A Laser Flash-Induced Kinetic Analysis of in Vivo Photosystem I Reduction by Site-Directed Mutants of Plastocyanin and Cytochrome c_6 in *Synechocystis* sp. PCC 6803†

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ABSTRACT: In cyanobacteria, plastocyanin and cytochrome c_6 are two soluble metalloproteins which can alternately serve as electron donors to photosystem I. From site-directed mutagenesis studies in vitro, it is well-established that both hydrophobic and electrostatic forces are involved in the interaction between the donor proteins and photosystem I. Hence, two isofunctional areas, a hydrophobic one in the north and an acidic one in the east, have been described on the surface of both electron donors. In this work, we have tested the relevance of such kinds of interactions in the photosystem I reduction inside the cell. Several plastocyanin and cytochrome c_6 site-directed mutant strains affecting both the acidic and hydrophobic regions of the two metalloproteins, which were previously characterized in vitro, have been constructed. The photosystem I reduction kinetics of the different mutants have been analyzed by laser flash absorption spectroscopy. Relevant differences have been found between the in vitro and in vivo results, mainly regarding the role played by the electrostatic interactions. Adding positive electrostatic charges to the acidic patch of plastocyanin and cytochrome c_6 promotes an enhanced interaction with photosystem I in vitro but yields the opposite effect in vivo. These discrepancies are discussed in view of the different environmental conditions, in vitro and in vivo, for the reaction mechanism of photosystem I reduction, namely, differential interaction of the electron donors with the thylakoidal membrane and kinetics of donor exchange.

Plastocyanin (Pc)[1] and cytochrome c_6 (Cyt) are two small soluble metalloproteins that act as alternative electron donors to photosystem I (PSI) in the photosynthetic electron transport chain (see refs *1* and *2* for recent reviews), although it has been recently demonstrated that they are also involved in the cyanobacterial respiratory chain as donors to the aa_3-type cytochrome *c* oxidase (*3*−*5*). The expression of one protein or another is regulated by the presence of copper in the medium: the copper protein Pc is expressed in the presence of this metal, whereas Cyt, a heme protein, is synthesized in the absence of copper (*6*).

The factors determining the efficiency of PSI reduction by Pc and Cyt have been extensively studied in vitro, a hierarchy of mechanisms with an increase in efficiency accompanying the replacement of Cyt with Pc in the evolution from cyanobacteria to plants (reviewed in ref *7*). From these studies, two isofunctional regions have been identified in both proteins: a hydrophobic area in the north pole of the molecule involved in the electron transfer and an acidic patch in the east region responsible for electrostatic interactions with PSI (*8*). In the cyanobacterium *Synechocystis*, a single oriented collisional mechanism has been reported for the in vitro reduction of PSI by both donors. This process involves repulsive electrostatic interactions without formation of any kinetically detectable electron-transfer complex (*9*). According to this reaction mechanism, the in vitro site-directed analysis of the process has shown that adding positive charges to the acidic east patch of both Pc and Cyt led to an increase in the efficiency of PSI reduction, whereas changes in the hydrophobic north side of Pc resulted in a less efficient process (*10*, *11*).

Previous studies have revealed relevant differences between the effects of diverse factors on the electron-transfer reactions in vitro and in vivo (*12*−*15*). Particularly, the large effects of charge mutations on the partners observed in vitro were almost abolished in vivo. The high ionic strength and molecular crowding inside the cell have been suggested to explain these discrepancies.

Recently, the kinetics of PSI reduction by Cyt or Pc in *Synechocystis* were analyzed in whole cells (*16*). Whereas the kinetic traces for PSI reduction by Pc correspond to a

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[1] Abbreviations: BCSA, bathocuproinedisulfonic acid; Cyt, cytochrome c_6; k_F and k_S, observed rate constants for the fast and slower phases, respectively, of biphasic kinetics; k_M, observed rate constant for monophasic kinetics; LAHG, light-activated heterotrophic growth; Pc, plastocyanin; *petE*, gene encoding plastocyanin; *petJ*, gene encoding cytochrome c_6; PSI, photosystem I; WT, wild-type.

