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Residues PsaB Asp612 and PsaB Glu613 of Photosystem I Confer pH-Dependent Binding of Plastocyanin and Cytochrome c_6

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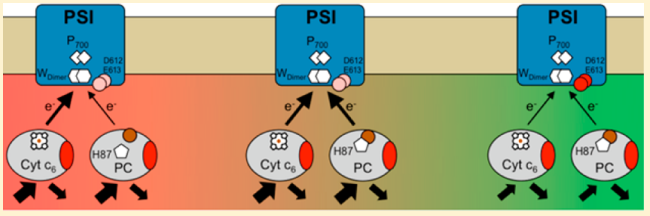
Supporting Information

ABSTRACT: The binding and electron transfer between plastocyanin (pc) or cytochrome c_6 (cyt c_6) and photosystem I (PSI) can be described by hydrophobic as well as electrostatic interactions. The two α helices, l and l' in PsaB and PsaA, respectively, are involved in forming the hydrophobic interaction site at the oxidizing site of PSI. To obtain mechanistic insights into the function of the two negatively charged residues D612 and E613, present in α helix l of PsaB, we exchanged both residues by site-directed mutagenesis with His and transformed a PsaB deficient mutant of *Chlamydomonas reinhardtii*. Flash-induced absorption spectroscopy revealed that PSI harboring the changes D612H and E613H had a high affinity toward binding of the electron donors and possessed an altered pH dependence of electron transfer with pc and cyt c_6. Despite optimized binding and electron transfer between the altered PSI and its electron donors, the mutant strain PsaB-D612H/E613H exhibited a strong light sensitive growth phenotype, indicating that decelerated turnover between pc/cyt c_6 and PSI with respect to electron transfer is deleterious to the cells.



The eukaryotic photosystem I (PSI), which is embedded in the chloroplast thylakoid membrane, is a light-driven plastocyanin:ferredoxin oxidoreductase. It accepts electrons from the luminal soluble, copper (Cu)-containing electron carrier plastocyanin (pc) and donates it to the stroma-localized soluble ferredoxin (fd). In cyanobacteria and under limited Cu availability conditions in green algae, pc can be substituted with a *c*-type heme containing cytochrome c_6 (cyt c_6).[1−4] The PSI core is built mainly by subunits PsaA and PsaB, harboring the P700 chlorophyll pair and [4Fe-4S] iron−sulfur cluster F_X among other cofactors. On the stromal side, PSI subunit PsaC contains [4Fe-4S] iron−sulfur clusters F_A and F_B as well as PsaD and PsaE form the interaction site for fd.[5]

The electron transfer reaction from pc to PSI is a well-studied process[6] and can be described by a protein−protein interaction model depicted by Sommer et al.[7] According to the model, the binding and electron transfer between pc/cyt c_6 and PSI can be described by hydrophobic as well as electrostatic interactions between the electron transfer partners.

The hydrophobic interaction site is formed by two α helices, l and l', stemming from loops j and j' in PsaB and PsaA, respectively. These two helices are arranged parallel to the membrane plane, each carrying one Trp residue, PsaB W627 and PsaA W652 forming with their indole side chains a sandwichlike structure above P700. Altering these residues to Phe by site-directed mutagenesis selectively abolished the formation of an intermolecular complex between PSI and the electron donors.[7,8]

The positively charged N-terminal domain of the PsaF subunit of PSI allows the electrostatic interaction between the electron donors and PSI as proven by several independent approaches.[9−12] Additionally, the negatively charged PsaB residue E613 is important for efficient unbinding of the oxidized pc from the PSI complex.[13] The alteration of PsaB E613 to Asn leads to a higher affinity for binding of pc to the modified PSI in comparison to wild-type PSI. The high-affinity binding of pc to PSI in turn slows the dissociation of oxidized pc from PSI. The involvement of PsaB E613 in the binding of pc is also supported by the currently released PSI crystal structures from pea.[14,15]

As photosynthetic electron transfer proceeds in the light, a proton gradient that fuels the thylakoid-embedded ATPase to generate ATP in the stroma is built up.[16,17] Thereby, the lumen becomes acidified to a pH of ∼6 and slightly below (for a review, see ref 18). Using EPR spin probes, it has been demonstrated that the luminal pH in *Vicia faba* can decrease to values as low as 5.4.[19] Accordingly, the violaxanthin de-epoxidase (VDE) that converts the carotenoid violaxanthin to zeaxanthin has its activation maximum at pH 5.2.[20] The decrease in the luminal pH also influences the electron transfer between pc and PSI as already shown by Hansson and co-workers,[21] demonstrating that the efficiency of the electron transfer decreases at pH <5.5.

