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Kinetic and Cross-Linking Studies on the Interactions of Negative Patch Mutant Plastocyanin from *Silene pratensis* with Photosystem I Complexes from Cyanobacteria, Green Algae, and Plants[1]

Takashi Hibino,* Byung Hyun Lee,† Tatsuo Yajima,‡ Akira Odani,‡ Osamu Yamauchi,‡ and Teruhiro Takabe*,§,2

*Department of Chemistry, Faculty of Science & Technology, Meijo University, Tenpaku-ku, Nagoya 468; †BioScience Center, School of Agricultural Science, Nagoya University, Chikusa-ku, Nagoya 464-01; ‡Department of Chemistry, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464-01; and §Research Institute of Meijo University, Tenpaku-ku, Nagoya 468

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The site-directed mutants of negative patches on silene plastocyanin (PC) were used to investigate the change of interactions between photosystem I (PSI) and PC during the course of evolution from cyanobacteria to plants. The net charges of two highly conserved negative patches (#42-45 and #59-61) on silene PC were systematically modified from −4 to +1. PSI complexes from cucumber and *Chlamydomonas reinhardtii* were efficient electron acceptors for silene PC. The increase of net charge on the negative patch (#42-45) of silene PC decreased the reduction rates of PSI from cucumber and *Chlamydomonas*, while the modification of the other negative patch (#59-61) had no effect. Though the addition of $MgCl_2$ decreased the reduction rate of cucumber PSI, the decrease was severely diminished in the case of *Chlamydomonas* PSI, and the reduction rate increased with increasing concentration of $MgCl_2$ when the net charge of the negative patch (#42-45) was modified to +1. The PSI complexes from *Anabaena variabilis* and *Synechosystis* sp. PCC 6803 were inefficient electron acceptors for silene PC and their rates were almost independent of the net charge of the negative patches, as well as the ionic strength of the reaction mixtures. Silene PC specifically cross-linked to the PsaF subunit of PSI complexes from cucumber, *Chlamydomonas*, *Anabaena*, and *Synechosystis* sp. PCC 6803. Modification of the negative patch (#42-45) inhibited the formation of cross-linked adducts in all the cases examined, whereas modification of the other negative patch (#59-61) had essentially no effect. Based on these results, the changes of electrostatic interactions between PC and PSI during the course of evolution from cyanobacteria to plants are discussed.

Key words: electron transfer, photosynthesis, photosystem I, plastocyanin, site-directed mutagenesis.

Plastocyanin (PC) is a copper protein (10.5 kDa) that functions as a mobile electron carrier between the cytochrome *b6/f* (Cyt. *b6/f*) complex and the photosystem I (PSI) complex within the electron transport chain of oxygen-evolving photosynthetic organisms (*1*). PC occurs in plants, algae, and cyanobacteria (*1-3*). Whereas higher plants utilize only PC as an electron carrier between the two complexes, cyanobacteria and eukaryotic algae can use either PC or Cyt. *c*6 (Cyt. *c*-553), of which the gene expression is differently regulated (*4-6*). PC from higher plants and green algae is an acidic protein, whereas PC can be either basic or acidic in cyanobacteria (*1-3*).

The PSI complex is the photochemical reaction complex that catalyzes the transport of electrons from PC to ferredoxin (*7*). More than 14 genes (*psaA-psaN*) have been proposed to encode the subunits of PSI complex (*7*). The PSI complex contains the photosynthetic pigments, the reaction center (P700), and five electron carriers (A_0, A_1, F_X, F_A, and F_B) that are bound to the PsaA, PsaB, and PsaC proteins (*7, 8*). The involvement of PsaD and PsaE subunits in the interactions between F_A/F_B of PsaC and ferredoxin has been demonstrated (*9-11*). The similarities of primary structures of individual subunits and overall reaction mechanisms of PSI complexes among cyanobacteria, green algae, and plants are well recognized (*7*). However, the functions of several subunits (PsaF-PsaN) are unknown or differ among these organisms. For example, PsaG, PsaH, and PsaN subunits and the light-harvesting chlorophyll *a/b* protein complexes (LHCPI) are absent in cyanobacteria PSI, whereas PsaM subunit has not been detected in plant PSI (*7*). In previous papers, we demonstrated that the PSI core complex from cucumber, which contains eight subunits (PsaA, PsaB, PsaC, PsaD, PsaE, PsaF, PsaG, and an unknown subunit), catalyzes the light-

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[2] To whom correspondence should be addressed.

Abbreviations: Cyt., cytochrome; EDC, *N*-ethyl-3-[3-(dimethylamino)propyl] carbodiimide; LHCPI, light harvesting chlorophyll *a/b* protein associated with photosystem I; PC, plastocyanin; PSI, photosystem I; P700, photosystem I reaction center chlorophyll.