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monophasic process, the Cyt−PSI interaction follows biphasic kinetics with a first fast component in the microsecond range which was absent in the in vitro experiments (*17*). The fast phase of PSI reduction has been typically assigned to a kinetic model involving transient complex formation before the electron-transfer step (*17*). Thus, as a general rule in cyanobacteria, it seems that Cyt interacts with PSI in vivo by following a mechanism more complex and more efficient than that of Pc (*18*).

To gain deeper insight into the reaction mechanism of PSI reduction under physiological conditions, we have constructed a series of *Synechocystis* mutant strains in which the WT copies of the *petE* (encoding Pc) or *petJ* (encoding Cyt) genes have been replaced by the modified genes of mutant proteins previously studied in vitro (*10*, *11*). The ability of the different mutated donors to carry out PSI reduction in vivo has been further analyzed by laser flash absorption spectroscopy.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions. Synechocystis sp. PCC 6803 cells were grown photoautotrophically in liquid BG-11 medium at 30 °C, either in the presence or in the absence of copper, as previously described (*16*). When necessary, copper was added at a concentration of 1 μM, whereas copper-depleted medium was supplemented with 300 μM bathocuproinedisulfonic acid (BCSA) as a chelating agent to eliminate any traces of this metal (*16*, *19*). Cell cultures with or without copper were pre-adapted to the new conditions by repeated incubation cycles, and cell growth was monitored by spectrophotometric measurements of the chlorophyll content (*20*). The Δ*petE* mutant was grown in the presence of kanamycin (50 μg/mL); the Δ*petJ* mutant was cultured in the presence of spectinomycin (5 μg/mL), and site-directed mutant strains were cultured in the presence of chloramphenicol (10 μg/mL). The whole protein concentration was determined as previously described (*21*). Light-activated heterotrophic growth (LAHG) experiments were carried out as described elsewhere (*16*, *22*) by adding 10 mM glucose to the standard solid medium.

Escherichia coli DH5α cells were grown in Luria-Bertani liquid or solid medium (*23*) supplemented, when required, with chloramphenicol (40 μg/mL), kanamycin (50 μg/mL), spectinomycin (100 μg/mL), or ampicillin (100 μg/mL).

DNA Manipulation. To construct *Synechocystis* strains carrying site-directed mutant copies of the *petE* gene, the Δ*petE* strain (*16*) was transformed as shown in Figure 1 (top). The pPlaF plasmid carrying the *petE* gene and flanking regions (*16*) was digested with *Sma*I, and the resulting 5.7 kb band was ligated with the 1.4 kb *Hin*cII−*Hin*cII band from the pRL479 plasmid carring the C.C2 cassette, which confers resistance to chloramphenicol (*24*), thus generating the pPlaCC2 plasmid. This plasmid was used as a template to insert the site-directed mutations: the 200 bp *Cel*II−*Bsa*I fragment of pPlaCC2, corresponding to the central part of the ORF of *petE*, was replaced with the 200 bp *Cel*II−*Bsa*I fragments of each ORF of WT and precisely mutated *petE* copies from plasmids previously developed (*10*). The resulting plasmids were tested by restriction and sequence analysis and used to transform the Δ*petE* strain by standard procedures (*25*). The transformants were selected and segregated on solid BG-11 medium in the presence of 1 μM copper, with increasing amounts of chloramphenicol from 10 to 80 μg/mL. The correct integration and segregation of the site-directed mutant copies were tested by Southern blotting by digesting the genomic DNA of isolated mutants (*26*) with *Pst*I and using, as a probe, the *Nhe*I−*Sma*I fragment from pPlaF radioactively labeled with ^{32}P with the Ready-To-Go commercial kit (Amersham). In a *Pst*I digestion of the genomic DNA, this probe hybridized with a 4.0 kb band in the case of Δ*petE* and with a 3.2 kb band in the case of WT and site-directed mutant strains.

