Changes in the Reaction Mechanism of Electron Transfer from Plastocyanin to Photosystem I in the Cyanobacterium *Synechocystis* sp. PCC 6803 As Induced by Site-Directed Mutagenesis of the Copper Protein†

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ABSTRACT: The kinetic mechanism of plastocyanin oxidation by photosystem I in the cyanobacterium *Synechocystis* sp. PCC 6803 is drastically changed by modifying the metalloprotein by site-directed mutagenesis. The mutations herein considered concern four specific residues, two in the east face and the other two in the hydrophobic patch of plastocyanin. The first set of mutants include D44A, D44K, D47A, and D47R, as well as the double mutants D44A/D47A and D44R/D47R; the second set consists of L12A and K33E. The kinetic efficiency of all these mutant plastocyanins has been analyzed by laser-flash absorption spectroscopy. The plastocyanin concentration dependence of the observed electron transfer rate constant (k_{obs}) is linear with most mutant plastocyanins, as with wild-type plastocyanin, but exhibits a saturation plateau at high protein concentration with the double mutant D44R/D47R, which suggests the formation of a plastocyanin−PSI transient complex. The effect of ionic strength on k_{obs} varies from the wild-type plastocyanin to some of the mutants, for instance D44K, for which the salt concentration dependence of k_{obs} is just the reverse as compared to the wild-type protein. The ionic strength dependence of k_{obs} with D44R/D47R exhibits a bell-shaped profile, which is similar to that of green algae and higher plants. These findings indicate that the double mutant D44R/D47R follows a reaction mechanism involving not only complex formation with PSI but also further reorientation to properly accommodate the redox centers prior to electron transfer, as is the case in most evolved species, whereas the wild-type copper protein reacts with PSI by following a simple collisional kinetic model.

Plastocyanin (Pc)[1] is a small single-copper protein (molecular mass, 10.5 kDa) which functions as a mobile electron carrier in the thylakoid lumen of oxygen-evolving photosynthetic organisms. Actually, Pc is reduced by cytochrome *f*—which is part of the membrane-embedded cytochrome b_6f complex—and further oxidized by the chlorophyll molecule $P700^+$ in photosystem I (PSI)—which is likewise immersed in the photosynthetic membranes [cf. Gross (1996) and Navarro *et al.* (1997) for recent reviews]. It is interesting to note that Pc is acidic in higher plants and green algae, with an isoelectric point of 3.5−4, but in cyanobacteria it can be either almost neutral, as is the case in *Synechocystis*, with an isoelectric point close to 6, or basic, as in *Anabaena*, with an isoelectric point of 8−9 (Sykes, 1991; Hervás *et al.*, 1993; Medina *et al.*, 1993).

As regards the mechanism of electron transfer from Pc (as well as from cytochrome c_6, which replaces the copper protein in some species of green algae and cyanobacteria) to PSI, we have recently reported that it has evolved from the simple one-step kinetic model of prokaryotic organisms such as *Synechocystis* sp. PCC 6803 to the three-step reaction of eukaryotic species such as green algae and higher plants. Whereas the former involves just an electrostatically oriented collision between the donor protein and PSI, the latter requires both formation of a transient complex and rearrangement of redox centers inside the complex before electron transfer takes place (Hervás *et al.*, 1995, 1996).

On the other hand, the 3D structure of Pc has been solved using proteins isolated from several higher plants and green algae [see Redinbo *et al.* (1994) for a review], and more recently from the cyanobacterium *Anabaena variabilis* (Badsberg *et al.*, 1996). Two electron transfer sites on Pc have been proposed: site 1 (or the so-called "hydrophobic" patch or "north site"), which is located in a flat region around the copper ligand His87, with the Cu atom close to the protein surface; and site 2 (or the so-called "east site"), which is referred to as the acidic patch in eukaryotic organisms because it includes aspartic and glutamic residues at positions 42−45 and 59−61 surrounding solvent-exposed Tyr83 (Gross, 1996; Navarro *et al.*, 1997).