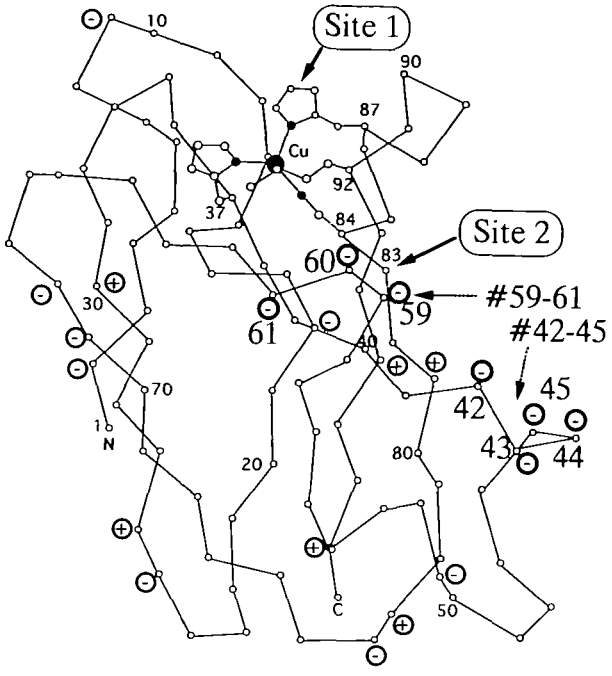


Fig. 1. **The α-carbon chain structure of poplar PC.** The acidic and basic amino acid residues are denoted by ⊖ and ⊕, respectively. The ligands to the copper (His37, Cys84, His87, Met92), Tyr83, the acidic patches at residues #42-45 and #59-61, and two potential binding sites are shown.

driven electron transfer from PC to ferredoxin, and the PsaF subunit is important for efficient electron transfer from PC to P700 (*12, 13*). Depletion of PsaF from PSI complex (*12, 14*) and cross-linking experiments (*12, 15, 16*) support the existence of specific interactions between the basic PsaF subunit and the acidic patch of PC. On the other hand, directed deletion of the *psaF* gene in the cyanobacterium *Synechocystis* sp. PCC 6803 (*17*) and *in vitro* kinetic analysis of the purified PSI complex (*18*) showed that the absence of PsaF in PSI complex did not affect the reduction rate of $P700^+$ by PC or Cyt *c*-553. These results clearly indicate that the PsaF subunit is not involved in the interaction between PC (Cyt *c*-553) and PSI in cyanobacteria. The molecular mechanisms of the different operating modes of PsaF subunits between plants and cyanobacteria are unknown.

Since we had constructed site-directed mutants of a higher plant, *Silene pratensis* (white campion) PC, in which the net charge on negative patches (Site 2, #42-45 and #59-61 of Fig. 1) was modified from -4 to $+1$ (*19*), it was of interest to make a comparative kinetic study of PSI reduction by various mutants of silene PC. In this study, evolutionarily distinct organisms were used for the preparation of PSI complexes, namely, *Anabaena*, *Synechosystis* sp. PCC 6803, *Chlamydomonas*, and cucumber. The isoelectric pHs of the respective PCs are different: 4.3 in cucumber (*20*), 4.3 in *Chlamydomonas (4)*, 5.6 in *Synechosystis* sp. PCC 6803 (*21*), and 7.6 in *Anabaena* (*20*). The local net charges of negative patches in mutant silene PCs cover the range of local net charges of negative patches in these organisms. From the kinetic study of PSI reduction by silene PC mutants, the structural changes of the oxidizing sides of PSI complexes during the course of evolution are discussed. Cross-linking experiments were also carried out to investigate the role of PsaF subunits in these organisms.

MATERIALS AND METHODS

Growth of Chlamydomonas reinhardtii, Anabaena variabilis, and Synechosystis sp. PCC 6803—Chlamydomonas reinhardtii (IAM c-9) and *Anabaena variabilis* were from the collection of the Institute of Applied Microbiology of Tokyo University. *Anabaena* and *Synechosystis* sp. PCC 6803 were grown photoautotrophically at 30°C in BG11 with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-3-propanesulfonic acid (HEPES), pH 8.0. *Chlamydomonas* was grown in a clostridium medium with metals at 22°C under continous light (200 lux).

Preparation of Mutant Silene PC and PSI Complexes—The expression and purification of negative patch mutants from *Silene pratensis* were carried out as previously described (*19, 22*). PSI complexes from cucumber were prepared as previously described (*13*). Preparation of *Chlamydomonas* PSI complexes was carried out as follows. After harvesting of the cell culture (1 liter), cells were washed with 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM EDTA, and 0.3 M sucrose (buffer A), and resuspended in the same buffer. The cell suspension was broken in a Bead Beater (Biospec Products) at 0°C by means of 5 pulses of 15 s each with 1 min cooling intervals. Unbroken cells were removed by centrifugation at $10,000 \times g$ for 15 min. The thylakoid membranes were pelleted by centrifugation at $186,000 \times g$ for 20 min and suspended with cold water to give 1 mg Chl/ml. PSI complexes were extracted with 0.45% Triton X-100 and purified according to the methods used for the preparation of cucumber PSI complexes (*13*). PSI complexes from *Anabaena* and *Synechosystis* sp. PCC 6803 were prepared by the method of Rogner *et al.* (*23*) in which the thylakoid membranes were treated with 1% β-dodecylmaltoside.