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To obtain further insights into the role of the two negatively charged residues, D612 and E613, in electron transfer and binding between the two electron donors and PSI, we took advantage of site-directed mutagenesis to mutate both residues to His and transformed a PsaB deficient mutant of *Chlamydomonas reinhardtii*.[22] Interestingly, PSI harboring the changes D612H and E613H possesses a high affinity toward binding of the electron donors and an altered pH dependence in accepting electrons from the soluble donors.

EXPERIMENTAL PROCEDURES

Strains and Media. Wild-type and mutant strains were grown as described previously.[23] For isolation of PSI and pc Tris-acetate-phosphate (TAP) medium, for the isolation of cyt c_6 copper deficient TAP medium was used. The growth tests were conducted with solidified (1.5% agar) TAP medium for photoheterotrophic and high-salt medium (HSM) for photoautotrophic growth.

Isolation of PSI Particles. Thylakoid membranes where isolated from whole cells according to published procedures.[24] The isolated membranes were diluted to a chlorophyll concentration of 0.8 mg/mL and solubilized with 0.9 and 1.5% (w/v) dodecyl β-maltoside for the wild-type and altered photosystems, respectively, by incubating for 20 min on ice. The photosystem complexes were separated using a linear sucrose density gradient,[25] and PSI was concentrated afterward using ultrafiltration columns with a size exclusion of 100000 molecular weight.

Heterologous Expression and Isolation of Plastocyanin. pc was heterologously expressed as described by Hulsker and co-workers.[26] The codon-optimized *petE* gene was delivered by GenScript and ligated into expression vector pET17b (Invitrogen). The 47-amino acid transit peptide of *petE* was replaced by a single methionine.

Isolation of Plastocyanin and Cytochrome c_6 from *C. reinhardtii*. Plastocyanin and cyt c_6 were isolated according to the methods of refs 2, 27, and 28. The concentration of both electron donors was determined spectroscopically as described previously for oxidized pc[29] and reduced cyt c_6.[4]

Growth Tests. Cells from log phase cultures were diluted to the same cell numbers (10^6 cells/mL), and 20 μL was spotted onto agar plates. The plates were kept under high light (450 μE m^{-2} s^{-1}), normal light (40 μE m^{-2} s^{-1}), and low light (5 μE m^{-2} s^{-1}). The plates were kept between 2 (e.g., high light, TAP) and 7 (e.g., low light, HSM) days under certain light conditions, depending on the growth rate and the medium used. Anaerobic growth was established using the GENbag aner anaerobiosis system (BioMérieux).

Generation of the Mutant Strain. Point mutations D612H and E613H in PSI subunit PsaB were introduced by site-directed mutagenesis.[30] The mutation was confirmed by sequencing and the expression of PSI by Western blot analysis. The mutant PsaB-D612H/E613H is also designated as T26 in the following.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Western Blot Analysis. SDS–PAGE (13% acrylamide gel) was conducted as described previously.[31] Proteins were transferred onto a nitrocellulose membrane by semidry electroblotting, and the immuno detection was performed using anti-PsaD and anti-PsaF antibodies in a 1:1000 dilution.

Single-Flash Absorbance Spectroscopy. The kinetics of flash-induced absorbance changes at 817 nm were measured as described previously,[7,8,11,32] using a cuvette containing 200 or 50 μL of the sample with an optical path length of 10 or 3 mm, respectively. Relative amplitude rel$A(1)$ of the fast kinetic phase of the P700 reduction kinetics is described by

$$\mathrm{rel}A(1) = f \times \frac{[\mathrm{D}]}{[\mathrm{D}]K_\mathrm{D}} \quad (1)$$

where $[\mathrm{D}]$ represents the reduced donor concentration, K_D the dissociation constant, and f an empirical factor ($f < 1$) that relates the amplitude $A(1)$ observed after the flash to the fraction of PSI in a complex with the reduced donor prior to the flash. The chlorophyll content was measured according to the protocol published by Porra.[33]

$NADP^+$ Photoreduction Measurements. The light-driven, PSI-dependent reduction of $NADP^+$ was conducted according to the protocol by Finazzi and co-workers.[13] To apply saturating light at the sample, a halogen lamp with a light intensity of $\sim 10^4$ μE m^{-2} s^{-1} was used.

