The Luminal Helix l of PsaB Is Essential for Recognition of Plastocyanin or Cytochrome c₆ and Fast Electron Transfer to Photosystem I in Chlamydomonas reinhardtii*

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Received for publication, November 5, 2001, and in revised form, December 11, 2001
Published, JBC Papers in Press, December 14, 2001, DOI 10.1074/jbc.M110633200

At the luminal side of photosystem I (PSI) in cyanobacteria, algae, and vascular plants, proper recognition and binding of the donor proteins plastocyanin (pc) and cytochrome (cyt) c₆ are crucial to allow subsequent efficient electron transfer to the photooxidized primary donor. To characterize the surface regions of PSI needed for the correct binding of both donors, loop j of PsaB of Chlamydomonas reinhardtii was modified using site-directed mutagenesis and chloroplast transformation. Mutant strains D624K, E613K/D624K, E613K/W627F, and D624K/W627F accumulated <20% of PSI as compared with wild type and were only able to grow photoautotrophically at low light intensities. Mutant strains E613N, E613K, and W627F accumulated >50% of PSI as compared with wild type. This was sufficient to isolate the altered PSI and perform a detailed analysis of the electron transfer between the modified PSI and the two algal donors using flash-induced spectroscopy. Such an analysis indicated that residue Glu₆₁₃ of PsaB has two functions: (i) it is crucial for an improved unidirectional complex of cyt c₆ to PSI, and (ii) it orients the positively charged N-terminal domain of Psaf in a way that allows efficient binding of pc or cyt c₆ to PSI. Modification of Trp₆₂₇ to Phe completely abolishes the formation of an intermolecular electron transfer complex between pc and PSI and also drastically diminishes the rate of electron transfer between the donor and PSI. This mutation also hinders binding and electron transfer between the altered PSI and cyt c₆. It causes a 10-fold increase of the half-time of electron transfer within the intermolecular complex of cyt c₆ and PSI. These data strongly suggest that Trp₆₂₇ is a key residue of the recognition site formed by the core of PSI for binding and electron transfer between the two soluble electron donors and the photosystem.

In Chlamydomonas reinhardtii, the multiprotein complex photosystem I (PSI) is a light-driven oxido-reductase that transfers electrons from the soluble luminal donors plastocyanin (pc) and cytochrome (cyt) c₆ to the soluble stromal acceptor ferredoxin. The eukaryotic PSI reaction center is a membrane-bound complex consisting of 13–14 polypeptide subunits (1). Depending on the relative availability of copper in the culture medium, Chlamydomonas can replace the type I copper protein pc with a class I-type cyt c₆ (2, 3). The two large PSI subunits, PSaA and PsaB, which carry the photochemical reaction center, each contain 11 transmembrane helices, of which helices k and m are connected by the luminal loop j (Fig. 1). The 2.5 Å x-ray crystal structure of PSI from Synechococcus elongatus (4) reveals that a part of the loop forms an α-helix l that is oriented parallel to the membrane close to the primary donor, P700.

In eukaryotic organisms, docking of the soluble luminal donors pc and cyt c₆ to PSI depends mainly on two different recognition sites, which are: (i) negative charges on the surface of the donor attracted by the positively charged N-terminal domain of the Psaf subunit of PSI (5–7), and (ii) the “northern part” of the donors interacting with an as yet undefined site of PSI (8).

The amino acid sequence of helix l is highly conserved among different species (Fig. 1). It is assumed that it could be involved in docking of the soluble donors (9). Sun et al. (10) generated site-directed mutants in the luminal loop of the Psaf protein from Synechocystis sp. PCC 6803. Indeed, a double mutant (W622C/A623R) was strongly affected in the interaction between the altered PSI and the electron donors pc and cyt c₆. In contrast to eukaryotic organisms, in cyanobacteria, efficient binding and electron transfer between PSI and pc or cyt c₆ do not depend on the Psaf subunit because the specific deletion of the psaf gene in cyanobacteria did not affect photoautotrophic growth (11), and the in vivo measured electron transfer rate between cyt c₆ and PSI was the same as that in wild type (12).