Cross-Linking between PC and PSI Complexes—The cross-linked complexes between PSI and PC were prepared according to a previous method (*13, 24*). PC (10 μM) and PSI complex (0.5 mg Chl/ml) were incubated for 30 min at 25°C in the light, in the presence of 5 mM *N*-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), 5 mM $MgCl_2$, and 20 mM 3-(*N*-morpholino)propanesulfonic acid buffer (MOPS), pH 6.5. The reaction was quenched with 0.1 M ammonium acetate, then the mixture was diluted with water, centrifuged at $200,000 \times g$ for 1 h, and used for SDS-PAGE.

Kinetics and Other Methods—Kinetic experiments on the electron transfer of PC with PSI were performed using a Union Giken single-beam spectrometer equipped with a Union RA-401 stopped flow instrument and a flash lamp for excitation (*19, 22*). The flash-induced absorbance change of P700 was measured at 697 nm. The polypeptide composition was determined by SDS-PAGE as previously described (*13, 19*). The concentration of acrylamide for cucumber and *Chlamydomonas* PSI complexes was 15% (v/v). The polypeptide composition of *Anabaena* PSI was determined by using Tricine-SDS-PAGE as previously described (*12*). All the experiments were done at 25°C.

RESULTS

Electron Transfer from Wild-Type Silene PC to Photooxidized P700 Isolated from Cucumber, Chlamydomonas reinhardtii, Anabaena variabilis, and Synechosystis sp. PCC 6803—The native PSI complexes were isolated from cucumber, *Chlamydomonas*, *Anabaena*, and *Synechosystis* sp. PCC 6803 as described in "MATERIALS AND METHODS." For the reduction of P700 by PC, the existence of two different reduction phases, *i.e.*, the electron transfer between the bound PC-PSI complex and the bimolecular reaction between free PC and PSI, has been reported in higher plants and in some eukaryotic algae (*15, 25-29*), although the association constant of the former phase was very large (50-170 μM) (*26-28*). In the cases of cyanobacteria and in heterologous systems such as the reactions between plant PSI and algal PC or plant PSI and mutant PC, absence of the former phase was reported (*17-19, 27-30*). In this study, we observed pseudo-first-order kinetics for the reduction of PSI complexes from cucumber, *Chlamydomonas*, *Anabaena*, and *Synechosystis* sp. PCC 6803 by the wild-type silene PC as well as the mutant silene PC (data not shown). The observed pseudo-first-order rate constants were linear with respect to the PC concentration up to 50 μM for all four PSI complexes, as shown in Fig. 2 and the reactions were analyzed as second-order. In the absence of $MgCl_2$, silene PC could most efficiently reduce the $P700^+$ of cucumber PSI complex. Compared to the case of cucumber PSI, silene PC donated electrons to the *Chlamydomonas* PSI at a reduced rate (26%) although these reactions were significantly affected by ionic strength. PSI complexes from *Anabaena* and *Synechosystis* sp. PCC 6803 were much more inefficient electron acceptors for silene PC and the rates were less than 1% of that of the reaction catalyzed by the cucumber PSI complex. It should be noted that the *Anabaena* PSI complex was reduced more efficiently than the *Synechosystis* PSI complex.

Electron Transfer from Negative Patch Mutants of Silene PC to Photooxidized P700 Isolated from Cucumber, Chlamydomonas reinhardtii, Anabaena variabilis, and Synechosystis sp. PCC 6803—As shown in Table I, the net charges on the negative patch (#42-45) of cucumber, silene, and *Chlamydomonas* PCs are all −4, whereas those of *Anabaena* and *Synechosystis* sp. PCC 6803 are −1 and −2, respectively. In *Chlamydomonas* PC, Asp53, and Glu85 are

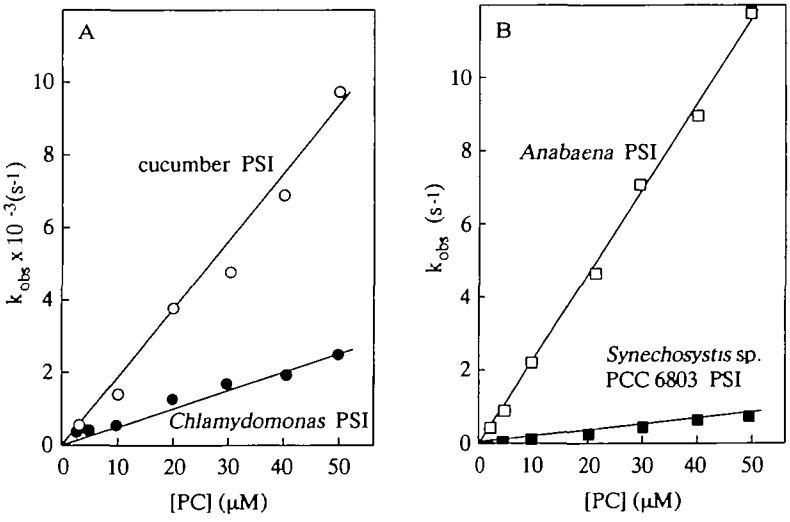


Fig. 2. Silene PC concentration dependence of the observed pseudo-first order rate constant for PSI reduction. The reaction mixture contained 0.25 μM P700, 2.5 mM ascorbate, 0.1 mM methylviologen, 0.05% Triton X-100, 1 mM $MgCl_2$, 1 mM Tris-HCl (pH 8.0), and the indicated silene PC. A: ○, cucumber PSI complex; ●, *Chlamydomonas* PSI complex. B: □, *Anabaena* PSI complex, ■, *Synechosystis* sp. PCC 6803 PSI complex.