Experiments with Pc modified either by chemical means or by site-directed mutagenesis, as well as with Pc cross-linked with both cytochrome *f* and PSI, have supplied relevant information on its interaction with the two redox partners. Actually, the hydrophobic patch containing Gly10 and Leu12 has been proposed to be crucial for the interaction with PSI, whereas electron transfer itself would take place via the surface-exposed imidazole ring of His87 (Nordling *et al.*, 1991; Haehnel *et al.*, 1994; Sigfridsson *et al.*, 1996). In site 2, Tyr83 would be involved in Pc interaction with

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[1] Abbreviations: k_{bim}, bimolecular rate constant for the overall reaction; k_{inf}, bimolecular rate constant extrapolated at infinite ionic strength; k_{obs}, observed pseudo-first-order rate constant; Pc, plastocyanin; PSI, photosystem I; WT, wild-type.

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cytochrome *f* and/or electron transfer, while the acidic east patch would serve as the binding area with both cytochrome *f* and PSI. The PsaF subunit of PSI—which is positively charged in eukaryotic organisms—has been proposed to be essential in higher plants for Pc docking (Wynn & Malkin, 1988; Hippler *et al.*, 1996); in cyanobacteria, however, the role of PsaF is not so clear (Chitnis *et al.*, 1991; Hatanaka *et al.*, 1993).

In this work, site-directed mutagenesis is used as a powerful tool to extend the studies of structure−function relationships in Pc from eukaryotic to prokaryotic organisms such as the cyanobacterium *Synechocystis*. The interest of this Pc comes not only from its rather simple reaction mechanism (see above) but also from the fact that its east patch is not so acidic as in eukaryotic proteins (Navarro *et al.*, 1997). In addition, the *pet*E gene coding for *Synechocystis* Pc has already been cloned and correctly expressed in *E. coli* (Hervás *et al.*, 1993), and its 3D structure has recently been solved by X-ray crystallography (Romero *et al*., manuscript in preparation). We have thus designed a number of Pc molecules modified by mutagenesis of specific residues either in the eastern face or in the hydrophobic patch, the kinetic mechanism of PSI reduction by such mutant Pcs being further analyzed by laser-flash absorption spectroscopy.

EXPERIMENTAL PROCEDURES

DNA Techniques. The mutant genes were constructed using the polymerase chain reaction (PCR) in two steps (Giebel & Spritz, 1990). The template used in such a two-step protocol was the *pet*E gene, which was cloned in pBluescriptII (SK+) (Stratagene) as described in Hervás *et al.* (1993). For each PCR, we used 1 ng of linearized template cutted with *Eco*RV, 200 μM dNTPs, 100 pmol of each primer, and the enzyme and buffer of Expand High Fidelity System (Boehringer) in a reaction volume of 50 μL. PCRs were run in 30 cycles of 1 min at 94 °C (denaturation), 1 min at 46 °C (primer annealing), and 1 min at 72 °C (primer extension). External primers to the gene carrying *Bam*HI and *Eco*RI restriction sites and mutagenic primers were from Pharmacia.

In the first PCR step, an external primer and the mutagenic primer were used. The resulting product was purified using DEAE-cellulose membranes to isolate fragments from agarose gels according to Sambrook *et al.* (1989). In the second PCR step, the product of the first PCR and the other external primer were used. Conditions were the same as above. The resulting PCR product was purified in the same way. The DNA fragment was cloned using the pGEM-T cloning kit (Promega). The expression vector was pBluescriptII (SK+), and the mutated genes were cloned using the *Bam*HI/*Eco*RI sites. Nucleotide sequence analysis was carried out by the dideoxy chain termination method (Sanger *et al.*, 1977) using a sequenase version 2.0 T 7 DNA polymerase kit (USB). Other molecular biology protocols were standard (Sambrook *et al.*, 1989).