In vitro measurements revealed that even at high concentrations of pc or cyt c₆ no difference in electron transfer rates could be measured between the donor proteins and PSI isolated from the wild type or the Psaf-deficient Synechocystis mutant (6). These differences can be explained by the absence of the specific eukaryotic N-terminal recognition site in the cyanobacterial Psaf protein that is required for the binding of pc and cyt c₆ to PSI (6, 7, 13).

To elucidate the role of loop j and especially that of helix l of the PsB protein in a eukaryotic system we performed a combinatorial site-directed mutagenesis approach taking advantage of a Psab-deficient mutant of C. reinhardtii (14). Of seven mutant strains, three allowed isolation of PSI sufficient for a further functional characterization. Electron transfer between the altered PSI particles and the donors pc and cyt c₆ has been investigated using flash-induced absorption spectroscopy. The results suggest features of the luminal surface of PSI that are

*This work was supported by a grant from the State of Baden-Wuerttemberg (Landesforschungszentrum “Evolutionäre Dynamik komplexer makromolekularer Interaktionen in pflanzlichen Zellorganellen”) (to M. H.) and Grant SFB388 A1 (to Wolfgang Haehnel). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The Journal of Biological Chemistry
Vol. 277, No. 8, Issue of February 22, pp. 6573–6581, 2002
Printed in U.S.A.

This paper is available online at http://www.jbc.org

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Sequence Alignment:

Chlamydomonas reinhardtii 599 KHLIWLQGQNV AQFDESSSTYL MGWLRYIYLW NSSQLINGYN PFGMNLS 650
Synechococcus PCC7002 604 KHLGIWQGQNV AQFNKSTYL MGWFYDIYMA NSAQLINGYN PYGNLNL 655
Aanabaena variabilis 604 KHLGIWQGQNV AQFNSTSTYL MGWFYDIYMA NSAQLINGYN PYGNLNL 655
Spinacia oleracea 597 KHLIWLQGQNV SQFDESSTSTYL MGWLRYIYLW NSSQLINGYN PFGMNLS 648

Fig. 1. Schematic topographical model of helices in PsaB of C. reinhardtii and sequence alignment of loop j connecting helices k and m with corresponding regions in different organisms. Helix I was predicted by using algorithms of Refs. 35 and 36. Highly conserved residues Glu613, Asp624, and Trp627 (shown in bold letters) were changed.

crucial for the optimized binding equilibria of both donors. Furthermore, differences between both donors in the recognition and requirements for a correct docking and electron transfer can be identified. The data presented here will be discussed in light of the new structural information available for cyanobacterial PSI (4).

EXPERIMENTAL PROCEDURES

Strains and Media—C. reinhardtii wild type and mutant strains were grown as described previously (15). Tris acetate phosphate medium and high-salt medium were solidified with 2% Bacto agar (Difco) and supplemented with 150 μM/l streptomycin (Sigma) when required.

Nucleic Acid Techniques—Procedures for the preparation of recombinant plasmids and DNA sequencing were performed as described previously (16). Escherichia coli DH5α was used as bacterial host. The site-directed change of PsaB Glu613 to Asn and Lys was carried out in a single tube PCR as described by Picard et al. (17). We used degenerated mutagenic oligonucleotide N (5′-CAATCGATAA/C/CTCGTCTACT-3′) together with two oligonucleotides X (5′-GTTGCTCTAGATGCTCGT-3′) and P (5′-ACATATTAAAGAAGA-3′) complementary to the flanking regions about 150 bp upstream and downstream from the mutagenic site containing cleavage sites I and XbaI, respectively. As template DNA, we used pKR162, a kind gift from K. Redding containing psaB coding sequence and 5′-untranslated region and also containing a 3′ untranslated region and also containing an aadA expression cassette conferring spectinomycin and streptomycin resistance to C. reinhardtii (18). The resulting DNA fragments were cloned into pBluescript SK, amplified in bacteria, and XbaI/PacI-digested. After gel purification, the fragments were then cloned into pKR162, replacing the original sequence.