TABLE I. **Amino acid sequences of negative patch region of PC.** Underlining indicates the modified amino acid. For the calculation of net charges on the negative patches, the charge of His is assumed as +0.5.

Species	40	41	42	43	44	45	46	47	53	Net charge of (#42-45)
Cucumber	Val	Phe	Asp	Glu	Asp	Glu	Ile	Pro		−4
Silene wild-type	Leu	Phe	Asp	Glu	Asp	Glu	Val	Pro		−4
M1 Asp42Asn	Leu	Phe	Asn	Glu	Asp	Glu	Val	Pro		−3
M2 Glu43Lys	Leu	Phe	Asp	Lys	Asp	Glu	Val	Pro		−2
M3 Asp42Asn +Glu43Lys	Leu	Phe	Asn	Lys	Asp	Glu	Val	Pro		−1
M4 Glu43Lys +Asp44Lys	Leu	Phe	Asp	Lys	Lys	Glu	Val	Pro		0
Chlamydomonas	Val	Phe	Asp	Glu	Asp	Ala	Ile	Pro	Asp	−4
Anabaena variabilis	Val	Phe	Asp	Ala	Ala	Leu	Asn	Pro		−1
Synechosystis sp. PCC6803	Val	Phe	Ala	Ala	Asp	Gln	Val	Asp		−2

Species	57	58	59	60	61	62	63	85	Net charge of (#59-61)
Cucumber	Met	Asn	Glu	Glu	Asp	Leu	Leu		−3
Silene wild-type	Met	Pro	Glu	Glu	Asp	Leu	Leu		−3
M5 Glu59Lys	Met	Pro	Lys	Glu	Asp	Leu	Leu		−1
M6 Glu59Lys +Glu60Lys	Met	Pro	Lys	Lys	Asp	Leu	Leu		+1
Chlamydomonas	—	—	Arg	Asp	Asp	Tyr	Leu	Glu	−2
Anabaena variabilis	Leu	Ser	His	Lys	Gln	Leu	Leu		+1.5
Synechosystis sp. PCC6803	Leu	Ser	His	Lys	Gln	Leu	Ala		+1.5

close to the negative patches (#42-45) and (#59-61), respectively (Fig. 1) (*31*). Therefore, they are included in the respective patches. The M2 and M3 mutants of silene PC have the same net charges on the negative patch (#42-45) as those of *Synechosystis* sp. PCC 6803 and *Anabaena*, respectively. The net charges on the other negative patch (#59-61) of cucumber, silene, and *Chlamydomonas* PCs are −3, −3, and −2, respectively, whereas those of *Anabaena* and *Synechosystis* sp. PCC 6803 are both 1.5 and close to that of the M6 mutant. Thus, the local net charges on negative patches (#42-45 and #59-61) of mutant silene PCs cover the range of local net charges on the negative patches of PCs from these four organisms.

Figure 3 shows the effects of modification of negative patches on silene PC upon the photoreduction of PSI complexes from cucumber, *Chlamydomonas*, *Anabaena*, and *Synechosystis* sp. PCC 6803. As shown in Fig. 3A, the reduction rate of cucumber PSI complex decreased exponentially upon the modification of one of two highly conserved negative patches (#42-45), whereas modification of the other negative patch (#59-61) had only minor effects. The modification of silene PC also affected the reduction rate of *Chlamydomonas* PSI in a similar way (Fig. 3A). These results indicate that the negative patch (#42-45) on silene PC is important for efficient electron transfer to the *Chlamydomonas* PSI as well as to the cucumber PSI. On the other hand, we observed that the modification of negative patches (#42-45 and #59-61) on silene PC did not affect the reduction rates of PSI complexes from *Anabaena* and *Synechosystis* sp. PCC 6803. These results indicate that the negative patches (#42-45 and #59-61) of silene PC are not involved in the interactions with PSI complexes from *Anabaena* and *Synechosystis* sp. PCC 6803.

Ionic Strength Dependence of Electron Transfer from PC to Photooxidized P700⁺ Isolated from Cucumber, Chlamydomonas reinhardtii, Anabaena variabilis, and Synechosystis sp. PCC 6803—Since the reaction between PC and PSI is predominantly electrostatic in a number of evolutionarily differentiated organisms (*12, 19, 25-30*), we examined the effects of modification of negative patches on silene PC upon the ionic strength dependence of this reaction. As shown in Fig. 4A, the reduction rate of cucumber PSI by wild-type PC first increased with increasing concentration of $MgCl_2$, reached the maximum at 3-5 mM $MgCl_2$, and then decreased. The decrease of PSI reduction rate with increase of $MgCl_2$ concentration was diminished by the modification of the negative patch (#42-45), indicating the importance of electrostatic interaction in this reaction. The reduction rate of *Chlamydomonas* PSI by wild-type PC was also increased by addition of $MgCl_2$ and then decreased, as shown in Fig. 4B. However, the decrease at higher concentrations of $MgCl_2$ was significantly reduced upon modification of the negative patch (#42-45). Consequently, the optimum concentration of $MgCl_2$ shifted to higher values upon increase of net charges on the negative patch (#42-45). In the case of M4 mutant, the rate increased monotonously with increasing concentration of $MgCl_2$ (Fig. 4B). On the other hand, the modification of the negative patch (#59-61) did not affect the ionic strength dependence of the reduction rates of either cucumber or *Chlamydomonas* PSI complex (Fig. 4, C and D), which is reasonable in view of the results of Fig. 3, since the negative patch (#59-61) of silene PC is not involved in the reduction of $P700^+$.