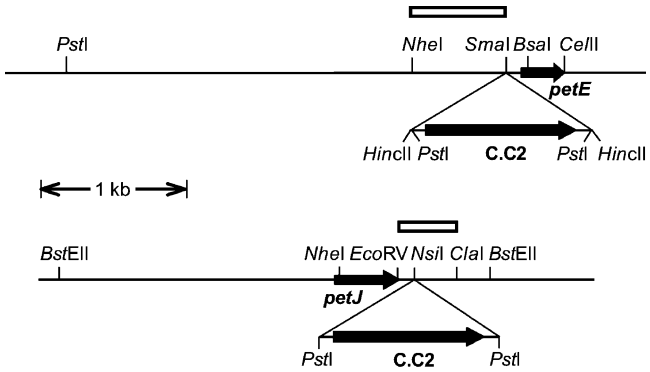

FIGURE 1: Strategy designed to obtain site-directed mutant strains for the *petE* (top) and *petJ* (bottom) genes in *Synechocystis* cells. Solid arrows denote ORFs, and open segments stand for probes used in Southern blot analysis.

In a similar fashion, the Δ*petJ* strain (*16*) was used to obtain the strains with WT and site-directed mutant copies of the *petJ* gene. The 2.4 kb *Sal*I−*Sac*II fragment from pCytF (*16*) was ligated to pBluescript II SK(+) (Stratagene) digested with *Sal*I and *Sac*II. The resulting plasmid was named pACC. A 1.4 kb *Pst*I−*Pst*I band from pRL479 carrying the C.C2 cassette was ligated to the *Nsi*I-digested pACC plasmid, thus generating the pCitCC2 plasmid (Figure 1, bottom). To obtain the constructs with the WT and site-directed mutant copies of the *petJ* gene, the 350 bp *Nhe*I−*Eco*RV fragment from the pCitCC2 plasmid was replaced with the 350 bp *Nhe*I−*Eco*RV fragments of each ORF of WT and site-directed mutant *petJ* copies from plasmids previously developed (*11*), and the resulting plasmids were used to transform the Δ*petJ* strain. Transformants were selected and segregated on solid BG-11 in the absence of copper with increasing amounts of chloramphenicol from 10 to 80 μg/mL. To test the correct integration and segregation of transformed strains, Southern blot experiments were performed as follows: genomic *Bst*EII-digested DNA from these strains was hybridized with the *Cla*I−*Eco*RV fragment from the ^{32}P-labeled pCytF plasmid as a probe, giving a band of 1.7 kb in the case of Δ*petJ* and a band of 4.3 kb in the case of the WT and site-directed mutants.

All DNA manipulating enzyme procedures followed the manufacturers' instructions.

RNA Isolation and Northern Blot Analysis. Total RNA isolation from *Synechocystis* liquid cultures was performed by breaking the cells in the presence of glass beads (0.2−0.3 mm in diameter, Sigma) as described elsewhere (*27*). Probes for Northern blots were the *Bam*HI−*Eco*RI fragment from the pBS-Pc plasmid (*28*) in the case of the *petE* gene and the *Eco*RI−*Hin*DIII fragment from the pBS-Cyt plasmid (*11*) in the case of the *petJ* gene. Both were radioactively

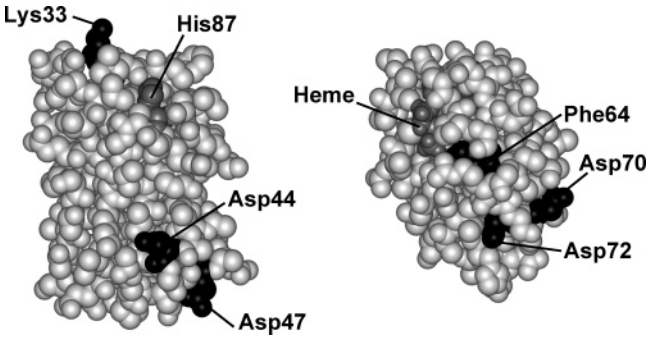

FIGURE 2: Space-filling representations of *Synechocystis* plastocyanin (left) and cytochrome c_6 (right) showing the residues mutated. The molecules are oriented with their electrostatic patches, or east sites, to the front and the hydrophobic patches, or north sites, at the top. The mutated residues are depicted in black. The electron-transfer ports for both proteins, His87 at plastocyanin and the heme group at cytochrome c_6, are also indicated. The numbering in plastocyanin corresponds to that of the spinach protein.

labeled as described above for Southern blots. As a control, in all cases filters were stripped and rehybridized with the *Hin*DIII−*Bam*HI 580 bp probe from the pAV1100 plasmid that contains the constitutively expressed RNase-P RNA gene (*rnpB*) from *Synechocystis* (*29*).