Fluorescence. Photoheterotrophically grown cells from log phase were diluted to the same chlorophyll content (10 μg/mL) and dark-adapted for 20 min before each measurement. Fluorescence was measured using a Maxi-Imaging PAM chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany). The effective photochemical quantum yield of photosystem II was measured [$Y(\mathrm{II}) = (F_\mathrm{m}' - F)/F_\mathrm{m}'$, where F_m' represents the maximal and F the steady-state level of fluorescence emission].

pK_a Calculation. pK_a values were calculated using MCCE 2.4[34] and DELPHI version 4.[35,36] PSI subunits PsaA, PsaB, and PsaF taken from Protein Data Bank entry 1YO9[37] were embedded into a 30 Å hydrophobic and low-dielectric slab using IPECE, as reported previously.[38] Rotational freedom, i.e., conformer sampling, was limited to residues on the luminal side of PSI. This restriction was imposed because of the technical limitation on the number of atoms that can be handled by MCCE and Delphi. For the sake of simplicity, all cofactors, except chlorophylls and carotenoids, were deleted from the structure.

RESULTS

Double Mutation D612H/E613H in PsaB Leads to Enhanced Light Sensitivity. To investigate the role of residues PsaB D612 and E613 in binding and electron transfer between pc and PSI, we performed site-directed mutagenesis. The DNA coding sequences for D612 and E613 in the *psaB* gene were altered each to encode His. The *psaB* plasmid encoding the D612H and E613H mutations and containing the *aadA* gene[39] (encoding aminoglycoside adenyl transferase and conferring resistance to spectinomycin or streptomycin) was transformed into a PsaB deficient *C. reinhardtii* mutant strain.[22] After transformation, several colonies were obtained by selection on spectinomycin-containing medium. One of the transformants, T26, was further analyzed in depth. SDS–PAGE fractionation of whole cells and subsequent immunoblot analyses using anti-PsaD antibodies revealed that the PsaB-D612H/E613H transformant possessed ~75% of PSI as compared to the wild type (WT) (Figure 1A). On the other hand, amounts of PsaD and PsaF in PSI particles that have been isolated by sucrose density centrifugation were comparable as assessed by immunoblotting after separation of PSI particles by SDS–PAGE (Figure 1B). In growth test experiments, the mutant strain showed a severe light sensitive

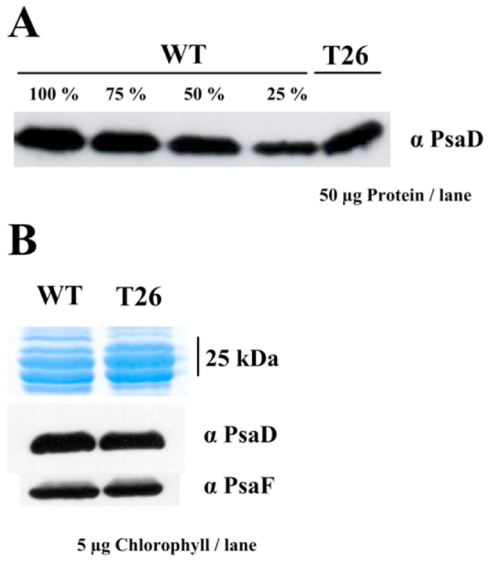

Figure 1. (A) Western blot analysis of whole cells loaded on equal protein and a dilution series for the wild type (WT). The mutant has ~25% less PSI than the WT. (B) Western blot analysis for PSI particles shows the same amount of PsaD and PsaF in the WT and mutant. The bands around 25 kDa on the Coomassie-stained gel represent the light-harvesting proteins that where serving as a loading control.