Production and Purification of Recombinant Proteins. E. coli DH5α (Bethesda Research Laboratories) transformed cells were grown in standard Luria Broth (LB) medium (Sambrook *et al.*, 1989) supplemented with 100 μM $CuSO_4$. Cells from 10 L of culture were collected, and the periplasmic fraction was extracted according to the method of Hoshino and Kageyama (1980) modified by Eftekhar and Schiller (1994). The cell pellet was suspended in 0.2 M $MgCl_2$, 50 mM Tris−HCl, pH 8, to a final concentration of 0.15 g of cells mL^{-1}. This suspension was alternatively incubated at 37 °C for 10 min and at 0 °C for 15 min up to 5 times. The spheroplasts were sedimented by 10 min centrifugation at 30000*g*. Solid ammonium sulfate was added to the supernatant up to 50% saturation. After centrifugation, ammonium sulfate was again added to the resulting supernatant up to 100% saturation, and the solution was centrifuged to concentrate the proteins. The final pellet was suspended in 2 mM Tris−HCl, pH 8, extensively washed through an Amicon YM3 membrane in an Amicon pressure cell to eliminate salts, and applied to a DEAE-cellulose (DE-52) column. Proteins were eluted with a 0−0.2 M NaCl gradient, the Pc-containing fractions being pooled, dialyzed against 5 mM Tris−acetate, pH 8, and chromatofocused in the pH range 4−7. Concentration and purity of the resulting Pc fractions were determined spectrophotometrically using an absorption coefficient of 4500 M^{-1} cm^{-1} at 597 nm and an absorbance ratio A_{275}/A_{597} of 2.2 for the pure protein (Hervás *et al.*, 1993). Pure protein preparations were concentrated and stored at −80 °C. The overall procedure yielded 5−10 mg of mutant Pc from a 10 L culture of *E. coli* cells.

Redox Titrations. Redox titrations were performed in a dual-wavelength spectrophotometer as described by Ortega *et al.* (1988). The differential absorbance changes at 597 nm minus 500 nm were monitored in the presence of the redox mediators menadione, diaminodurol, and *p*-benzoquinone at 20 μM final concentration. The errors in determining midpoint potentials were less than 5 mV.

Preparation of PSI Particles. PSI particles were isolated from *Synechocystis* cells by β-dodecyl maltoside solubilization as described by Rögner *et al.* (1990) and modified by Hervás *et al.* (1994). The chlorophyll/P700 ratio of the resulting PSI preparations was 130/1. The P700 content in PSI samples was calculated from the photoinduced absorbance changes at 820 nm using the absorption coefficient of 6.5 M^{-1} cm^{-1} determined by Mathis and Sétif (1981). Chlorophyll concentration was determined according to Arnon (1949).

Laser-Flash Absorption Spectroscopy. Kinetics of flash-induced absorbance changes in PSI were followed at 820 nm as described by Hervás *et al.* (1995). Unless otherwise stated, the standard reaction mixture contained, in a final volume of 0.2 mL, 20 mM buffer (Tricine−KOH, pH 7.5, or MES, pH 5.5), 0.03% β-dodecyl maltoside, an amount of PSI-enriched particles equivalent to 0.36 mg of chlorophyll mL^{-1}, 0.1 mM methyl viologen, 2 mM sodium ascorbate, 10 mM $MgCl_2$, and wild-type (WT) or mutant Pc at the indicated concentration. In the studies of the ionic strength effect, the standard reaction mixture contained 100 μM mutant Pc and NaCl or $MgCl_2$ at the indicated concentration in Tricine−KOH buffer, pH 7.5. All the experiments were performed at 25 °C in a 1-mm path length cuvette. Each kinetic trace was the average of six measurements with 30 s spacing between flashes. For most experiments, the estimated error in the observed rate constants was less than 10%, based on reproducibility and signal-to-noise ratios.