Other Techniques. Isolation of Pc and Cyt from *Synechocystis* WT cells and immunoblotting experiments were carried out as previously described (*16*). Proteins were immunodetected with polyclonal antibodies raised against *Synechocystis* Pc or Cyt (*16*).

For the kinetic analysis of in vivo PSI reduction, cells of *Synechocystis* carrying the WT or mutated genes were harvested by centrifugation at different stages of the culture. The in vivo reduction of PSI was followed by laser flash absorption spectroscopy in PSII-inactivated samples as previously described (*16*). Each value for kinetic constants was obtained from three to five different cultures. The structure of Cyt from *Synechocystis* was modeled by using SwissPDB Model, with the structure of Cyt from *Synechococcus elongatus* (PDB entry 1C6S) as a template.

RESULTS AND DISCUSSION

Synechocystis petE and petJ Site-Directed Mutant Strains. We have previously reported a detailed site-directed mutagenesis analysis of in vitro electron transfer from Pc and Cyt to PSI in *Synechocystis* (*10*, *11*). On the basis of these results, we have selected a number of mutants to perform an in vivo study on the effect of replacing surface residues in both Pc and Cyt on PSI reduction, comparing the results to those previously obtained in vitro, the replaced groups being shown in Figure 2 for both proteins. The selected mutants were D44A, D44K, D47R, and the double mutant D44R/D47R in the case of Pc and F64A, D70R, and D72R for Cyt. All these mutations are located in the acidic isofunctional patch of both proteins (Figure 2). In addition, the mutant K33E, with the charge located at the hydrophobic area of Pc which is close to the copper atom, has also been investigated (Figure 2). It has been previously reported that the redox potentials of the mutant proteins studied here are similar to those measured for the WT proteins (*10*, *11*).

The cyanobacterial cells were transformed with the plasmids carrying the specific site-directed mutations for *petE* or *petJ*, and the resulting strains were selected as transformants corresponding to each *petE* or *petJ* mutant. In the case of Pc, the respective mutant strains are named MP-K33E, MP-D44A, MP-D44K, MP-D47R, and MP-D44RD47R. In the case of Cyt, strains carrying the different mutations are named MC-F64A, MC-D70R, and MC-D72R. As a control, similar constructions carrying the WT *petE* and *petJ* genes were used to transform the Δ*petE* and Δ*petJ* strains, respectively (pPlaCC2 and pCytCC2 plasmids, respectively), resulting in reversion strains MP-WT and MC-WT. Site-directed mutant strains for Δ*petE* and Δ*petJ* genes were selected in the presence of chloramphenicol (80 μg/mL) and either in the presence or in the absence of 1 μM copper, respectively; in the case of the *petJ* mutants, 300 μM BCSA was also added to the medium. The correct integration and segregation of every mutant was checked by Southern blot (Figure 3), with the WT, Δ*petE*, and Δ*petJ* strains being used as controls. The lengths of the resulting bands and the absence of other bands were as expected for completely segregated mutants (see Experimental Procedures and Figure 1).