phenotype under photoautotrophic conditions (HSM, minimal medium), whereas under photoheterotrophic conditions (TAP, acetate-containing media), the cells were only slightly more sensitive than wild-type cells. This growth phenotype was evident on copper sufficient or deficient plates (Figure 2A). Interestingly, anaerobic growth conditions could rescue the light sensitive photoautotrophic phenotype of the PsaB-D612H/E613H transformant (T26) (Figure 2B), suggesting that the formation of ROS caused the light sensitivity of the mutant strain as already described for other mutants with defects in PSI-dependent electron transfer.[40,41] For further analyses, the quantum yield of PSII [Y(II)] was determined by fluorescence spectroscopy using dark-adapted cells (Figure 1 of the Supporting Information). As expected, the PSII quantum yield is significantly lower in the mutant than in the WT. Moreover, the PSII quantum yield is more diminished in strain T26 with increasing light intensities than in the WT, suggesting that PSII in the mutant is more susceptible to photodamage than in the WT. This implies that photoinhibition of PSII could be a cause of the light sensitivity of transformant T26, as already described for other PSI oxidizing side mutants.[40,41]

Electron Transfer between pc and PSI from the WT and PsaB-D612H/E613H. We used single-turnover flash excitation to determine the second-order electron transfer rate for the electron transfer between pc or cyt c_6 and PSI in vitro at different pH values with an increasing ionic strength

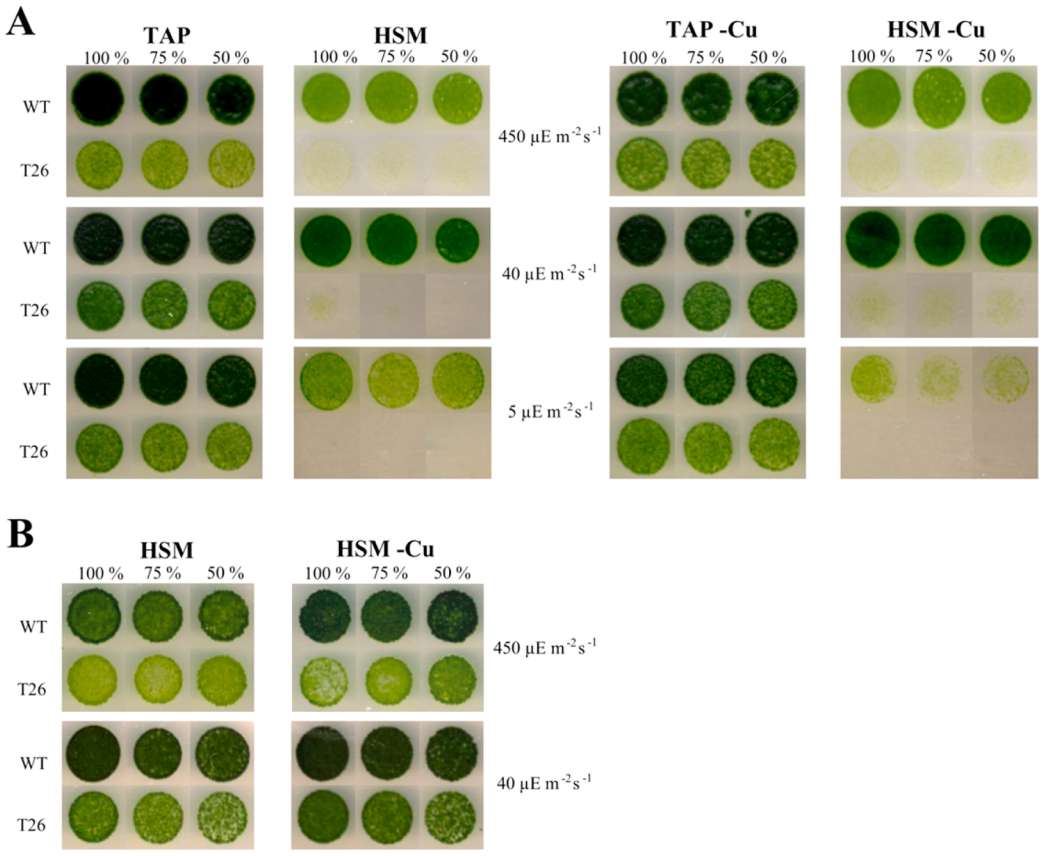

Figure 2. Growth test performed on agar plates to check the growth phenotype under different light intensities in combination with photoheterotrophic (TAP) and photoautotrophic (HSM) conditions. To check whether the electron donor has an influence on the phenotype, copper deficient medium was also used to induce the expression of cyt c_6. (A) The results show that the mutant has impaired growth under photoheterotropic conditions, whereas it shows almost no growth under photoautotrophic conditions, in a manner independent of the light intensity and electron donor. (B) Under anaerobic photoautotrophic conditions, it is possible to rescue the growth phenotype for all the light intensities that were used, in a manner independent of the electron donor pc (copper sufficient medium) and cyt c_6 (copper deficient medium).

