub>/P700<sup>+</sup> recombination, or a faster rate of reduction of P700<sup>+</sup> by F<sub>X</sub> because of the subunit modification. Such alternatives could be resolved by measurement of the rate of forward electron transfer between A<sub>1</sub> and F<sub>X</sub>. We therefore removed subunit PsaC by deletion of the psaC<sup>2</sup> gene of PS I in the cyanobacterium Synechocystis sp. PCC 6803 and measured both the rate of reduction of P700<sup>+</sup> and the rate of oxidation of A<sub>1</sub>.

Forward Electron Transfer from A<sub>1</sub> to F<sub>X</sub> in PsaC<sup>−</sup> PS I—The PsaC<sup>−</sup> mutant cells grew under heterotrophic conditions and assembled the core subunits of PS I in which light induced electron transfer from P700 to A<sub>1</sub>. The reduction of F<sub>X</sub> was substantially reduced, as seen from direct measurement of the extent of electron transfer from A<sub>1</sub> to F<sub>X</sub>. The oxidation of A<sub>1</sub> measured as a decrease in absorption at 380 nm was slow and proceeded at the range of t<sub>90</sub> = 5–200 μs. At this time range the quinone was oxidized mostly by A<sub>1</sub>/P700<sup>+</sup> recombination. A small fast absorption decrease due to the oxidation of A<sub>1</sub> with t<sub>90</sub> < 20 ns might have occurred, but if it happened, it was obscured by an absorption increase at 380 nm that took place almost at the same time range (possibly because of singlet excited Chl; see above). Some reduction of F<sub>X</sub> must have taken place, because about 10% of P700<sup>+</sup> reduction proceeded at t<sub>90</sub> of ~1 ms, which is typical for P700<sup>+</sup>/F<sub>X</sub> recombination. These results were substantially different from earlier study on thylakoids from the PsaC<sup>−</sup> mutant, where most of P700<sup>+</sup> reoxidation was reported to be because of P700<sup>+</sup>/F<sub>X</sub> recombination at a t<sub>90</sub> of ~1 ms (24). As the electronic bandwidth of the detection system was limited to 10 kHz, faster phases of P700<sup>+</sup> reduction might have escaped detection in this work (24). More recently, it was found that the inactivation of the F<sub>AB</sub> clusters by C14A or C51A mutations (25) caused the dissociation of PsaC. From the results that were presented in the characterization of these mutants, it can be seen that only ~30% of the F<sub>X</sub> was reduced as monitored by the extent of P700<sup>+</sup>/F<sub>X</sub> recombination (t<sub>90</sub> ~ 1 ms). The remaining 70% of the total reduction of P700<sup>+</sup> took place with t<sub>90</sub> of 20 to 200 μs and was mainly because of P700<sup>+</sup>/A<sub>1</sub><sup>−</sup> recombination. These results are more in harmony with the results we obtained in isolated PS I from the PsaC<sup>−</sup> mutant. Analysis of our data yielded 86% of total P700<sup>+</sup> reduction with t<sub>90</sub> of 5 to 200 μs, 9% with t<sub>90</sub> ~ 1 ms and 5% with t<sub>90</sub> > 100 ms (see Table I).

A Comparison between PsaC-depleted PS I Preparations—Our results on PS I from the psaC<sup>−</sup> mutant are strikingly different from a previous report on an urea-dissociated PS I core preparation, where the kinetics of A<sub>1</sub><sup>−</sup> reoxidation had been reported to be virtually the same as in intact PS I (9). Therefore, we reexamined an urea-dissociated PS I core under the same experimental conditions as the psaC<sup>−</sup> mutant. About half of A<sub>1</sub><sup>−</sup> (monitored at 380 nm) was oxidized by forward electron transfer and could be fitted by two exponential phases of t<sub>90</sub> of 15.6 ns (38%) and 126 ns (62%). The remaining half of A<sub>1</sub><sup>−</sup> recombined with P700<sup>+</sup> with kinetics in the 10-μs to 1-ms range (see “Results”). The kinetic constants of forward electron transfer in the urea core preparation were somewhat different from those measured in intact wild type PS I, namely t<sub>90</sub> of 8.3 ns (19%) and 180 ns (81%). Taking into account the signal-to-noise ratio and additional measurements at other wavelengths (not shown), we consider the difference as significant. This result indicated that the removal of F<sub>AB</sub> caused enhanced the forward electron transfer to F<sub>X</sub>. In earlier measurements of urea-treated PS I, the submicrosecond kinetics of A<sub>1</sub><sup>−</sup> oxidation were resolved into a single exponential phase with t<sub>90</sub> of about 180 ns and were considered to be the same as in untreated PS I (9). The different results in the present study might be attributed to the use of a superior measuring system, which gave better resolution in our experiments.

Before discussing possible origins of the different behavior of PS I from the psaC<sup>−</sup> mutant and the urea-dissociated PS I core, we would like to point out that removal of the extrinsic sub-
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units PsaC, PsaD, and PsaE could be expected to modify electron transfer from A$_1^-$ to F$_X$.