Kinetic Analyses. Data collection was as described previously (Hervás *et al.*, 1995). Oscilloscope traces were treated as sums of several exponential components. Exponential analyses were performed using the Marquardt method

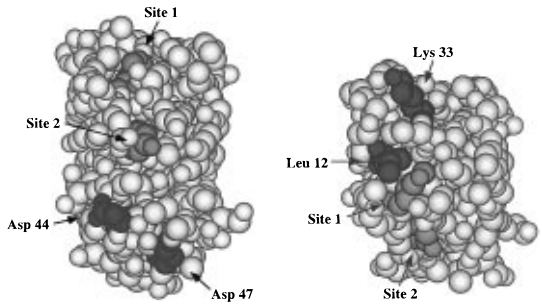

FIGURE 1: East (left) and north view (right) of the tertiary structure of *Synechocystis* plastocyanin. His87 and Tyr83 are located in the proposed electron transfer sites 1 and 2, respectively. The residues modified by mutagenesis are marked in darker color. The pictures, which were prepared using the program MacImdad, version 5.2, are based on the structure of the triple mutant A42D/D47P/A63L plastocyanin from *Synechocystis* sp. PCC 6803, which has recently been solved by X-ray crystallography at 2.1 Å resolution (Romero *et al.*, manuscript in preparation).

with the software devised by P. Sétif. Kinetic analyses were carried out according to the different reaction mechanisms previously proposed (Hervás *et al.*, 1995, 1996).

RESULTS

Reduction of photooxidized P700 in PSI particles from the cyanobacterium *Synechocystis* sp. PCC 6803 has been shown to follow a simple oriented collisional reaction mechanism, no intermediate complex formation being observed either with Pc or with cytochrome c_6 as the electron donor protein (Hervás *et al.*, 1994). Here we have analyzed the effect of specific mutations in Pc on such a reaction mechanism. Actually, four residues (two in the east face, the other two in the hydrophobic patch) have been mutated (Figure 1). Aspartate residues at positions 44 and 47 have been replaced by either neutral or positively charged amino acids.[2] Whereas Asp44 is a highly conserved residue located in the east patch near Tyr83, Asp47 is a proline residue in all organisms, except in *Synechocystis*, that is located on the southeast face just at the beginning of the only short α-helix of the Pc molecules. Mutant *Synechocystis* Pcs herein considered include the single mutants D44A, D44K, D47A, and D47R, as well as the double mutants D44A/D47A and D44R/D47R. Modifications at the hydrophobic patch include the residues Leu12 and Lys33, the former being closer than the latter to His87 (Figure 1). In order to analyze both the steric and electrostatic effects in this region, the mutants designed have been L12A and K33E, respectively. As can be seen in Table 1, the replacement of any of these four surface residues does not significantly affect the redox potential value of the copper center, thus indicating that the observed differences in the reaction mechanism (see below) cannot be attributed to changes in the driving force. The electronic absorption spectrum of Pc is likewise not affected by the mutations herein considered (not shown).

The kinetics of PSI reduction by any mutant Pc are monoexponential, with no fast phase, as is the case with WT Pc (Hervás *et al.*, 1994). The oscilloscope traces obtained with D44R/D47R and L12A, as well as with WT Pc, are shown in Figure 2. Even though the three kinetics can be well fitted to single exponential curves, the photooxidized P700 decay is much slower with L12A than with the double mutant or WT Pc.

Figure 3 shows that the observed pseudo-first-order rate constants (k_{obs}) of PSI reduction at pH 7.5 depend linearly on the concentration of added metalloprotein, not only with

[2] In order to facilitate the identification of residues, the numbering of amino acids of *Synechocystis* plastocyanin herein used is that corresponding to the metalloprotein from higher plants.

Table 1: Midpoint Redox Potential Values of Wild-Type and Mutant Plastocyanins (E_0), Bimolecular Rate Constants for the Overall Reaction of PSI Photooxidation by the Different Metalloprotein Molecules (k_{bim}), and Differences in the Interaction Energy of Such Electron Transfer Reactions with Mutant Plastocyanins As Compared with That of the WT Plastocyanin ($\Delta\Delta G$) (For Further Explanations, See the Text)