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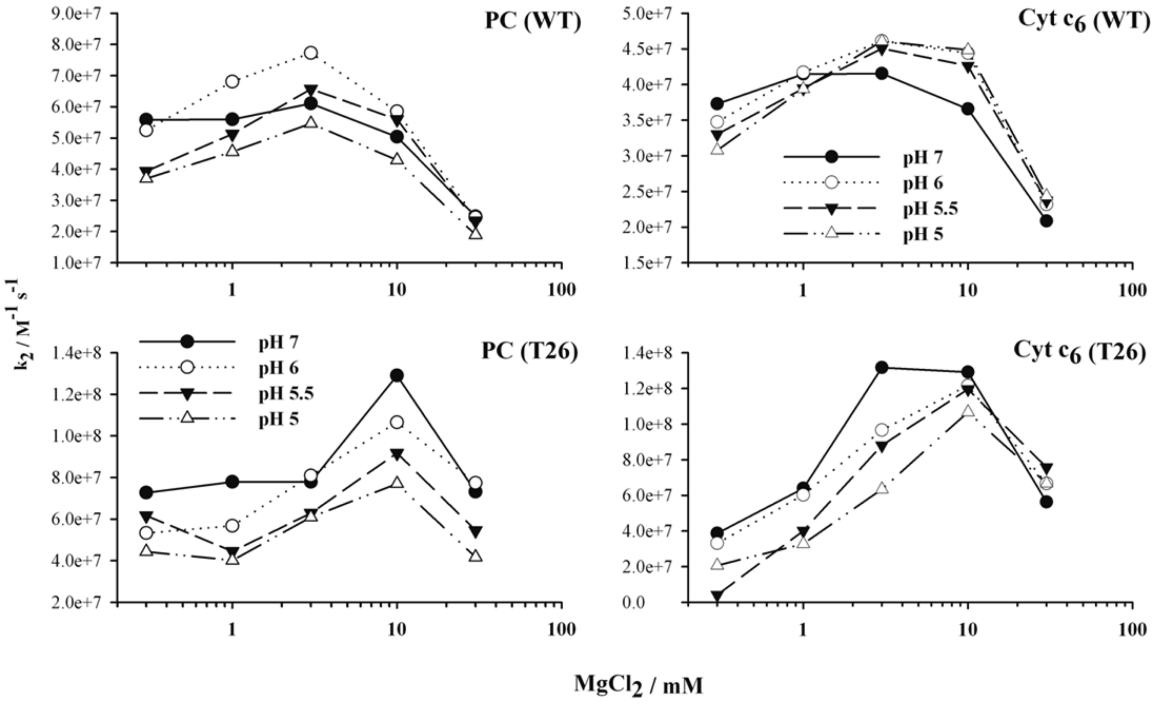

Figure 3. Second-order rate constant of reduction of $P700^+$ by pc or cyt c_6 at different $MgCl_2$ concentrations with a changing pH. To increase the ionic strength, small amounts of a concentrated $MgCl_2$ solution were added. The pH of each sample was measured after the spectroscopic measurements to ensure the correct pH. The optimal salt concentration is ~3 mM for WT and 10 mM for the altered PSI, whereas the optimal pH values are pH 6 and 7, respectively.

Table 1. Second-Order Rate Constants (k_2) and Dissociation Constants (K_D) for pc or cyt c_6 and PSI[a]

	PC			cyt c_6		
	k_2 (M^{-1} s^{-1})	K_D (μM)	f	k_2 (M^{-1} s^{-1})	K_D (μM)	f
WT, pH 7	6.10×10^7	96	0.52	4.15×10^7	81[b]	0.68[b]
WT, pH 6	7.72×10^7	–	–	4.61×10^7	–	–
WT, pH 5.5	6.56×10^7	–	–	4.50×10^7	–	–
WT, pH 5	5.46×10^7	56	0.46	4.61×10^7	–[c]	–[c]
T26, pH 7	1.29×10^8	16	0.74	1.29×10^8	26	0.70
T26, pH 6	1.07×10^8	–	–	1.22×10^8	–	–
T26, pH 5.5	9.17×10^7	–	–	1.19×10^8	–	–
T26, pH 5	7.69×10^7	11	0.67	1.07×10^8	–[c]	–[c]