According to non-adiabatic electron transfer theory (59), the electron transfer rate, $k_{et}$, is proportional to the square of electronic coupling ($T_{DA}$) between electron donor and acceptor and to the Franck-Condon factor (F.C.), as illustrated by Equation 1.

$$k_{et} = \frac{2\pi}{\hbar} |T_{DA}|^2 \text{ (F.C.)} \quad \text{(Eq. 1)}$$

$T_{DA}$ is not expected to change upon removal of the extrinsic subunits, unless this removal would cause structural changes in the remaining PS I core complex that would modify the distance between A$_1$ and F$_X$. The Franck-Condon factor can be expressed in a classical treatment of nuclear motion (59), as shown below in Equation 2, where $k_B$ is the Boltzmann constant, $T$ the absolute temperature, $\Delta G^0$ the standard free energy change of the electron transfer reaction, and $\lambda$ the reorganization energy.

$$\text{(F.C.)} = \frac{1}{\sqrt{\pi \lambda k_B T}} \exp \left[ -\frac{(\Delta G^0 + \lambda)^2}{4\lambda k_B T} \right] \quad \text{(Eq. 2)}$$

Removal of the extrinsic subunits could affect $\Delta G^0$ and hence the electron transfer rate in the following two ways: 1) The Coulomb interactions between permanent charges on these subunits and the transferred electron would disappear, and a modified charge distribution may be established in the remaining core complex. This would affect the $\Delta G^0$ (see below). 2) Replacing the extrinsic subunits by highly polarizable water should facilitate charge accumulation on F$_X$ (from two to three negative charges upon reduction) and hence decrease $\Delta G^0$ (see Ref. 60 and 61) for such a dielectric effect on the reduction potential of a [4Fe-4S] cluster in nitrogenase. According to Equations 1 and 2, decreasing $\Delta G^0$ should increase the rate of electron transfer from A$_1^-$ to F$_X$ (the opposite case would only occur in the "inverted region" ($-\Delta G^0 > \lambda$), which can be safely excluded for this electron transfer (16)).

The reorganization energy $\lambda$ generally increases with increasing static dielectric constant of the environment of electron donor and acceptor (59). Hence we expect an increase of $\lambda$ upon replacing the extrinsic subunits by water. As $|\Delta G^0| < \lambda$ for electron transfer from A$_1^-$ to F$_X$ (59), increasing $\lambda$ should decrease the rate of this electron transfer (see Equation 2). A lack of modification of the electron transfer rate as reported previously (9) could occur only if the effects of the change in $\lambda$ and of the change in $\Delta G^0$ would cancel.

In summary, it is not surprising that removal of the extrinsic subunits PsaC, PsaD, and PsaE modified the oxidation kinetics of A$_1^-$; it is surprising, however, that a much stronger modification was observed upon removal of these subunits by mutagenesis than upon removal by urea treatment. We would like to consider two possible origins for this difference.

First, the two methods to remove the extrinsic subunits affected the reaction free energy of electron transfer from A$_1^-$ to F$_X$ differently, such that it was down hill ($\Delta G^0 < 0$) in urea-treated PS I whereas it was up hill ($\Delta G^0 > 0$) in the PsaC$^-$ mutant in the latter case, electron transfer from A$_1^-$ to F$_X$ would occur only with low yield, and charge recombination between A$_1^-$ and P700$^+$ would be the dominating process, as observed. Interestingly, our immunoassay (Fig. 2b) showed that some of the PsaC subunit remained attached to PS I after urea treatment, whereas no PsaC was detected in the PsaC$^-$ mutant. One may therefore suggest that electron transfer from A$_1^-$ to F$_X$ is up hill in the complete absence of PsaC but down hill in the presence of PsaC lacking iron-sulfur clusters F$_A$ and F$_H$. As outlined above, the dielectric effect of replacing the extrinsic subunits by water is rather expected to facilitate reduction of F$_X$. However, this effect may be overcompensated by the change in Coulomb interactions upon removal of the extrinsic subunits. In fact, from the crystallographic structure of PS I from S. elongatus (5), it can be seen that the removal of subunits PsaC, PsaD, and PsaE results in the braking of six salt bridges between these subunits and PsaB and PsaA. The charged amino acids remaining on the surface of PsaB and PsaA are four aspartic acids D440B, D555B, D566B, and D568A, lysine K702B, and arginine R561A (Fig. 10). The potential induced on F$_X$ by these surface charges can be roughly estimated using an expression derived (62) for the potential of a charge at the interface between a flat membrane and an aqueous phase, illustrated by Equation 3, shown below, where $\phi_m$ and $\phi_a$ are the potentials in the membrane and the aqueous phases at a distance $r$ of a charge $q$, $\epsilon_0$ is the permittivity of free space, $\epsilon_m$ is the relative permittivity or dielectric constant of the membrane phase, and $\epsilon_a$ is the dielectric constant of the aqueous phase.

$$\phi_m = \frac{2q}{4\pi \epsilon_0} \left( \frac{\epsilon_r}{\epsilon_m} + \frac{1}{\epsilon_a} \right) \quad \text{(Eq. 3)}$$

It was assumed that $\epsilon_a = 78$ and $\epsilon_m = 3.9$. When $\epsilon_m \ll \epsilon_a$ the potential in both the membrane and aqueous phases is twice the value predicted from the Coulomb's law for a single charge $q$ in the bulk aqueous phase.