	E_0, pH 7.5	$k_{bim} \times 10^{-6}$ (M^{-1} s^{-1})		$k_{inf}{}^a \times 10^{-6}$ (M^{-1} s^{-1}),	$\Delta\Delta G$ (kJ/mol)	
plastocyanin	(mV)	pH 5.5	pH 7.5	pH 7.5	pH 5.5	pH 7.5
WT	360	9.9	8.6	10.6	0	0
D44A	336	9.5	9.5	9.4	0.1	−0.2
D44K	357	16.4	15.7	8.3	−1.2	−1.5
D47A	349	18.4	11.8	12.5	−1.5	−0.8
D47R	342	9.2	8.5	8.7	0.2	0.03
D44A/D47A	352	15.9	14.4	7.6	−1.2	−1.3
D44R/D47R	336	382[b]	471[b]	7.6	−9.0	−9.9
K33E	336	3.4	3.2	10.8	2.6	2.4
L12A	357	2.4	2.8	4.9	3.5	2.8

[a] k_{inf} stands for the bimolecular rate constant extrapolated to infinite ionic strength. [b] These two values stand for the bimolecular rate constants of complex formation between reduced plastocyanin and PSI, which were estimated according to the formalism described in Meyer *et al.* (1993).

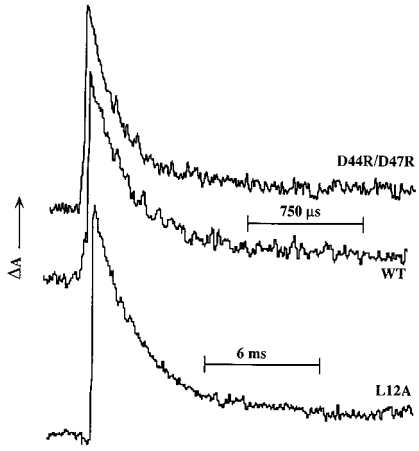

FIGURE 2: Kinetic traces showing *Synechocystis* PSI reduction by wild-type and mutant plastocyanins. Copper protein concentration was 200 μM, and the pH value was 7.5. All kinetic traces were well fitted to single exponential curves.

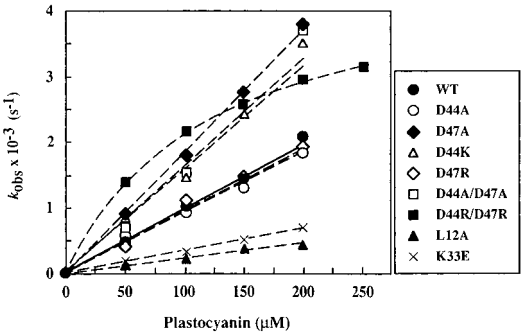

FIGURE 3: Dependence upon plastocyanin concentration of the observed rate constant (k_{obs}) for *Synechocystis* PSI reduction by wild-type and mutant plastocyanins. Experimental conditions were as in Figure 2.

WT Pc but also with all mutant Pcs except with D44R/D47R. Such a linear copper protein concentration dependence can be interpreted by assuming that there is no formation of any transient Pc−PSI complex, thereby confirming previous data for WT Pc suggesting a simple collisional reaction mechanism. An exception is the double mutant D44R/D47R, for which the dependence of k_{obs} upon donor protein concentration exhibits a saturation profile that indicates the formation of a bimolecular [$Pc_{red}\cdots PSI_{ox}$] complex prior to electron transfer, according to the following minimal two-step kinetic mechanism:

$$Pc_{red} + PSI_{ox} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} [Pc_{red}\cdots PSI_{ox}] \xrightarrow{k_{et}} Pc_{ox} + PSI_{red}$$

for which k_{obs} extrapolated to infinite Pc concentration, that is, to saturating values, stands for the electron transfer rate constant (k_{et}).