For the site-directed mutagenesis of PsaB Trp627 to Phe and PsaB Asp624 to Lys, we performed PCR using pKR162 as template, oligonucleotide X, and the degenerated mutagenic oligonucleotides F (5′-ACC-ATTAAATTTGAAAGTGT/TAAC/C/AT/G/AGG/G/ATGTCAC-CTAACA-3′) or K (5′-ACATATTAAATTTGAAAGTGT/TAAC/C/AT/G/AGG/G/ATGTCAC-CTAACA-3′) together with two oligonucleotides X (5′-GTTGCTCTAGATGCTCGT-3′) and P (5′-ACATATTAAAGAAGA-3′) complementary to the flanking regions about 150 bp upstream and downstream from the mutagenic site containing cleavage sites I and XbaI, respectively. As template DNA, we used pKR162, a kind gift from K. Redding containing the PsaB coding sequence and 5′-untranslated region and also containing an aadA expression cassette conferring spectinomycin and streptomycin resistance to C. reinhardtii (18). The resulting DNA fragments were cloned into pBluescript SK, amplified in bacteria, and XbaI/PacI-digested. After gel purification, the fragments were then cloned into pKR162, replacing the original sequence.

All mutations were verified by sequencing using the ABI310 capillary system.

Chloroplast Transformation and Analysis of Transformants—Chloroplast transformation in C. reinhardtii was carried out as described previously (19) using a helium-driven PDS-1000/He particle gun (Bio-Rad) with 1100 psi rupture discs (Bio-Rad). M10 tungsten particles (2.5 mg; Bio-Rad) were coated with 2 μg of the appropriate DNA as described previously. Cells were electroporated as described (17) and scored three times with 500 μl of absolute ethanol and resuspended in 25 μl of absolute ethanol by short sonication; 7 μl of the suspension were used per transformation. ΔPsaB cells lacking psaB were grown at 25 °C in liquid Tris acetate phosphate medium in the dark, and 4 × 10^6 cells were dispersed per Tris acetate phosphate plate containing 150 μg/ml spectinomycin. Once the plates were dry, the cells were bombarded with the DNA-coated particles. The bombarded cells were kept under low light (5 μE m^-2 s^-1) for 2 weeks. The appearing transformants were restreaked on fresh Tris acetate phosphate plates containing spectinomycin and used for further investigations.

Isolation of pc and cyt c_6—The isolation of pc and cyt c_6 followed previously published procedures (3, 20), with modifications as described in Ref. 21. The concentrations of pc and cyt c_6 were determined spectrophotometrically using an extinction coefficient of 4.9 mM^-1 cm^-1 at 597 nm for the oxidized form of pc (22) and 20 mM^-1 cm^-1 at 552 nm for the reduced form of cyt c_6 (2).

Isolation of Thylakoid Membranes and the PSI Complex—The isolation of thylakoid membranes purified by centrifugation through a sucrose step gradient and the isolation of PSI particles were as described previously (21, 23). Chlorophyll concentrations were determined as described in Ref. 24.

SDS-PAGE and Western Analysis—SDS-PAGE (15.5% T, 2.66% C) was carried out according to Ref. 25. After the electrophoretic fractionation, the proteins were electroblotted onto nitrocellulose and incubated with antibodies as described previously (26). Immunodetection was carried out according to Ref. 26. To quantify the amount of PSI, wild type thylakoids were diluted with thylakoids from the ΔpsaB strain (lacking PSI), resulting in fractions that contained 100%, 50%, 25%, and 0% PSI. Equal amounts of thylakoid proteins from these fractions and from mutant thylakoids were separated by SDS-PAGE and analyzed by immunoblotting using PsaF-specific antibodies to estimate the PSI content in the different strains. The blots were also probed with light harvesting complex II antibodies to verify equal loading.

Cross-linking Procedure—Cross-linking was performed as described in Ref. 21.