Taking into account the distances between F$_X$ and the six surface charges, their cumulated potential in the center of F$_X$ according to Equation 3 is $-68$ mV. Similarly, the potential in the centers of the phylloquinones Q$_K$A and Q$_K$B is obtained as $-24$ and $-39$ mV, respectively. Hence, the surface charges present because of the complete lack of PsaC, PsaD, and PsaE in the PsaC$^-$ mutant may well induce a significant decrease of the driving force of electron transfer from A$_1$ to F$_X$.

Second, a large fraction of F$_X$ was lost or damaged in the PsaC$^-$ mutant samples used in the transient absorption experiments. This seems unlikely, because several experiments (quantification of acid-labile sulfide and iron, x-ray absorption fine structure analysis, lack of effect of F$_X$ reconstitution treatment, partial recovery of long-lived charge separation upon rebinding of the extrinsic subunits, and reconstitution of F$_X$ and F$_D$) indicate that F$_X$ is present and potentially functional in isolated PS I from the PsaC$^-$ mutant. We cannot completely exclude, however, that F$_X$ is more fragile in these samples than in urea-treated PS I and may suffer some lost or damaged during repetitive excitation in the transient absorption experiments. Possibly, the detergent treatment used to extract PS I from the membrane might cause some sensitivity of F$_X$ when it is not protected by the extrinsic subunits. Such an effect might also explain the slightly higher yield of electron transfer to F$_X$ in thylakoids from the PsaC$^-$ mutant (24) as compared with isolated PS I from this mutant.

The Effect of Intrasubunit Interactions on Forward Electron Transport—Many experiments demonstrated that changes in the environment of the binding region of F$_X$ affected electron transport in PS I. In this work, we have shown for the first time by direct measurements of the quinone oxidation that modification of amino acid in the F$_X$ binding domain affected the forward electron transfer. In the mutant C565S/D566E, the major component of the oxidation kinetics of A$_1^-$ fits an exponential decay with a half-time of 3 $\mu$s, compared with 180 ns in the wild type. Yet 85% of P700$^+$ was as long-lived in the mutant (half-life times of 26 and 74 ms) as in the wild type. Hence, we attribute the 3-$\mu$s phase of A$_1^-$ oxidation in the mutant mostly to forward electron transfer to F$_X$ and further to F$_{AB}$. Only 15% of P700$^+$ was reduced with microsecond kinet-
ics ($t_{eq} = 3.2$ and $16 \mu$s), which indicate charge recombination with $A_t^-$ and which were not observed in the wild type. The slight reduction in the yield of long-lived charge separation in isolated PS I from the C565S/D566E mutant, as compared with the wild type, could not explain by itself the large decrease in growth rate of mutant cells under photoautotrophic conditions. It is possible that the mutations C565S/D566E lowered the redox potential of $F_X$, shifting the redox equilibrium between $A_t$ and $F_X$. It is also possible that this shift in the redox equilibrium might cause a 20-fold decrease in the rate of the forward electron transfer to $F_X$ in the mutant C565S/D566E and therefore resulted in a decreased growth rate. In other cases that were reported of mutants in the $F_X$ binding domain, the bacteria could not grow photoautotrophically but assembled a modified PS I (5, 63). Presumably, in these mutants, the rate of reduction of $F_X$ should be even slower than the rate measured in mutant C565S/D566E. The changes in the redox equilibrium between $A_t$ and $F_X$ in these PsbA mutants resulted in a substantial slow down in the rate of the electron transfer via $F_X$ that prevented photoautotrophic growth of the cell.

The Possible Physiological Implications—Based on the data presented in this work and other results we propose that $F_X$ has a major role in controlling electron transfer through PS I. The redox potential of $F_X$ is determined in turn by electronic interaction between the iron-sulfur clusters. Reduction or removal of clusters $F_{A+A}$ may cause a lowering of the redox potential of $F_X$ and an inhibition in its reduction, resulting in a fast recombination of reduced phyloquinone ($A_t$) with oxidized P700. This control switch has physiological importance in prevention of photooxidation damage that could result from reduction of oxygen by reduced $F_X$ and $F_{A+A}$. Recently selective photoactivation of PS I under high light intensity was shown to cause damage to this complex because of the formation of oxygen radicals (26). In the absence of superoxide dismutase the damage was located at $F_{A+A}$ and was suggested to be the result of formation of radicals formed during the reduction of oxygen by the iron-sulfur clusters (64).

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Control of Electron Transport in Photosystem I by the Iron-Sulfur Cluster $F_X$ in Response to Intra- and Intersubunit Interactions
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