Mutagenesis of Prochlorothrix Plastocyanin Reveals Additional Features in Photosystem I Interactions

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Three surface residues of plastocyanin from Prochlorothrix hollandica have been modified by site-directed mutagenesis. Changes have been made in methionine 33, located in the hydrophobic patch of the copper protein, and in arginine 86 and proline 53, both located in the eastern hydrophilic area. The reactivity toward photosystem I of single mutants M33N, P53A, P53E, R86Q, R86E, and the double mutant M33N/P14L has been studied by laser flash absorption spectroscopy. All the mutations yield increased reactivity of plastocyanin toward photosystem I as compared with wild type plastocyanin, thus indicating that in Prochlorothrix electron donation to photosystem I is not optimized. The most drastic increases in the intracomplex electron transfer rate are obtained with mutants in methionine 33, whereas replacing arginine 86 only modestly affects the plastocyanin-photosystem I equilibrium constant for complex formation. Mutations at position 53 also promote major changes in the association of plastocyanin with photosystem I, yielding a change from a mechanism involving complex formation to a simpler collisional interaction. Molecular dynamics calculations indicate that mutations at position 33 promote changes in the H-bond network around the copper center. The comparative kinetic analysis of the reactivity of Prochlorothrix plastocyanin mutants toward photosystem I from other cyanobacteria reveals that mutations M33N, P53A, and P53E result in enhanced general reactivity.

Plastocyanin (Pe) is a soluble type-I copper metalloprotein (molecular mass, ~10.5 kDa) located inside the thylakoid lumen of photosynthetic organisms and acting as a mobile electron carrier between the membrane-embedded cytochrome b6f and photosystem I (PSI) complexes (1, 2). In eukaryotic Pe, two active sites have been identified: site 1, an hydrophobic flat region around the copper binding area, and site 2, a charged region referred to as the acidic patch in plants and eukaryotic algae because it includes aspartate and glutamate residues. Whereas site 1 is involved in vitro in hydrophobic interactions of Pe with both redox partners and in the electron transfer event itself, site 2 is responsible for the electrostatic interactions and molecular recognition with complementary positive areas both in PSI and cytochrome f (3). However, the relevance of such electrostatic interactions in vivo can be different, as is the case for the interaction between cytochrome f and Pe (4). In cyanobacteria, site 2 can be either negatively or positively charged (5). All these differences between distinct organisms have led to different reaction mechanisms for PSI reduction (5–7).

Prochlorophytes represent a diverse group of cyanobacteria containing both chlorophyll a and b (8, 9). Recently, an analysis of the interaction of Pe with PSI from the prochlorophyte Prochlorothrix hollandica, both for WT and mutated Pe (10, 11), revealed that Prochlorothrix Pe reacts with PSI by forming a transient complex with PSI that is stabilized by means of hydrophobic interactions. The solution structure of Prochlorothrix Pe has been solved by NMR spectroscopy (12), showing that this Pe contains an altered hydrophobic patch due to the presence of three unique residues, Tyr-12, Pro-14, and Met-33 (corresponding to the conserved Gly-10, Leu-12, and Asn-31 as numbered in the spinach protein). Single (Y12G, Y12F, Y12W, and P14L) and double (Y12G/P14L) mutations in the hydrophobic patch of Prochlorothrix Pe do not alter the constant of complex formation with PSI, but the electron transfer rate constant is significantly affected (11). Most interesting changes are obtained with mutants at Pro-14: reversion of the “unique” Pro-14 of Prochlorothrix Pe to the “standard” leucine of all other Pcs enhances the reactivity of Pe toward PSI, thereby indicating that Prochlorothrix Pe interaction with PSI is not optimized (11). More recently, a computational simulation of Prochlorothrix Pe-PSI docking suggested that Tyr-12 in Pe participates in hydrogen bonding with an asparagine residue in the PsaA polypeptide of PSI. This model also shows a short a-helix in Pe around position Pro-53 interacting with a small b-sheet extension of the PsaA polypeptide in PSI (13).

In this study, we have extended our previous studies of Prochlorothrix PSI reduction by Pe to analyze the reactivity of Prochlorothrix Pe mutants at Met-33, in the hydrophobic patch, as well as at Pro-53 and Arg-86, in the electrostatically charged area. An arginine residue in an equivalent position to Arg-86 in Prochlorothrix Pe seems to be a specific feature of prokaryotic Pe, and it has been shown that this residue plays a fundamental role in the interaction with PSI (5, 14). The laser-flash absorption spectroscopy analyses herein reported indicate that
replacing Met-33 by the standard asparagine (as is the case for the replacement of Pro-14 by leucine) as well as Pro-53 substitution make the copper protein react much more efficiently with PSI, whereas Arg-86 replacement only promotes moderate effects on (Pc-PSI) complex formation.