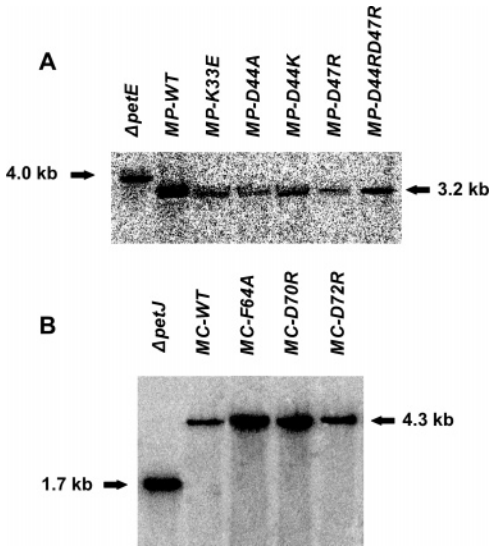

FIGURE 3: Southern blot of site-directed mutant strains of *petE* and *petJ* genes. *Synechocystis* wild type (WT) and Δ*petE* and Δ*petJ* mutants were used as controls. Genomic DNAs were digested with appropriate restriction enzymes and hybridized with either the [^{32}P]-dCTP-labeled *Nhe*I−*Sma*I fragment of the pPlaF plasmid (A) or the [^{32}P]dCTP-labeled *Cla*I−*Eco*RV fragment of the pCytF plasmid (B). The band length is expressed in numbers of kilobase pairs. See the text for details.

To test the ability of every strain to synthesize the mutated protein, Western blot experiments were performed. The *petE* mutant strains were grown in the presence of copper to test the expression of mutated Pc, whereas *petJ* mutant strains were cultured in the absence of the metal to allow the synthesis of mutated Cyt. As shown in Figure 4, both MP-WT and MC-WT reversion mutant strains, as well as most site-directed mutants, were able to produce normal levels of the mutant proteins, thus not only corroborating the correct integration of the constructions in the genome of the cyanobacteria but also showing that these integrations did not affect the regulation of the expression of both Pc and Cyt. The only exception was the MC-D70R strain, for which a dramatic decrease in the Cyt expression levels was observed (Figure 4).

Northern blot analysis of the MC-D70R strain showed no deficiencies in gene transcriptions, in the case of either *petJ*

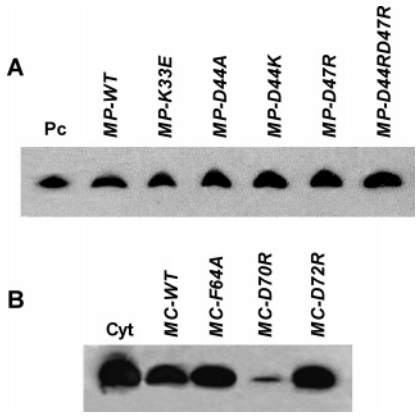

FIGURE 4: (A) Immunodetection of plastocyanin (Pc) in site-directed mutants of *petE* gene strains cultured in the presence of copper. (B) Immunodetection of cytochrome c_6 (Cyt) in site-directed mutants of *petJ* gene strains cultured in the absence of copper. Cell extracts with 100 μg of total protein were loaded into each gel lane, with 0.1 μg of purified Pc and Cyt as controls. Polyclonal antibodies against Pc (A) and Cyt (B) were used.

or *petE* genes (not shown), thus suggesting that the low level of Cyt expression in this strain is due to an unidentified post-transcriptional process. There is maybe the inefficient translation in *Synechocystis* of the CGA codon for arginine 70 or the instability of the D70R mutant protein inside the cell, although the purified recombinant protein was shown to be as stable as the WT (*11*).

All the *petE* site-directed mutants and the MP-WT strains were as efficient as WT *Synechocystis* cells when growing under phototrophic conditions in the presence of copper (not shown). The same results were obtained in the case of *petJ* mutants and MC-WT strains when cultured in the absence of this metal. These findings indicated that mutations did not significantly affect the global photosynthetic electron flow rate in the cell; this result is as expected because it is well-established that the rate-limiting process in the photosynthetic electron flow is the plastoquinone−cytochrome b_6f interaction (*30*). Similar results were obtained in LAHG experiments, thus indicating that the global respiratory rate was not affected by the mutations.

In Vivo Reduction of PSI in Mutant Strains. Figure 5 shows kinetics traces of in vivo PSI reduction in *Synechocystis* strains carrying the reverted WT genes and some of the mutated Pc and Cyt. For an equivalent amount of cells, similar amplitudes of the signals corresponding to PSI were obtained, although slightly different signal-to-noise ratios could be observed depending on the strains and culture conditions. Ascorbate was present in the reaction cell to ensure the total reduction of the donor protein pool (*16*). Thus, the interaction between the donor protein and PSI can be directly measured with no interference arising from differences in the redox status of Pc and Cyt pools, or in the relative content of PSI (*16*, *18*).