[a]The PSI particles as well as pc and cyt c_6 were isolated from WT and altered PSI (T26) from the mutant strain. f represents an empirical factor ($f < 1$), which relates the amplitude A(1) observed after the flash to the fraction of PSI forming a complex with the reduced donor before the flash. [b]Values taken from ref 7. [c]Not possible to evaluate the measurement because the cyt c_6 started to precipitate at pH 5 and higher concentrations.

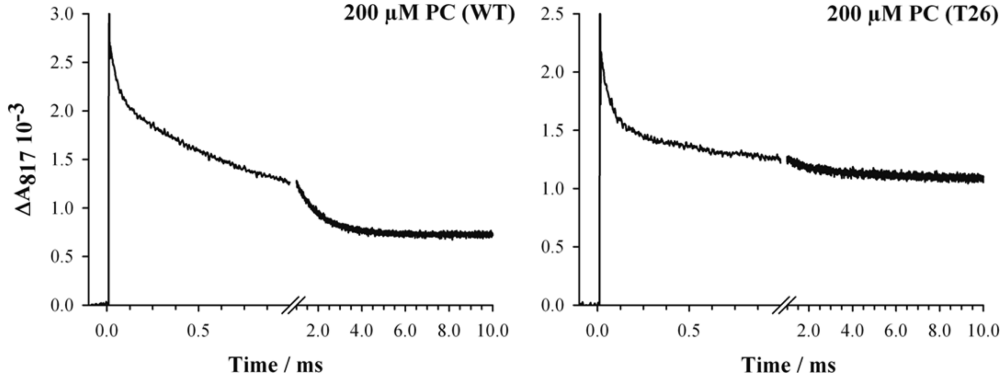

Figure 4. Absorbance transient at 817 nm of the fast kinetic for the reduction of WT and altered PSI in the presence of 200 μM pc. The first-order phase shows a half-time of ~5 μs in both cases, whereas the relative amplitude [$A(1)$] is greater in altered PSI than in the WT.

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(Figure 3). The maximal electron transfer reaction rate between WT PSI and pc or cyt c_6 was observed at a $MgCl_2$ concentration of 3 or 3−10 mM, respectively, whereas the altered PSI PsaB-D612H/E613H showed the highest rate at 10 mM $MgCl_2$ for both electron donors (Table 1). The optimal electron transfer between the donors and WT or altered PSI was found at pH 6 or pH 7, respectively, resulting in second-order electron transfer rates of 7.7×10^7 M^{-1} s^{-1} for pc and 4.6×10^7 M^{-1} s^{-1} for cyt c_6 for the wild type and 1.3×10^8 M^{-1} s^{-1} for pc and 1.3×10^8 M^{-1} s^{-1} for cyt c_6 for altered PSI under optimal conditions (Table 1). Thus, the pH optima for the electron transfer between PSI and its two soluble donors are strongly dependent on the presence of D612 and E613.

Figure 4 shows the absorbance transients at 817 nm induced by a laser flash for WT and PsaB-D612H/E613H PSI particles in the presence of 200 μM pc (pH 7) and the optimal $MgCl_2$ concentration. For both the WT and mutant PsaB-D612H/E613H, the time course of $P700^+$ reduction can be attributed to three kinetic components. The fast component with a half-life of 3−4 μs and a variable amplitude $A(1)$ reflects a first-order electron transfer reaction that is independent of the donor concentration (Table 1a,b of the Supporting Information). The second component, $A(2)$, exhibits a half-life that is dependent on the donor concentration and decreases with increasing donor concentrations. At the same time, $A(2)$ decreases while $A(1)$ congruently increases at increasing donor concentrations. The third very slow component, $A(3)$, is due to PSI complexes that lost their PsaF subunit in the course of PSI particle isolation,[28] and its amplitude corresponds to 25−40% of the total PSI signal. We will further consider only kinetic components $A(1)$ and $A(2)$ and a kinetic model that has been described to account for the binding and electron transfer between the donors and PSI.[32] In this model, a simple dissociation equilibrium, which also takes the redox equilibrium into account, can account for the concentration dependence of the $A(1)$ and allows the calculation of the dissociation constant K_D.[32] Taking advantage of the formula (eq 1) described by Drepper et al.,[32] we calculated the pH-dependent changes in the K_D values for the electron transfer reaction between WT or mutant PsaB-D612H/E613H and pc or cyt c_6. Interestingly, the resulting K_D values determined for the altered PSI were 6 times lower at pH 7 and 5 times lower at pH 5 than the K_D values obtained for the WT, reflecting an increased affinity for pc or cyt c_6 when residues D612 and E613 were substituted with His (Table 1). However, it appears that the changes of D612 and E613 to His render mutant cells light sensitive, although they allow a stronger binding and faster second-order electron transfer between PSI and its donors (Figure 3), pointing to the fact that rapid unbinding of oxidized pc is important for the overall electron transfer reaction.