Flash Absorption Spectroscopy—Kinetics of flash-induced absorbance changes at 817 nm were measured essentially as described previously (13, 27). The measuring light was provided by a luminescence diode (Hitachi HE8404SG; 40 mW; full width at half-maximum, 30 nm) supplied with a stabilized battery-driven current source. The light was filtered through a 817-nm interference filter (full width at half-maxi-
light intensities equivalent to or higher than 700 μE m⁻² s⁻¹, respectively. In Western blot analyses of SDS-PAGE fractionated thylakoids using PSI-specific antibodies, such as anti-PsaD or anti-PsaF antibodies, the amount of PSI in these strains could be estimated to be <20% of the amount of PSI found in wild type thylakoids (Table I). For mutant strains E613N, E613K, and W627F, these estimations revealed that >50% PSI as compared with wild type accumulated in the mutant thylakoids. However, strains E613K and W627F died under photoautotrophic or heterotrophic conditions at light intensities equivalent to or higher than 700 μE m⁻² s⁻¹ (Table I). These growth phenotypes are comparable with the phenotype observed for the PsaF-deficient mutant. Mutant strain E613N showed a strong light sensitivity under photoautotrophic conditions and light intensities equivalent to or higher than 700 μE m⁻² s⁻¹ (Table I). The higher amounts of PSI in these three strains enabled us to isolate the altered PSI complexes and to perform detailed functional studies using flash-induced absorption spectroscopy.

Mutation E613N in PsaB Leads to Enhanced Binding of pc and cyt c₆ at PSI. — The electron transfer from pc or cyt c₆ to PSI, whereas Mutation E613K Drastically Diminishes Binding — The electron transfer from pc or cyt c₆ to PSI isolated from wild type and mutants E613N and E613K was investigated using excitation by single turnover flashes. It should be noted that residue Glu613 is located in the inter-
helical-loop region of loop $j$. Fig. 2 shows the absorbance transients at 817 nm induced by a laser flash for PSI particles in the presence of 20 $\mu$M cyt $c_6$. In the cases of wild type and mutant E613N, the time course of the P700$^-$ reduction can be deconvoluted into three kinetic components (for wild type, see also Fig. 6). The fast component with a constant half-life of 3–4 $\mu$s and a variable amplitude $A(1)$ reflects a first-order electron transfer, the rate of which is independent of the concentration of the donor proteins. This phase can be explained by an electron transfer reaction within a preformed complex between the donor and PSI. The half-time of the fast phase, which was also identified in the kinetics of P700$^-$ reduction using pc as electron donor (data not shown), is found to be the same for wild type and mutant E613N PSI. The intermediate component with an amplitude $A(2)$ shows a half-life that decreases with increasing concentration of reduced donor protein, as known for second-order reactions between soluble reactants (see Fig. 3). Amplitude $A(1)$ increases with increasing concentration of reduced donor protein at the expense of $A(2)$ (see Fig. 3). The third very slow component with an amplitude of about 25 and 40% of the total signal for PSI isolated from wild type and mutant E613N, respectively, has an electron transfer rate constant in the range of $7–9 \times 10^5$ M$^{-1}$ s$^{-1}$ for pc or cyt $c_6$, which is comparable with the values found for electron transfer between both donors and PSI from the PsaF-deficient mutant under similar conditions (21). In the case of mutant E613K, the time course of the P700$^-$ reduction can be deconvoluted into only one kinetic component, with a second-order rate constant of about $5 \times 10^5$ M$^{-1}$ s$^{-1}$, which is again comparable with the values found for electron transfer between both donors and PSI from the PsaF-deficient mutant. Therefore, the very slow electron transfer between mutant PSI E613K and cyt $c_6$ or pc (Fig. 2, Table II) indicates that binding and electron transfer are independent of PsaF in this mutant.

For further analysis of PsaF-dependent functional electron transfer, we will consider mainly the two kinetic components $A(1)$ and $A(2)$. Drepper et al. (27) described a kinetic model for the binding and electron transfer between pc and PSI that also takes into account the redox equilibrium of the electron transfer. In this model, a simple dissociation equilibrium of the complex between the reduced donor protein ([D]) and PSI was used to describe the concentration dependence of the amplitude $A(1)$. An estimate of the dissociation constant ($K_D$) can be determined using the following equation:

![Fig. 4. Salt dependences of the second-order rate constant of P700$^-$ reduction by 20 $\mu$M pc or cyt $c_6$. For conditions, see Fig. 2. Small amounts of concentrated MgCl$_2$ solutions were added to increase ionic strength.](image-url)
Helix l of PsAB and Recognition of pc and cyt c at PSI