As previously described for WT *Synechocystis* cells and other cyanobacteria (*16*, *18*), kinetic traces for Pc−PSI interaction can be well-fitted to monophasic kinetics (Figure 5A), whereas Cyt−PSI interaction follows biexponential curves (Figure 5B). The monophasic kinetics have been assigned to a single oriented collisional mechanism, whereas the biphasic ones can be explained according to kinetic models that involve transient complex formation. From the

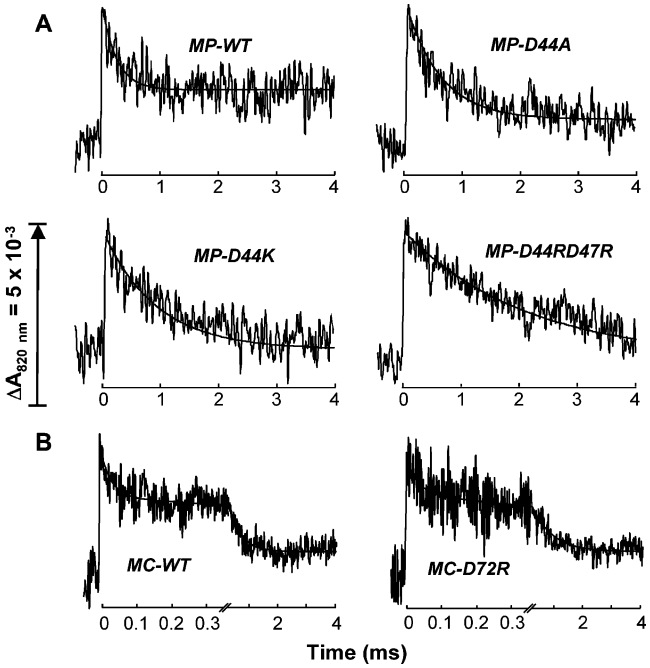

FIGURE 5: Kinetic traces showing in vivo PSI reduction in *petE* (A) and *petJ* (B) site-directed mutant strains. The cells were grown either in the presence (A) or in the absence (B) of copper. The reaction cell contained an amount of cells equivalent to a total chlorophyll content of 150−300 μg/mL. Absorbance changes were recorded at 820 nm.

Table 1: Observed Rate Constants for the in Vivo Reduction of PSI in the Pc Site-Directed Mutant Strains and Bimolecular Rate Constants for the in Vitro PSI Reduction in the Presence of 250 mM NaCl

	in vivo k_M[a] ($\times 10^{-3}$ s^{-1})	in vitro[b] k_{250}[c] ($\times 10^{-7}$ M^{-1} s^{-1})
MP-WT	2.8 ± 0.5	0.97
MP-K33E	3.6 ± 0.6	0.58
MP-D44A	1.8 ± 0.6	0.97
MP-D44K	1.5 ± 0.4	0.96
MP-D47R	1.3 ± 0.3	0.87
MP-D44RD47R	0.9 ± 0.6	0.83

[a] Observed rate constant for monophasic kinetics. [b] Data from ref *10*. [c] Bimolecular rate constant in the presence of 250 mM NaCl.

Table 2: Observed Rate Constants for the in Vivo PSI Reduction in the Cyt Site-Directed Mutant Strains and Bimolecular Rate Constants for the in Vitro PSI Reduction in the Presence of 250 mM NaCl

	in vivo		in vitro[a]
	k_F[b] ($\times 10^{-4}$ s^{-1})	k_S[c] ($\times 10^{-3}$ s^{-1})	k_{250}[d] ($\times 10^{-7}$ M^{-1} s^{-1})
MC-WT	3.9 ± 1.6	2.2 ± 0.3	1.18
MC-F64A	3.8 ± 1.5	1.8 ± 0.5	0.92
MC-D72R	3.6 ± 1.8	1.4 ± 0.3	2.04

[a] Data from ref *11*. [b] Observed rate constant for the fast phase of biphasic kinetics. [c] Observed rate constant for the slow phase of biphasic kinetics. [d] Bimolecular rate constant in the presence of 250 mM NaCl.