In contrast to the cases of cucumber and *Chlamydomonas*, the reduction rates of PSI complexes from *Anabaena* and *Synechosystis* sp. PCC 6803 were rather insensitive to the concentration of $MgCl_2$ (Fig. 5, A and B). The rates increased slightly at low concentrations of $MgCl_2$ (< 10 mM). The modification of negative patches (#42-45 and #59-61) on silene PC did not affect the ionic strength dependence of the reduction rate of PSI complexes from *Anabaena variabilis* and *Synechosystis* sp. PCC 6803.

Cross-Linking Experiments between the Negative Patch Mutant PC and PSI Complexes—Previously, it has been shown that the PC specifically cross-links to the PsaF subunit of plant PSI (*12, 15*). The specific cross-linking between cytochrome *c*-553 and PSI from *Synechococcus* sp. PCC 7002 has also been reported (*16*). Therefore, it was of interest to examine whether or not the negative patch mutants can cross-link to the PSI complexes from various organisms. The cross-linked adducts were immunologically detected by using antibodies raised against spinach PC. Figure 6A shows that the substitution of Asp42 by Asn slightly inhibited the formation of the cross-linked adduct, and further modification completely inhibited the cross-

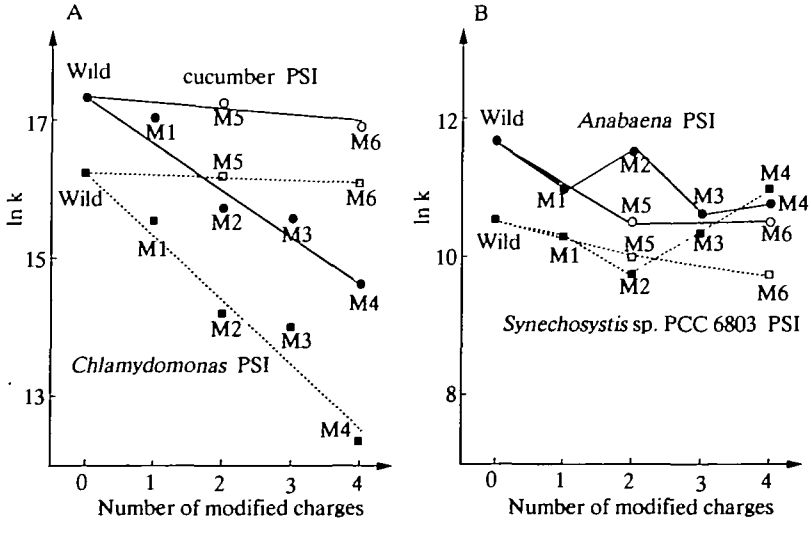
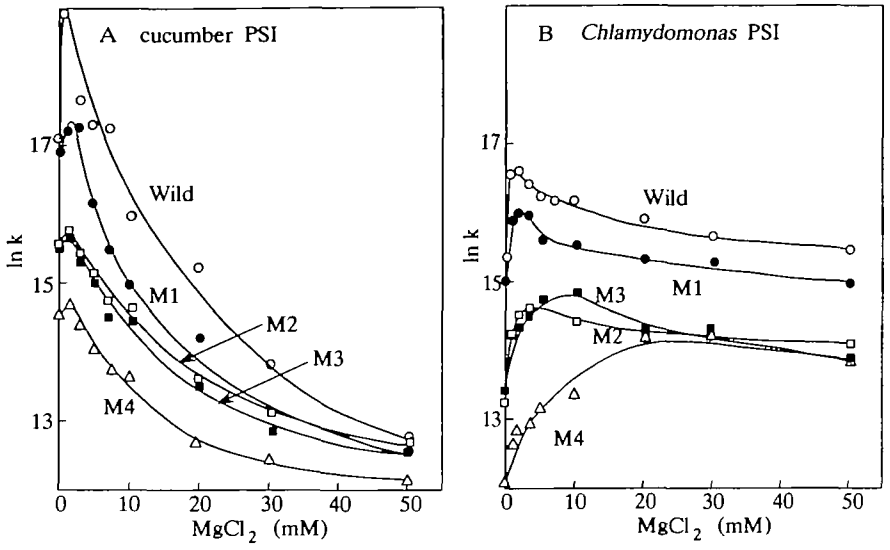


Fig. 3. Effect of total net charges on the PC surface on the second-order rate constants for the electron transfer from silene PC to $P700^+$. A: circle, cucumber PSI complex; square, *Chlamydomonas* PSI complex. B: circle, *Anabaena* PSI complex, squire, *Synechosystis* sp. PCC 6803 PSI complex. The second-order rate constants were measured under the conditions of 1 mM $MgCl_2$ and 10 mM Tris-HCl pH 8.0.