TABLE I
Growth properties of wild type, the PsAB-deficient mutant, and the ΔpsAB transfectants with an altered PsB protein on TAP and HSM plates

| Strains          | 10 μE m⁻² s⁻¹ | 60 μE m⁻² s⁻¹ | 700 μE m⁻² s⁻¹ | PSI content |
|------------------|---------------|---------------|---------------|-------------|
|                  | HSM | TAP | HSM | TAP | HSM | TAP |                |
| WT               | +   | +   | +   | +   | +   | +   | 100%           |
| E613N            | +   | +   | +   | +   | +   | +   | >50%           |
| E643K            | +   | +   | +   | +   | +   | +   | >50%           |
| W627F            | +   | +   | +   | +   | +   | +   | >50%           |
| E643K/W627F      | +   | +   | +   | +   | +   | +   | >50%           |
| D694K            | +   | +   | +   | +   | +   | +   | >50%           |
| E643K/D6424K     | +   | +   | +   | +   | +   | +   | >50%           |
| D694K/W627F      | +   | +   | +   | +   | +   | +   | >50%           |
| 3bF (ΔpsAB)      | +   | +   | +   | +   | +   | +   | >50%           |

* TAP, Tris acetate phosphate; HSM, high-salt medium; WT, wild type.

Table II
Properties of the electron transfer from pc and cyt c to PSI from wild type, the PsAB-deficient mutant and the ΔpsAB transfectants with an altered PsB protein

The second-order rate constants k₂ and the dissociation constants K_d determined for wild type and the PsAB-deficient strain 3bF are taken from Refs. 7 and 21.

| Strains          | Plastocyanin | Cytochrome c |
|------------------|--------------|--------------|
|                  | k₂           | K_d          | t₁/2(off)  | k₂    | K_d    | t₁/2(off) |
|                  | (10⁻⁶ s⁻¹)   | (μM)         | (ms)       | (10⁻⁶ s⁻¹) | (μM) | (ms) |
| WT               | 9            | 83           | 0.09       | 3.4   | 83    | 0.25  |
| PsaB E613N       | (0.3 mM)     | (f = 0.69)   |            | (0.3 mM) | (f = 0.66) |        |
| PsaB E613K       | 11           | 29           | 0.22       | 8.2   | 7.7   | 1.1   |
| ΔpsAB            | 0.22         | >1000        | n.d.       | 0.34  | >1000 | n.d.  |
| PsaB W627F       | 0.74         | >1000        | n.d.       | 1.6   | 177   | 0.24  |

* The second-order rate constant k₂ determined from linear regression of the observed rate constant of the donor-dependent kinetic phase as shown in Fig. 3B and Fig. 4A also gives an estimate of the on-rate for the formation of the complex. All kinetic constants refer to conditions close to the optimal concentrations of MgCl₂ as indicated in parentheses.

The dissociation constant K_d of the active complex is estimated from the amplitude of the fast kinetic component as shown in Fig. 3A and Fig. 7A.

The half-life of the active complex, t₁/2 = ln(2)/(k₂), where k₂ is the rate of dissociation of the donor from the photosystem estimated by using the approximate relation k₂ = K_d × k₆. For a detailed discussion of the limits of these estimates and a comparison of their results to a more refined kinetic analysis, see Ref. (27).

WT, wild type; n.d., not determined.

The dissociation constants of 29 and 7.7 μM as well as the second-order rate constants of 11 and 8.2 × 10⁻⁶ s⁻¹ for pc and cyt c, respectively, are significantly smaller for mutant PSI E613N than for the wild type (Table II). The determination of k₂ and K_d values for binding of pc and cyt c to PSI isolated from mutant E613N and the dissociation equilibrium of the complex, respectively, implies that the unbinding of the donors from altered PSI is about 3 times slower compared with wild type PSI. As mentioned above, mutation of residue Glu₆₁₃ in PsAB to Lys has a dramatic effect on the binding of pc and cyt c to the PSI complex. Their second-order rate constants for reduction of PSI are decreased by 2 orders of magnitude, which is comparable with the electron transfer reactions of both donors with the PsAB-deficient PSI. To further analyze the electron transfer properties of mutants E613N, E613K, and W627F, we investigated the salt dependence of the electron transfer between the mutant PSI and the two donors.