The bimolecular rate constants (k_{bim}) for the overall reaction of PSI, which are obtained from the linear plots in Figure 3, are summarized in Table 1, both at pH 5.5 and at pH 7.5. As can be seen, the mutants D44A and D47R behave like WT Pc, but the replacement of the negative charge at position 44 by a positive residue, as in D44K, or just the removal of the negative charge at position 47, as in D47A, accelerates the reaction to make the bimolecular rate constant double that with WT Pc. D44A/D47A exhibits the same bimolecular rate constant as the single mutant D47A, indicating that it is this mutation, and not that at position 44, which originates the phenotype. It must be noted that the k_{bim} values with D44R/D47R in Table 1 stand for the bimolecular rate constants of complex formation between reduced Pc and photooxidized PSI, that is k_{12} in the above scheme, and are thus kinetically different from the k_{bim} values with other Pcs. Changes of specific amino acids at the hydrophobic patch induce a significant effect on the reactivity of the mutants, thereby making the bimolecular rate constant decrease by a factor of 3−4 for L12A and K33E. As can also be seen in Table 1, there is almost no effect of pH on the bimolecular rate constant with all Pc molecules, with the only exception of D47A, for which k_{bim} at pH 7.5 is 64% of that at pH 5.5.

Taking into account the electrostatic nature of the interaction of Pc with PSI, a comparative analysis of the effect of ionic strength on k_{obs} has been performed with all mutant Pcs. Figure 4 shows that k_{obs} with WT Pc increases with increasing ionic strength to reach a nearly limiting value; this can be explained by assuming that ionic strength weakens repulsive electrostatic interactions between Pc and PSI. The removal of just one negative charge on the east or southeast face of Pc, as is the case when replacing Asp44 or Asp47 by alanine, leads to mutants showing ionic strength dependences similar to that of WT Pc. However, the simultaneous removal of such two negative charges in the double mutant D44A/D47A makes the repulsive interaction with PSI rather insignificant as inferred from the fact that there is practically no dependence of k_{obs} on ionic strength.

The results obtained not only after removal of the negative charges at positions 44 and 47 but also by replacing them by positive residues are very interesting. In fact, the ionic strength dependence of k_{obs} with D47R is similar to that with WT Pc, thus suggesting that the electrostatic charge at this

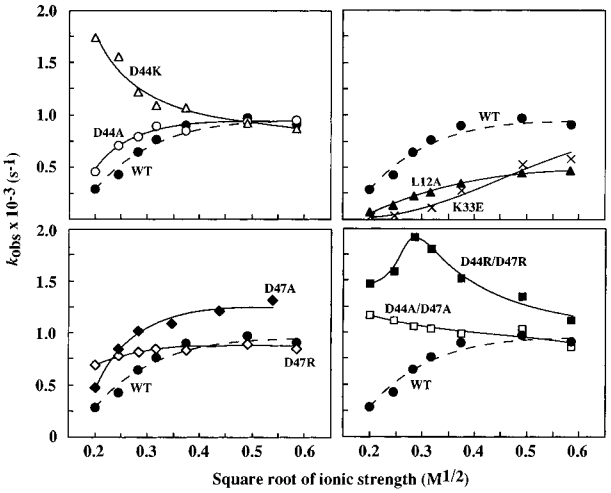

FIGURE 4: Effect of ionic strength on the observed rate constant (k_{obs}) for *Synechocystis* PSI reduction by wild-type and mutant plastocyanins. Experimental conditions were as in Figure 2. For comparative purposes, the WT data are shown in the four panels.

position has practically no effect on the interaction of Pc with PSI. In the mutant D44K, however, the replacement of Asp44 by a positive residue just reverses the ionic strength dependence, thereby making the Pc−PSI interaction stronger at lower ionic strength and decreasing with increasing salt concentration. Noteworthy are the kinetic properties of the double mutant D44R/D47R, for which the ionic strength dependence of k_{obs} shows a broad bell-shaped profile: the rate constant increases with increasing ionic strength, reaches a maximum value, and decreases thereafter. Such a biphasic dependence, which has previously been observed in eukaryotic organisms such as spinach and the green alga *Monoraphidium braunii* (Hervás *et al.*, 1992), has been ascribed to the formation of a "frozen" Pc−PSI complex at low ionic strength that is not optimized for electron transfer, whereas increasing ionic strength would induce the weakening of electrostatic interactions and would thus allow the rearrangement of redox partners within the transient complex so as to optimize electron transfer.