linking reaction between silene PC and cucumber PSI. The modification of the negative patch (#59-61) on silene PC had only a minor effect on the formation of cross-linked adduct. Essentially similar results were observed for the cross-linking between silene PC mutants and the *Chlamydomonas* PSI complex (Fig. 6B). These results are consis-



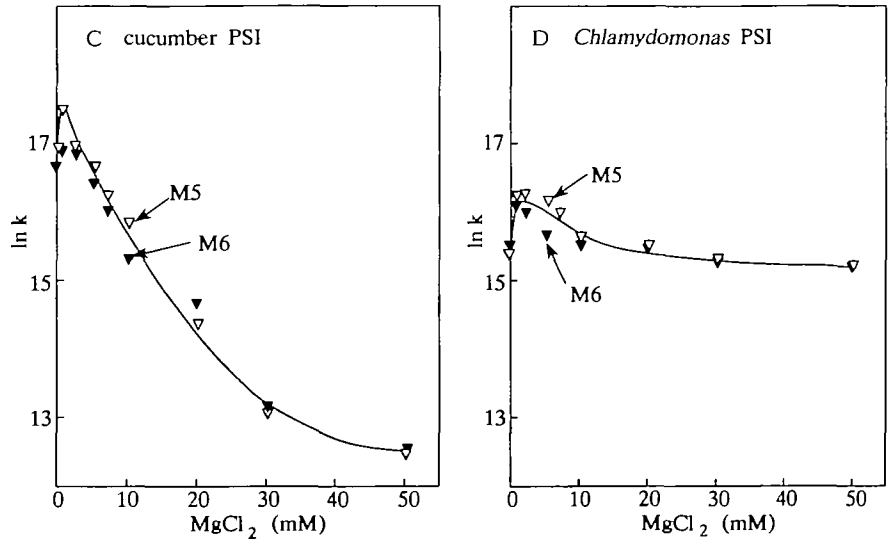


Fig. 4. Ionic strength dependence of the second-order rate constants for the photoreduction of P700 by silene PC. A: cucumber PSI complex; wild-type (○), M1 (●), M2 (□), M3 (■), and M4 (△) mutants. B: *Chlamydomonas* PSI complex; wild-type (○), M1 (●), M2 (□), M3 (■), and M4 (△) mutants. C: cucumber PSI complex; M5 (▽) and M6 (▼). D: *Chlamydomonas* PSI complex; M5 (▽) and M6 (▼). The reaction mixture contained 0.25 μM P700, 2 μM PC, 2.5 mM ascorbate, 0.1 mM methylviologen, 0.05% Triton X-100, 1 mM Tris-HCl (pH 8.0), and the indicated amount of $MgCl_2$.

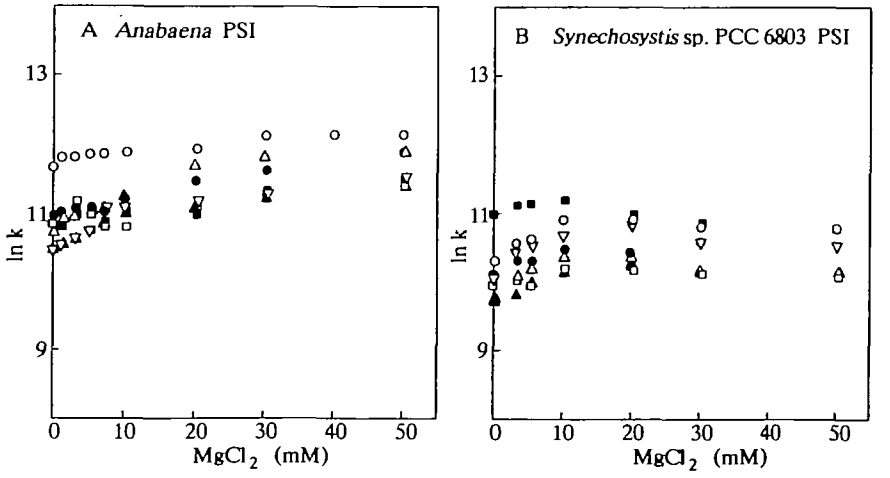


Fig. 5. Ionic strength dependence of the second-order rate constants for the photoreduction of P700 by silene PC. A: *Anabaena* PSI complex; wild-type (○), M1 (●), M2 (□), M3 (■), M4 (△), M5 (▲), and M6 (▽). B: *Synechosystis* sp. PCC 6803 PSI complex; wild-type (○), M1 (●), M2 (□), M3 (■), M4 (△), M5 (▲), and M6 (▽) mutants. The reaction mixture was the same as that of Fig. 3.