Salt Dependence of Electron Transfer from pc and cyt c to PSI

Isolated from Mutants E613N, E613K, and W627F—to analyze the role of electrostatic interactions in the reactions between the altered PSI and pc or cyt c, we measured the second-order rate constant of P700⁻ reduction by pc and cyt c as a function of the MgCl₂ concentration (Fig. 4). It was shown that the electron transfer rate constants from both donors to wild type PSI decreased at salt concentrations higher than 3 mM MgCl₂, whereas the electron transfer rates increased with...
increasing salt concentration for the PsaF-deficient PSI (21). For mutants E613N and W627F, the electron transfer rates from pc or cyt $c_6$ to PSI show an optimum between 1 and 3 mM MgCl$_2$ and a decrease by >1 order of magnitude at higher concentrations. The optimal second-order rates are found for PSI E613N with pc and cyt $c_6$ at 30 and 9 × 10$^6$ M$^{-1}$ s$^{-1}$, respectively, and for PSI W627F with pc and cyt $c_6$ at 0.9 and 1.75 × 10$^5$ M$^{-1}$ s$^{-1}$, respectively. It is noteworthy that the optimum second-order rate constant for the altered PSI W627F in the interaction with pc is about 1 order of magnitude slower than that with wild type or mutant E613N PSI (Fig. 4, see also below). The electron transfer rates from pc or cyt $c_6$ to mutant PSI E613K increase with increasing salt concentrations, displaying an optimum between 100 and 300 mM MgCl$_2$. The optimal second-order rates are found at values of 1.9 and 3.4 × 10$^6$ M$^{-1}$ s$^{-1}$ for pc and cyt $c_6$, respectively. The shape of the salt dependence and the optimal electron transfer rates measured for interaction between the two donors and mutant PSI E613K are very similar to what was found with the PsaF-deficient PSI (21), thus suggesting that mutation PsaB E613K drastically disturbs the interaction between the donors and the positively charged N-terminal domain of PsaF. However, another possible explanation could be that the PsaF subunit is lost during the isolation of mutant E613K PSI, although it is present in thylakoids of the mutant. To analyze this question, equal amounts of protein from enriched PSI particles isolated from wild type or mutant E613K were fractionated by SDS-PAGE and analyzed by immunoblotting using anti-PsaF antibodies. These immunoblots show that PsaF is present in PSI from mutant E613K at almost equal amounts as compared with the amount of PsaF present in a wild type PSI preparation (data not shown). To check whether PsaF is reachable in the isolated altered PSI E613K particles for the soluble donors, we performed cross-linking experiments.

**Cross-linking of pc or cyt $c_6$ to PsaF Is Diminished with PSI from Mutant Strains E613K and W627F—**The interactions between PsaF subunit and pc or cyt $c_6$ were examined by cross-linking studies, using purified PSI particles from the E613N, E613K, and W627F transformants and from wild type (Fig. 5). The cross-linked products were fractionated by SDS-PAGE and identified by immunoblotting using PsaF antibodies (Fig. 5). The cross-linking products between pc or cyt $c_6$ and PsaF were found at about 29 kDa. It was shown in a previous study that the cross-linked products consist of either PsaF and pc or PsaF and cyt $c_6$ (21). The data confirm that the PsaF subunit is present in the E613K PSI particles. However, the cross-linking efficiency is clearly diminished for both donors. Cross-linking of pc or cyt $c_6$ to PsaF in PSI particles isolated from mutant W627F is also strongly reduced. This result indicates that efficient cross-linking depends not only on the electrostatic interaction between the donors and PsaF but also on a direct contact between the donors and the core of PSI.