EXPERIMENTAL PROCEDURES

Expression and Characterization of Mutant Pc—Mutant and WT *P. hollandica* Pc were expressed as inclusion bodies in *Escherichia coli* BL21(DE3) pLYsS (Novagen, Madison, WI), as previously described (15). Pc preparations were analyzed by absorption and far-UV circular dichroism spectroscopy as described in Babu et al. (15). Redox titration of Pc preparations was monitored by absorption spectroscopy at 602 nm in increasing ratios of ferrocyanide/ferricyanide as previously described (16). Construction of mutant Pc in our expression system employed the Stratagene QuickChange kit, and custom mutagenic primers were obtained from Invitrogen. The primer sequences directing the following mutations were obtained: for M33N, 5'-CGCGCGTGAAGCCGCTGGGCTGGTCGGC-3'; R66E, 5'-CGCCCAACAGGCGCTGGTCGGC-3'; P53A, 5'-CGCGCGTGAAGCCGCTGGTCGGC-3'; P53E, 5'-CGCGCGTGAAGCCGCTGGTCGGC-3'. Double mutant M33NP14L employed the P14L mutation present as a template for the M33N primer (11).

PSI Purification and Kinetic Studies—PSI particles from *Prochlorothrix* were obtained by β-dodecyl maltoside solubilization as described by Rögner et al. (17) and modified in Navarro et al. (11). 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 Sétil (18). Chlorophyll concentration was determined according to Arnon (19). The chlorophyll/P700 ratio of the resulting preparation was 150/1. Spinach, *Synechocystis*, and *Anabaena* PSI were purified as previously described (6).

Kinetics of flash-induced absorbance changes in PSI were followed at 820 nm as described in Hervás et al. (6), except that the setup was optimized by replacing the measuring light by an attenuated laser diode (820 nm, 150 mW, model LD 1361 from Laser 2000 Ltd.). Unless otherwise stated, the standard reaction mixture contained, in a final volume of 0.2 ml, 20 mM Tricine-KOH, pH 7.5, 0.03% -dodecyl maltose, 10 mM MgCl\(_2\), an amount of PSI-enriched particles equivalent to 0.35 mg of chlorophyll m\(^{-1}\) cm\(^{-1}\), 0.1 mM methyl viologen, 2 mM sodium ascorbate and Pc at the indicated concentration. All the experiments were performed at 22°C in a 1-mm path-length cuvette. Each kinetic trace was the average of 5–10 independent measurements with 30 s spacing between flashes. For most experiments, the estimated error in the observed rate constants (k\(_{\text{obs}}\)) was less than 10%, based on reproducibility and signal-to-noise ratios. Data collection and analysis were as previously described (6, 20).

Molecular Dynamics Simulations—Mutations were introduced into the NMR solution structure of the WT *Prochlorothrix* Pc via SWISS-PDB Viewer (21) and saved as a (Protein Data Bank) file. Only those rotamers lacking steric clashes with other amino acids were selected. Charges of most atoms were taken from the AMBER 6.0 force field (22). Charges and other force field parameters for the copper atom and its ligands were obtained from NMR structural data and compared with similar Pc simulations in the literature (23, 24). To preserve the geometry of the copper site, the bonds between the copper atom and its ligands were treated as covalent (25). Topology and parameter files for both mutants were generated using the xLeap module of the AMBER 6.0 package. Hydration was treated explicitly by including the protein and a 50 Å water box according to the TIP3P model (26). Proteins were subjected to 1000 steps of steepest descent energy minimization to relax large steric overlaps or electrostatic inconsistencies. Next, the system was equilibrated for 30 ps with 500 kcal/mol constraints applied on the protein structure. The temperature was gradually increased from 100 K to 300 K. During the equilibration phase the potential, kinetic, and total energies were monitored. The water box reached density 1.00 after 4 ps. After equilibration, the system was minimized during 1000 steps of the steepest descent algorithm. Next, the molecular dynamics was run for 1000 ps at 300 K, during which the data were collected every 1000 fs. No constraints were applied on the Pc secondary structure to allow for the better exploration of conformational space. Non-bonded van der Waals interactions were cut off beyond 9 Å. The long-range electrostatic interactions were treated with the Particle Mesh Ewald method (27). The SHAKE option (28) was used to constrain all bonds, which enables a 2-fs time step. The 950 ps of data were used for analysis by the CARNAL block of AMBER.