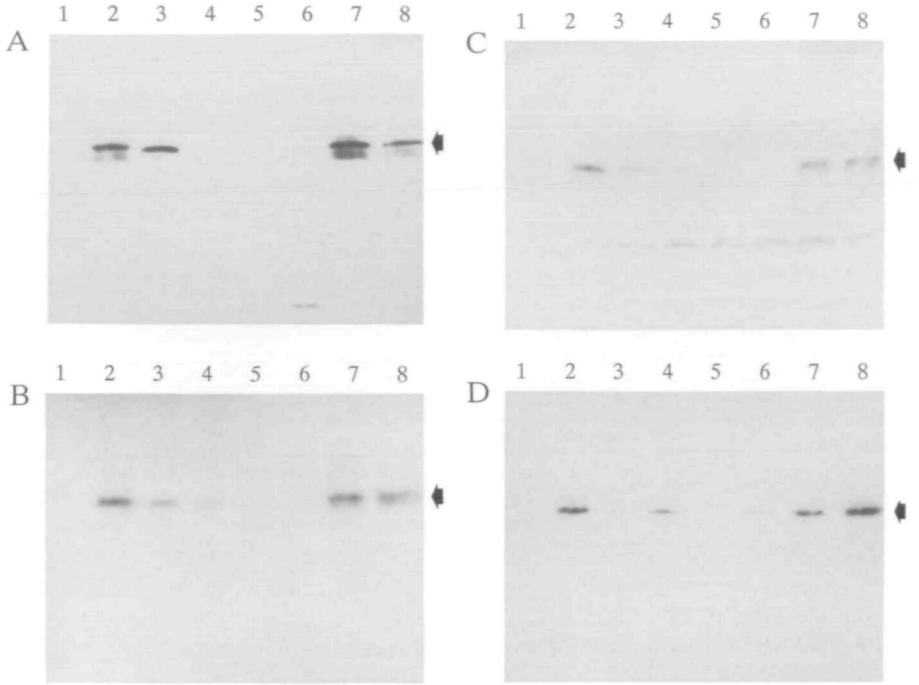


Fig. 6. **Immunological detection of cross-linked adducts between silene PC and PSI complex using the antibodies raised against spinach PC.** A, cucumber PSI complex; B, *Chlamydomonas* PSI complex; C, *Anabaena* PSI complex; D, *Synechosystis* sp. PCC 6803 PSI complex. The cross-linking reactions were carried out by using the same amounts of PC (10 μM) and PSI (0.5 mg chlorophyll/ml) in all the cases. Lane 1, PSI+EDC; lane 2-8, PSI+EDC+PC. Lane 2, wild-type PC; lane 3, M1; lane 4, M2; lane 5, M3; lane 6, M4; lane 7, M5; lane 8, M6.

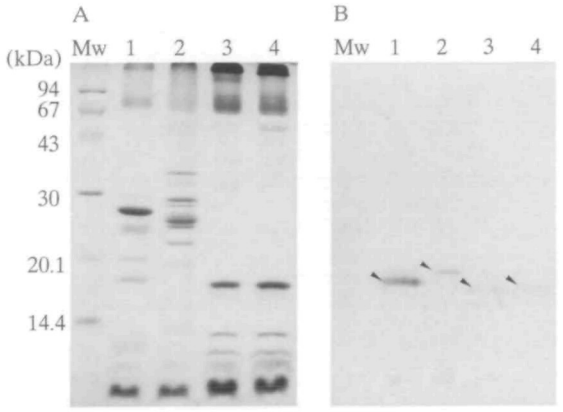


Fig. 7. **Cross-reactivities of the antibodies raised against the spinach PsaF subunit with the PSI complexes from cucumber (lane 1), *Chlamydomonas* (lane 2), *Anabaena* (lane 3), and *Synechosystis* sp. PCC 6803 (lane 4).** A, SDS-PAGE of PSI complexes stained with Coomassie Brilliant Blue. B, immunological detection using the antibodies raised against the spinach PsaF.

tent with the kinetic results, which showed that only one of two negative patches (#42-45) of silene PC interacts with the PsaF subunit of both cucumber and *Chlamydomonas* PSI complexes.

Figure 6, C and D, shows that the silene PC specifically cross-reacted with the subunits of PSI complexes from both *Anabaena* and *Synechosystis* sp. PCC 6803. The formation of cross-linked adducts was inhibited with increasing net charges on the negative patch (#42-45), but not on the negative patch (#59-61) of silene PC. These facts suggest that the negative patch (#42-45) of silene PC specifically cross-links to the PSI complexes from *Anabaena* and *Synechosystis* sp. PCC 6803.

Cross-Reactivity of the Antibody against Spinach PsaF Subunit to Those from Cucumber, Chlamydomonas reinhardtii, Anabaena variabilis, and Synechosystis sp. PCC 6803—Since the above results suggest that silene PC specifically cross-links to the PsaF subunits of plants, green algae, and cyanobacteria, we examined the immunological cross-reactivity of PsaF subunits in these four species. Protein profiles of the PSI complexes isolated from cucumber, *Chlamydomonas*, *Anabaena*, and *Synechosystis* sp. PCC 6803 are shown in Fig. 7. The cucumber and *Chlamydomonas* PSI complexes contain several polypeptides of LHCPI in the range of 21-25 kDa, whereas those from *Anabaena* and *Synechosystis* sp. PCC 6803 have no band in this region due to the absence of LHCPI and depletion of phycobilisomes. These polypeptide patterns are essentially similar to those reported previously (*23*), although additional bands are also observed. Western blotting analysis showed that the antibody raised against spinach PsaF subunit cross-reacted to all the PSI complexes (Fig. 7B). The intensities of PSI complexes from cucumber and *Chlamydomonas* were strong, whereas those of *Anabaena* and *Synechosystis* sp. PCC 6803 were weak. The mobility of the *Chlamydomonas* PsaF subunit in SDS-PAGE (19 kDa) is slightly smaller than that of the others (18 kDa). This is consistent with the fact that the *Chlamydomonas psaF* gene encodes a slightly longer mature PsaF polypeptide (18.3 kDa) than that of spinach (17.2 kDa) or *Synechosystis* sp. PCC 6803 (15.6 kDa) (*32-34*). These results indicate that the PsaF subunits from the four species have a structural similarity.