**Mutation W627F Abolishes Formation of an Electron Transfer Complex between pc and the Altered PSI—**The cross-linking results and the salt dependence of the second-order rate constant indicate that mutation W627F has a strong impact on the interaction between the altered PSI and cyt $c_6$ or pc. To further characterize the electron transfer reactions between pc or cyt $c_6$ and the mutant W627F PSI, we measured reduction of P700$^+$ at different donor concentrations (see Fig. 7). Fig. 6 shows absorbance transients at 817 nm of PSI particles from wild type (top panels) and the W627F transformant (bottom panels) induced by a laser flash in the presence of 120 μM pc (left panels) or 120 μM cyt $c_6$ (right panels). In the case of wild type, the time course of the P700$^+$ reduction can be deconvoluted into three kinetic components, as described above. In contrast, no fast phase A(1) can be observed for the time course of P700$^+$ reduction in the case of mutant W627F when pc functions as electron donor. A fast phase with a half-time of 30 μs can be deconvoluted from the flash-induced absorbance transient of mutant W627F PSI in the presence of 120 μM cyt $c_6$. This half-time is about 10-fold slower than the half-lives found for the intermolecular electron transfer complex formed between wild type PSI and pc or cyt $c_6$. The amplitude of the 30-μs phase increases with increasing concentration of reduced donor protein at the expense of A(2) (see Fig. 7), indicating that it reflects a first-order electron transfer reaction.

If a $f$ value of 0.66 is considered, as found for the reaction of cyt $c_6$ with wild type PSI, a dissociation constant of 177 μM can be estimated. However, we cannot exclude from our data that the maximum value $f$ of the relative amplitude is higher in the mutant than in the wild type. Assuming a value $f$ of 1.0 in the fit of the curve to the data in Fig. 7 would translate into a dissociation constant of 310 μM. The slower kinetic components of the time course of P700$^+$ reduction for pc or cyt $c_6$ show a rate constant that increases with increasing concentration of reduced donor protein (Fig. 7B). Evaluations of the second-order rate constants, which can be determined from the slopes of the curves in Fig. 7B at low concentrations ($k_{s} = \ln(2)/(t_{1/2} \times [D])$), result in values of 0.74 and 1.6 × 10$^7$ M$^{-1}$ s$^{-1}$ for pc and cyt $c_6$, respectively.

**DISCUSSION**

In previous studies, we could show that efficient electron transfer from both pc and cyt $c_6$ to PSI in eukaryotic organisms depends on PsaF (6, 7, 13, 21). In this study, we have taken advantage of a PsaB-deficient mutant (14) to modify luminal loop $j$ including helix l of PsaB using chloroplast transformation and site-directed mutagenesis. PSI particles from mutant strains containing a specific amino acid change in the PsaB protein, E613N, E613K, and W627F, were isolated together with PSI particles from wild type and used to characterize the electron transfer from purified pc and cyt $c_6$ to P700$^+$ in vitro. Our results indicate that helix l of PsaB is, in addition to the N-terminal domain of PsaF, a second structural element that is
essential for recognition of pc and cyt c6 to the core of PSI, leading to an intermolecular complex competent in fast electron transfer.

Mutation of Glu613 to Asn resulted in a clear acceleration of electron transfer rates as well as improved binding of both donors to PSI as compared with wild type. It is therefore likely that the negative charge provided by residue Glu613 is exposed on the surface of PSI. This view is supported by the data obtained from the three-dimensional structure of the cyanobacterial PSI at 2.5 Å resolution (4). In the structure of the cyanobacterial PSI, the side chain of Glu 617 corresponding to Glu613 in C. reinhardtii points into the lumenal space (Fig. 8).

Thus, the change of Glu to Asn could lead to a tighter binding of the donors to PSI in mutant E613N because this mutation decreases the electrostatic repulsion between the negatively charged donors and PSI. This in turn results in a slower unbinding of both donors from the reaction center (see Table II). Drepper et al. (27) proposed that the rate of unbinding of pc from PSI is limiting for the rate of electron transfer between PSI and the cyt b6/f complex. Such a limitation could be a possible explanation of why mutant E613N dies in strong light (see Table I).