traces shown in Figure 5, the values for the observed kinetic constants for such monoexponential curves (k_M) for PSI reduction by Pc (Table 1) and those for the observed rate constants for the fast (k_F) and slow (k_S) phases for PSI reduction by Cyt (Table 2) have been estimated with all mutants. In both cases, the in vitro second-order bimolecular constants at 250 mM NaCl (k_{250}) are shown for comparative

purposes (see below). These k_{250} values reflect the relative efficiency of the different mutant proteins in the interaction with PSI in vitro at near-physiological ionic strength, allowing a more appropriate comparison with the in vivo results. Under such conditions, a collisional bimolecular mechanism for the in vitro PSI reduction has been observed (*10*, *11*). Both the MP-WT and MC-WT reverted strains gave kinetic traces and observed rate constants similar to those previously reported for WT *Synechocystis* cells grown in the presence and absence of copper, respectively (*16*, *18*), thus validating the methodological approach applied here. As already published for the WT cells, no significant differences in the kinetic constants were observed among the cultures growing in either case (not shown).

With regard to Pc mutations, a decrease in the efficiency of PSI reduction directly correlated with the decrease in the size of the negative patch in the east side of Pc could be observed (Figure 5A and Table 1). Thus, the removal of the negative charge of Asp44 (MP-D44A strain) leads to a decrease in the k_M of up to 65% of that of MP-WT, but this decrease is even more pronounced (ca. 50%) when a negatively charged amino acid is replaced with a positively charged one (MP-D44K and MP-D47R strains). The most drastic effect is observed with the double mutant strain (MP-D44RD47R), where two negative residues have been replaced with positive ones, a decrease in the k_M to 30% of that of WT being obtained. This double mutant protein has the largest modification of the charged patch of any of the mutants tested, and accordingly, it shows the lowest electron-transfer efficiency. In contrast, adding negative charges to the north side of Pc in the MP-K33E strain leads to a PSI reduction slightly faster than that of the WT.

With regard to Cyt mutations, from the four mutants of Cyt previously studied in vitro (*11*), it was only possible to obtain the three mutant strains here assayed. Although it is not possible to attain general conclusions from this reduced number of available mutants, the results obtained with the MC-D72R mutant are in agreement with those described above for Pc. Thus, this Cyt mutant, in which a negative charge has been replaced with a positive one, showed a decrease in the k_S to 65% of that of the WT protein, although no significant differences are found for the fast phase component, either in the k_F value or in the relative amplitude of the fast phase (35%). This differential effect of the mutation on the fast and slow kinetic components indicates that the mutation is not affecting the electron-transfer process itself, but the ability of both partners to interact with each other.

The mutant MC-D70R shows a dramatic decrease in the k_S value (not shown), because of the inability of this strain to produce normal levels of Cyt (see above). However, the kinetic fast component seems to be maintained, although its small amplitude prevented a reliable estimation of k_F. This finding also confirms that Cyt mutations leave intact the electron-transfer process itself. Moreover, the kinetic behavior of the MC-D70R strain further supports the fact that no other donor can efficiently replace Pc and Cyt in reducing PSI (*16*). Finally, the F64A mutation, with no charge replacement, has no effect on PSI reduction in vivo (Table 2), as was also the case for in vitro PSI reduction by this mutant (*11*). Thus, Phe64, which is close to the heme group and was proposed to be the counterpart of Tyr83 in Pc, does not definitively appear to be involved in the electron transfer to PSI.

In Vivo versus in Vitro Kinetics. When data obtained for the same process in vitro and in vivo are compared, it is necessary to take into consideration the very different experimental conditions of both situations. First, the ionic strength inside the thylakoid is 0.2−0.3 M; second, the presence of the thylakoid membrane imposes additional electrostatic and hydrophobic interactions, along with a physical diffusional constraining factor, and third, the molecular crowding inside the cell can also impose severe limitations on the diffusion and encounter of protein partners.