By applying the formalism developed by Watkins *et al.* (1994) to our data on the ionic strength dependence of k_{obs}, we have determined the bimolecular rate constants extrapolated to infinite ionic strength (k_{inf}) as they facilitate the analysis of the intrinsic reactivity of the redox partners in the absence of electrostatic interactions. As shown in Table 1, k_{inf} with most mutant Pcs is very similar to that with WT Pc, thereby indicating that the changes in the reaction mechanism induced by mutations in the east and southeast faces are probably due to electrostatic effects but not to structural or redox changes.

Regarding mutations in the hydrophobic patch, Figure 4 shows that both L12A and K33E yield values for k_{obs} significantly lower than WT Pc. However, L12A exhibits a k_{inf} value 2 times lower than either WT Pc or K33E, thus suggesting that the mutation at position 12 must induce structural changes that are hindering the interaction between Pc and PSI. The lower efficiency of K33E at low ionic strength must be mainly due to the change in the electrostatic charge.

The effect of replacing charged amino acids on the interaction between Pc and PSI can be evaluated in a more adequate way by calculating the interaction energies ($\Delta\Delta G$),

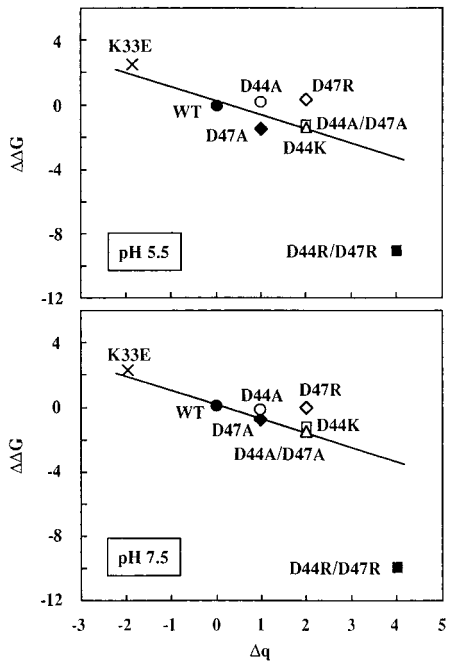

FIGURE 5: Interaction energy ($\Delta\Delta G$) between plastocyanin and PSI as a function of the difference in charge (Δq) between mutant and wild-type metalloproteins. The interaction energy values were those in Table 1.

as described by Kannt *et al.* (1996):

$$\Delta\Delta G = RT \ln \frac{k_{bim}(\text{WT})}{k_{bim}(\text{mutant})} \quad (1)$$

As shown in Figure 5, with most mutant proteins the interaction energy is linearly dependent on the net charge difference between mutant and WT Pc (Δq), both at pH 5.5 and at pH 7.5. There are, however, some mutant Pcs separate from the line. D47R, in particular, seems to deviate from the line whereas D47A perfectly matches it, thereby suggesting that another factor besides the change in charge is affecting the reaction rate, namely, the difference in size of the side chain of the arginine residue as compared to that of aspartic acid. D44R/D47R, in its turn, also deviates drastically from the line, but this finding is surely due to the different approach used for the calculation of k_{bim} (see above).

DISCUSSION

With regard to the residues involved in Pc−PSI interaction and electron transfer, a high number of site-directed mutagenesis analyses have been addressed in eukaryotic systems [see Gross (1996) for a review], but none had been done in cyanobacteria. The main aim of this work has thus been to elucidate the role of specific Pc amino acids in prokaryotic organisms, namely, the cyanobacterium *Synechocystis* sp. PCC 6803. The residues we have modified here by site-directed mutagenesis are located both in the hydrophobic and in the acidic patches as they have been reported to be involved in the interaction of eukaryotic Pc with PSI (see the introduction).