Fig. 6. Mutation of Trp627 to Phe in PsaB abolishes complex formation of PSI with pc but not with cyt c6. Absorbance changes at 817 nm induced by a laser flash in PSI particles from wild type and mutant W627F in the presence of 120 μM pc or 120 μM cyt c6; for conditions, see Fig. 2. The relative amplitudes and half-times of the different kinetic components were as follows: for wild type, A(1) = 0.25 and 0.24 with t1/2(1) = 3.5 × 10⁻⁶ s, A(2) = 0.4 and 0.54 with t1/2(2) = 6.7 × 10⁻⁵ s and 2 × 10⁻⁴ s, and A(3) = 0.35 and 0.22 with t1/2(3) = 1.5 × 10⁻¹ s and 3 × 10⁻² s for pc and cyt c6, respectively, and for mutant PSI W627F, a two exponential decay for pc with A(1) = 0.2 with t1/2(1) = 6 × 10⁻⁴ s, A(2) = 0.73 with t1/2(2) = 3.4 × 10⁻⁴ s, and a triphasic decay with A(1) = 0.09 with t1/2(1) = 3 × 10⁻⁷ s, A(2) = 0.24 with t1/2(2) = 3.5 × 10⁻⁴ s, and A(3) = 0.67 with t1/2(3) = 1.1 × 10⁻¹ s.
N-terminal domain of PsaF would be strongly impaired. This interpretation can explain why the salt dependence of electron transfer between both donors and the altered PSI E613K resembles the data obtained with PSI from the PsaF-deficient mutant. It may also explain why mutations E613N and E613K have opposite effects on binding and electron transfer between the altered PSI particles and the two donors. From these data, we can conclude that (i) residue Glu$^{613}$ supports unbinding of the two donors from PSI because of electrostatic repulsion, and (ii) residue Glu$^{613}$ orients the positively charged N-terminal domain of PsaF in a way that allows efficient binding of donors pe and cyt $c_6$ to PSI.

For eukaryotic organisms, site-directed mutagenesis of pc and analysis of binding and electron transfer between the altered pe and PSI (6, 8, 28, 29) already suggested that besides the long-range electrostatic interaction between the positively charged PsaF and the negative patches of pc, a second recognition site is required that brings the flat hydrophobic surface of pe in close contact with the core of PSI to allow efficient electron transfer from the copper center to P700$^-$. The fact that mutation PsaB W627F abolishes the formation of an intermolecular electron transfer complex between the altered PSI and pe suggests (i) interaction of negatively charged PsaB Glu$^{613}$ with the positively charged PsaF in Chlamydomonas, and (ii) participation of PsaB Trp$^{627}$ in binding of the electron donors.

![Fig. 7. Determination of the kinetic constants for the electron transfer between mutant PSI PsaB W627F and the two electron donors.](image)

![Fig. 8. Crystal structure of S. elongatus PSI at a resolution of 2.5Å with a focus on the luminal side.](image)
changes in distance between electron transfer partners (33). Thus, W627 is also important for binding and electron transfer of cyt c₆ to PSI and required for the formation of the intermolecular electron transfer complex competent in the 3-μs electron transfer.

A functional characterization of a cyanobacterial PSI complex carrying an algal-type PsA/F subunit has already suggested that cyt c₆ from C. reinhardtii is regarding its binding mechanism to PSI an evolutionary intermediate between cyt c₆ from S. elongatus and pc from C. reinhardtii (13). This view is substantiated with the data and interpretations presented above.

Interestingly, mutation E613K and mutation W627F cause a strong limitation of electron transfer between PSI and pc or cyt c₆, as already observed for the PsaF-deficient mutant. In comparison with the PsaF-deficient mutant, this restriction results in a strong limitation of electron transfer between PSI and pc or cyt c₆ to PSI.

In summary, we can conclude that lumenal loop j (especially helix l) is essential for efficient binding and fast electron transfer between PSI and pc or cyt c₆, as already observed for the PsA/F-deficient mutant. In comparison with the PsA/F-deficient mutant, this restriction results in an evolutionary intermediate between cyt c₆ from S. elongatus and pc from C. reinhardtii (13). This view is substantiated with the data and interpretations presented above.

Acknowledgments—We are very grateful to Dr. K. Redding for the kind gift of various plasmids and the ΔpsaB mutant strain of C. reinhardtii. We also thank Dr. J. D. Rochaix for the kind gift of antibodies. We are indebted to Dr. R. Bock for initial help in using the helium-driven particle gun. We acknowledge the support of Dr. W. Haehnel.

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J. Biol. Chem. 2002, 277:6573-6581.
doi: 10.1074/jbc.M110633200 originally published online December 14, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M110633200

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