PSI-Dependent $NADP^+$ Photoreduction. As an independent approach for measuring electron transfer between pc and PSI, we examined the in vitro $NADP^+$ photoreduction as described previously.[13,40] At low concentrations of the recombinant WT (1.5 μM), the $NADP^+$ photoreduction rate for the altered PSI was ~3 times higher than that of the WT, which is in line with the observed higher affinity for reduced pc as described above (Figure 5). However, this tendency was reversed at higher pc concentrations (80 μM), where the $NADP^+$ photoreduction rate for the altered PSI is now diminished in comparison to that of the WT (Table 2). Such a property has already been described for $NADP^+$ photoreduction of mutant PSI PsaB-E613N using endogenous WT

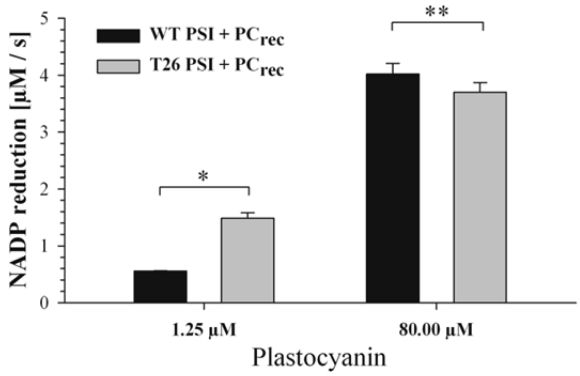

Figure 5. $NADP^+$ photoreduction measurements. WT and altered PSI in combination with recombinant WT pc at low (1.25 μM) and high concentrations (80 μM). The difference between WT and altered PSI (T26) is significant (*$P \leq 0.001$; **$P \leq 0.05$).

Table 2. $NADP^+$ Photoreduction Rates for WT and Altered PSI (T26) Particles in the Presence of 1.25 and 80 μM Recombinant Mutated and WT pc, Respectively

[PC] (μM)	WT PSI (μM/s)	T26 PSI (μM/s)
1.25	0.55 ± 0.01	1.49 ± 0.01
80	4.02 ± 0.19	3.70 ± 0.17

pc[13] and was interpreted in a way that the slow unbinding of oxidized pc from PSI E613N limits the forward transfer of an electron to $NADP^+$. A similar scenario can also be suggested for $NADP^+$ photoreduction in the presence of high pc concentrations and PSI from mutant PsaB-D612H/E613H.

DISCUSSION

The binding and electron transfer between pc and PSI is a well-described process,[6] where hydrophobic and electrostatic interactions are required for an efficient electron transfer reaction between the two partners. In the course of evolution, the dissociation constant for binding of pc to PSI was decreased nearly 100-fold, comparing the electron transfer reaction between cyanobacterial pc and PSI and plant pc and PSI.[6] This higher affinity is facilitated by the development of a novel plant N-terminal domain of PsaF that is missing in cyanobacteria (see the introductory section). On the other hand, evolution has not selected for an overly tight binding between the two partners. This has been demonstrated by Sommer et al.[8] and Finazzi and co-workers[13] as well as in this study and is evidenced by the fact that mutant strains PsaB-E613N[8,13] and PsaB-D612H/E613H exhibit an increased light sensitivity. In contrast to Asn, the two His residues at PsaB positions 612 and 613 might be protonated by acidification of the thylakoid lumen during photosynthetic electron transfer.