Mutations of residues Leu12 and Lys33 clearly indicate that the hydrophobic patch plays an important role in the interaction of *Synechocystis* Pc with PSI, as previously observed using eukaryotic Pcs (Nordling *et al*., 1991; Haehnel *et al*., 1994; Sigfridsson *et al*., 1996). However, the efficiency of L12A and K33E to donate electrons to PSI is not as low as that of similar eukaryotic mutant Pcs, in which the fast phase of electron transfer to PSI totally disappears (Haehnel *et al.*, 1994; Sigfridsson *et al.*, 1996). This finding can be easily explained by assuming that such specific effects are much more difficult to observe in the very simple oriented collisional mechanism of the *Synechocystis* Pc−PSI system.

The acidic patch of eukaryotic Pc appears to be clearly involved in the reaction with PSI (Haehnel *et al*., 1994; Sigfridsson *et al.*, 1996) by driving electrostatic interactions with the positively charged PsaF subunit (Hippler *et al*., 1996). In *Synechocystis*, however, there are repulsive electrostatic interactions between Pc and PSI, as inferred from the increase of the reaction rate constant with increasing ionic strength (Hervás *et al*., 1994). Actually, the site-directed mutagenesis studies reported herein of the two acidic residues at positions 44 and 47 indicate that this area of *Synechocystis* Pc plays an important role in such repulsive interactions. The effects observed are probably due to the global surface charge in this area of the metalloprotein but not to any specific residue, as deduced from the linear dependence of the interaction energy upon the net charge difference between mutant and WT Pc [cf. Kannt *et al*. (1996) and Figure 5]. In *Synechocystis*, the PsaF subunit does not seem to be directly involved in the interaction with Pc (Hippler *et al.*, 1996), but the electrostatic charges in the east face of Pc probably interact with certain areas in the PsaA/PsaB heterodimer of PSI (Fromme *et al.*, 1994), thus facilitating the correct orientation of the redox centers before electron transfer. The only exception to this behavior is that of the mutant D47R, in which some minor structural change in Pc, besides the change in electrostatic charge, seems to be involved as the D47A and D44A/D47A mutants stay in the straight line of the interaction energy dependence (see Figure 5).

The double mutant D44R/D47R is of special relevance among all the Pc molecules up to now modified in the east face. The replacement of just two negative charges by positive residues gives rise to a completely different kinetic behavior: in contrast to the WT protein, such a double mutant is able to form an electrostatic Pc−PSI complex prior to electron transfer, as is the case in most evolved organisms (Hervás *et al*., 1995, 1996). In other words, these two mutations appear to be just enough to redistribute the surface electrostatic potential of Pc and allow it to complex to PSI. Moreover, the bell-shaped salt dependence of the reaction rate constant observed with D44R/D47R suggests that there is a rearrangement of the two partners within the reaction complex before electron transfer takes place, as previously proposed in other Pc−PSI systems (Sigfridsson *et al.*, 1996; Hervás *et al*., 1995). Such a complex is not optimized as it does not exhibit any fast phase in the electron transfer to PSI, as described in the highly evolved kinetic systems of spinach and eukaryotic algae (Bottin & Mathis, 1985; Hervás *et al*., 1995). Actually, a comparison of the relevant kinetic parameters for *Synechocystis* D44R/D47R and spinach Pc clearly shows that the reaction rate constants for both complex association (K_A) and electron transfer (k_{et}) are significantly lower with the former: K_A is 9.5×10^3 M with the double mutant and 8.8×10^4 M with spinach Pc, whereas k_{et} is 3.9×10^3 s^{-1} with D44R/D47R and 6.3×10^4 s^{-1} with higher plant Pc.

We can conclude by proposing that the PsaF subunit, which appears to be critical for an optimized interaction in eukaryotic systems but not to play any significant role in *Synechocystis* (see the introduction), could, however, be able to form a transient complex with the double mutant D44R/D47R. This would uphold the hypothesis that the PsaF polypeptide, which was initially serving as a PSI structural element in primitive organisms, could finally learn to play a crucial role in the reaction mechanism of most evolved systems.

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