DISCUSSION

From the nucleotide sequences of *psaF* genes of spinach (*32*), *Chlamydomonas reinhardtii* (*33*), *Synechosystis* sp. PCC 6803 (*34*), and *Anabaena variabilis* (accession number X93923 in the GenBank/EMBL/DDBJ databases), we estimated the isoelectric pHs of mature PsaF as 9.5, 9.1, 6.1, and 5.0, respectively. Therefore, it was anticipated

that the values of electrostatic attraction energy between silene PC and PsaF subunits of PSI complexes from cucumber, *Chlamydomonas*, *Synechosystis* sp. PCC 6803, and *Anabaena* decrease in that order. Data presented above clearly demonstrated that the PSI complexes from cucumber and *Chlamydomonas* are efficient electron acceptors for silene PC, whereas the PSI complexes from *Anabaena* and *Synechosystis* sp. PCC 6803 are inefficient electron acceptors for silene PC. These data are consistent with the viewpoint that the net charges on the PsaF and PC are one of determinant factors of the PSI reduction rate. A similar conclusion was reached by Tamura *et al.* (*35*), and they described the importance of net charges on PC and the membrane surface around PSI complexes.

The data presented above also clearly indicated the importance of local charges on PC for the reduction of PSI. As shown in Fig. 3, the reduction rates of PSI complexes from cucumber and *Chlamydomonas* decreased upon the increase of net charge on the negative patch (#42-45) on silene PC, whereas their rates were independent of modification of the other negative patch (#59-61). These facts indicate the importance of the negative patch (#42-45) on PC, but not (#59-62), for the reduction of PSI. The reduction rates of PSI from cucumber and *Chlamydomonas* were similarly affected by the modification of the negative patch (#42-45) on silene PC. However, the effects of $MgCl_2$ on the reduction rates of PSI were different (see Fig. 4). Addition of higher concentrations of $MgCl_2$ decreased the reduction rate of cucumber PSI, whereas the decrease of reduction rate of $P700^+$ was inhibited, in the case of *Chlamydomonas* PSI, upon increase of net charges on the negative patch (#42-45). Since the net charges on PsaF from cucumber and *Chlamydomonas* are similar, these facts also support the significance of changes of local charges on the oxidizing sides of the PSI complexes in these two cases.

In contrast to the PSI reduction, cross-linking experiments demonstrated that silene PC specifically cross-linked to the subunit of PSI complexes from cucumber, *Chlamydomonas*, *Anabaena*, and *Synechosystis* sp. PCC 6803. Modification of the negative patch (#42-45) inhibited the formation of cross-linked adducts in all the cases examined, whereas modification of the other negative patch (#59-61) had essentially no effect. The results of cross-linking experiments are apparently inconsistent with the ionic strength effects on the PSI reduction from *Anabaena* and *Synechosystis* sp. PCC 6803. We interpreted this as due to the change of rate-determining steps in these two reactions. The cross-linking reactions were carried out at low pH and low ionic strength. Under these conditions, specific interactions between the negative patch (#42-45) on PC and the positive charges on the PsaF subunit are possible even if the total net charges on PsaF are different. Moreover, protein folding similarities of PsaF from spinach, *Chlamydomonas*, and *Synechosystis* sp. PCC 6803 have also been suggested. The amino acid sequence of PsaF from *Synechosystis* sp. PCC 6803 is considerably homologous to those from spinach (48% identity) and *Chlamydomonas* (50% identity). The overall homology reaches more than 60% if conservative replacements are considered, and the hydropathy profiles of the three PsaF subunits are almost identical. The conservation of the primary structure of the PsaF subunit in these organisms is consistent with the similar cross-linking pattern with silene PC.

Hitherto, two possible interaction sites have been proposed for the reduction of PSI by PC. One is the hydrophobic interaction between the helixes around the P700 (*7*) and hydrophobic patch surrounding His87 (Site 1 of Fig. 1) (*1*). The other is the electrostatic interaction between the negative patch (#42-45) on PC and positive charges on the PsaF subunit. In view of the weak electrostatic interactions between silene PC and PSI complexes from cyanobacteria, we propose that the hydrophobic environment favoring electron transfer *via* His87 of PC to $P700^+$ is the determinant factor for the reduction of cyanobacterial PSI complexes; this is consistent with the fact that their reduction rates are independent of changes of ionic strength (see Fig. 5). However, the electrostatic interaction is rate-determining for the reduction of PSI complexes from cucumber and *Chlamydomonas*, and these reactions are strongly affected by changes of ionic strength. Clearly, more study is necessary to understand the change of the molecular mechanisms of PSI reduction during the course of evolution from cyanobacteria to plants.

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