Residues PsaB D612 and PsaB E613 Are Responsible for the pH Dependence of the Electron Transfer between pc and PSI. To investigate the possible charge state of the two introduced His residues, we calculated their pK_a values (Table 3). The pK_a values of the two His residues in the PsaB-D612H/E613H mutant appear to be very low (1.455 for H612 and 3.867 for H613), pointing to the fact that both residues are not protonated, even when the luminal pH decreases to a low value such as 5.5, which is barely reached under physiological conditions (see above). In line with previous data obtained from altered PSI PsaB-E613N, and also for PsaB-D612H/E613H, high-affinity binding between

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Table 3. Calculated pK_a Values for psaB Residues D612 and E613 and the Altered PSI Containing Two Histidines at These Positions[a]

	pK_a 612	n (slope)	χ^2	pK_a 613	n (slope)	χ^2
E613				3.403	0.672	1.908
D612H/E613H	1.455	0.350	3.534	3.867	0.625	0.541
E613K				9.985	0.688	1.613

[a]Furthermore, the pK_a of the lysin residue in the previously described E613N[8] mutant was calculated. Because the structure was obtained from pea, the residues changed in the structure were PsaB N610 (D612 in *Chlamydomonas*) and PsaB E611 (E612).

PSI and pc is observed. This points to the fact that the introduced His residues remain likely nonprotonated, which is supporting our experimental findings. It is interesting to note that with PSI harboring the D612H and E613H changes, the rate of electron transfer between PSI and pc does not increase from pH 7 to 6 as observed for WT PSI. This indicates that residues PsaB D612 and PsaB E613 are probably partially protonated at decreasing pH, thereby weakening the charge repulsion between negatively charged residues provided by PsaB D612 and PsaB E613 and of pc and thus allowing faster electron transfer. The decrease in the electron transfer rate at pH 5.5 and 5 could be associated with the finding that the copper binding site of pc changes its conformation from a tetrahedral to a planar trigonal geometry at low pH, favoring the Cu^I structure and thereby suggesting that the protein might be redox-inactive.[42] Measurements of the midpoint potential of free pc at various pH values showed an increase with lower pH values, having a pK_a value for the whole protein of ~5.6.[29,43] The pK_a value of His87 appears to be 4.9 in spinach as measured by NMR spectroscopy.[44,45] Once His 87 binds to PSI, its pK_a value is shifted to a lower pH.[46] It is possible that pc loses copper from its active site at low pH. Notably for cyt c_6, such a pH-dependent inactivation is not reported and the rate of electron transfer between the donor and PSI is faster with WT PSI at pH <7.0 but slower with PSI from mutant T26. Thus, residues PsaB D612 and PsaB E613 appear to be responsible for the pH dependence of electron transfer between pc or cyt c_6 and PSI. Taken together, the data indicate that replacement of PsaB D612 and PsaB E613 with amino acids not providing charged side chains increases the affinity between the donor and PSI in a way that overall turnover, including binding, electron transfer, and unbinding of the oxidized donor, is slower than that of the WT, leading to light sensitivity and compromised growth of the respective mutant strains (see Figure 5). Under conditions where the luminal pH decreases, residues PsaB D612 and PsaB E613 will become partially deprotonated, which in turn will accelerate binding and electron transfer (see Table 1). However, this acceleration is not promoted to the extent that is seen for altered PSI harboring noncharged amino acids at these positions. A remaining negative charge at residues PsaB D612 and PsaB E613 is probably important for a more efficient unbinding of the oxidized donors as suggested previously.[8] Thus, the electron transfer reaction between pc or cyt c_6 and PSI is a fine-tuned, balanced reaction optimized for an in vivo physiological situation.

■ ASSOCIATED CONTENT

Supporting Information

One figure comparing the PSII quantum yields [Y(II)] of the WT and mutant and two tables listing the relative amplitudes and half-times from single-flash absorbance spectroscopy. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PSI, photosystem I; PSII, photosystem II; LHCII, light-harvesting antenna of PSII; CEF, cyclic electron transfer; TAP, Tris-acetate-phosphate medium; HSM, high-salt medium; μE, microeinsteins; WT, wild type; T26, PsaB-D612H/E613H mutant strain; pc, plastocyanin; cyt c_6, cytochrome c_6; fd, ferredoxin.

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