In *Synechocystis*, the in vitro reduction of PSI by both Pc and Cyt involves repulsive electrostatic interactions, and so the electron-transfer rate increases with ionic strength up to a saturating plateau (*9*). In agreement with this, an increase of the electron-transfer rate at low salt concentrations occurs when negative residues are replaced with positive ones in the east negative side of both donors (*10*, *11*). Thus, in vitro, the efficiency of PSI reduction at relatively low ionic strengths is related to the net charge of the mutation in the acidic patch of the donor protein; however, the bimolecular rate constants approach WT values at higher salt concentrations (*10*, *11*). On the other hand, mutations in the hydrophobic north side of Pc resulted in an impairment of the electron-transfer process itself (*10*).

Surprisingly, when the effect of mutations in the acidic patch of both donor proteins is studied in vivo, the tendency is just the opposite of that observed in vitro: the efficiency of PSI reduction in vivo decreases when positive charges are added. This effect seems to be additive, as the smaller rate constants are obtained with the double MP-D44RD47R mutant strain. The discrepancies between the results obtained in vitro and in vivo are also observed when comparing the in vivo results with the in vitro data at near-physiological ionic strength, i.e., at 250 mM NaCl, at which all the rate constants in vitro of the mutants approach WT values (Tables 1 and 2). Interestingly, the in vivo results with the MC-D72R Cyt mutant show a diminished rate for the slow phase of PSI reduction, but the initial fast phase is not affected by the mutation, either in the kinetic rate or in the amplitude, which indicates that the electron-transfer step and the equilibrium of preflash complex formation are not modified. Thus, only the ability of both proteins to encounter each other is modified.

Together, the in vivo results show that adding positive charges to the east acidic side of the donor proteins promotes a less efficient reduction of PSI, contrary to the effects previously observed in vitro. It is also interesting to note that the magnitude of the effects induced by the mutations is much higher in vitro than in vivo with respect to the WT proteins. Discrepancies between the effects of mutations on the electron-transfer process in vivo versus in vitro have been previously observed in other photosynthetic redox systems (*12*−*15*). Several factors have been invoked to explain these discrepancies. First, the electrostatic interactions do not seem to be as important in vivo as in vitro because of the shielding of charges by the high ionic strength inside the cell (*31*). Second, the thylakoid membrane could promote both packaging and diffusional motions of the soluble proteins in the thylakoid lumen. The interaction of the donors with the thylakoid membrane has also been argued to influence

electron-transfer reactions (*13*, *32*), and this interaction would be modified by charge replacement in mutated proteins. Third, it has been recently proposed, from site-directed mutagenesis of PSI, that in vivo PSI reduction in the green alga *Chlamydomonas* is limited by the release of oxidized Pc from the PSI complex (*15*). This effect could also explain both our results with mutant Pc, in terms of slower donor exchange induced by the charge mutations and the specific effect of the MC-D72R mutation on the slow diffusional kinetic component, without affecting the equilibrium of complex formation and the electron-transfer step (*15*). Thus, we have to invoke molecular crowding to explain why this effect seems not to apply in vitro.

With regard to the weaker effect induced by charge mutations in vivo, it is interesting to note that, in general, the charged residues at the electrostatic patches of both soluble donors and, in particular, those mutated herein are not conserved in cyanobacteria. In fact, the electrostatic pattern of both proteins varies in a parallel way from one organism to another (*7*). However, it has been recently reported (*33*) that such variations in the electrostatic properties of the electron donors do not correlate with complementary changes at the corresponding PSI docking site, thus indicating that the PSI−donor interactions are less specific in cyanobacteria than in eukaryotes, the PSI docking site being similar in the former organisms (*33*).

Finally, adding a negative charge to the hydrophobic north side of Pc in the MP-K33E mutant, which in vitro promotes a strong impairment of the Pc−PSI interaction, has shown almost no effect in vivo. Again, differences in salt concentration and/or the presence of the thylakoid membrane could explain these results.

To conclude, our results reveal the differences found when carrying out in vivo experiments with respect to in vitro conditions and thus show the relevance of studying biological processes in their physiological environment. Adding positive charges to the acidic patch of Pc and Cyt, which in vitro promoted an enhanced interaction with PSI, yields opposite effects in vivo; the mutations induce a decrease in the PSI reduction efficiency, but this does not significantly affect the overall photosynthetic metabolism.

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