Hydrogen bonding was defined by geometric criteria: the cut-off distance between hydrogen and acceptor was 3.5 Å (for sulfur, 4 Å) and the deviation of donor-acceptor distance from linearity was less than 60° (29). All H-bonds with occupancy greater than 25% were considered as maintained during the simulation.

RESULTS

The variant residue Met-33 at the hydrophobic patch of *Prochlorothrix* Pc (Fig. 1) was chosen to be mutated to the standard asparagine in other Pcs in order to study its role in the reactivity of Pc toward PSI, because it has been previously reported that replacement in this area of the also atypical Pro-14 by the standard leucine drastically enhances *Prochlorothrix* Pc electron transfer to PSI (11). A double mutant with reversion of both methionine and proline to the standard residues asparagine and leucine, respectively, was also constructed (Fig. 1). Proline at position 53, located in a loop proposed to interact with PSI (13), was replaced by either alanine or glutamic acid. Additionally, arginine at position 86 was replaced both by glutamine and glutamate (Fig. 1), because an equivalent arginine has been shown to be crucial in the donor-PSI interaction in other cyanobacteria (5).

Most of the mutations do not significantly alter the midpoint redox potential of the copper protein (ΔE\(_{\text{m}}\) = 20 mV; Table I), with the exception of the R86E mutant, whose redox potential is about 30 mV lower than WT. In general, changes in redox potential should slightly increase the driving force for electron transfer to PSI, except in the case of the M33N mutant, whose redox potential is about 20 mV higher than WT (Table I). All mutant oxidized Pc yield an absorption peak at 602 nm, identical to that of WT.

As previously described for the WT Pc (10, 11), the laser-flash-induced kinetic traces of PSI reduction by all the mutated Pcs are monoequponential (Fig. 2). However, the efficiency of the electron transfer process changes from one protein to another. Thus, as shown in Fig. 2 for some mutants, kinetic traces for PSI reduction by the P53A and M33N mutants are significantly faster as compared with WT Pc, whereas the R83E mutant shows slightly slower kinetics (Fig. 2). The dependence of the observed pseudo first-order rate constant (k\(_{\text{obs}}\)) upon donor protein concentration shows a saturation profile for all mutants at positions 33 and 86 but not for those at position 53, which present linear concentration dependences (Fig. 3). These findings suggest the formation of a bimolecular (Pc-PSI) transient complex prior to electron transfer for mutants at positions 33 and 86, as previously described for the WT system as well as...
for other mutants of *Prochlorothrix* Pc (10, 11). However, the behavior of the proteins mutated at position 53 can be better described by assuming just a second-order collisional process, with no formation of any detectable electron transfer complex (6).

From the saturation profiles in Fig. 3, and applying the formalism developed in Meyer et al. (20), it is possible to estimate both *K* _A_ (the equilibrium constant for complex formation) and *k*_2 (the intracomplex electron transfer first-order rate constant). The bimolecular second-order rate constant (*k*_1) for the interaction between P53A and P53E Pc and PSI can be directly calculated from linear dependences shown in Fig. 3. Table I shows the values for all these constants, as well as the previously reported values for the P14L mutant for comparative purposes (11). There are no significant changes in *K* _A_ for the mutants M33N and M33N/P14L as compared with WT Pc. Replacing Arg-86 by glutamine promotes a moderate decrease (to 60%) in *K* _A_ whereas the most drastic decrease is observed for the R86E mutant (to 15%), in which a negative charge is introduced. Concerning the electron transfer step, Table I shows that all the mutations significantly enhance the efficiency of Pc in donating electrons to PSI, with the most relevant increases being obtained for both mutants in Met-33, in which ~5 × increases in *k*_1 are observed (Table I). Although it is not possible to estimate a *k*_1 value for P53A and P53E mutants, the *k*_obs values shown in Fig. 3 at high Pc concentration (up to 8,500 s⁻¹) are much higher than any *k*_1 value from Table I.

To check whether the hydrophobic nature of the interaction between Pc and PSI in *Prochlorothrix* is altered by mutations, the kinetics of PSI reduction were also followed at high ionic strength. As can be seen in Table I, none of the mutants shows significant changes in *K* _A_ , *k*_1, or *k*_2 upon increasing salt concentration, as previously described for the WT and other mutated Pcs (10, 11).

It has been previously reported that *Prochlorothrix* WT Pc exhibits a very low reactivity in cross reactions with PSI from either spinach or the cyanobacteria *Anabaena* and *Synechocystis* (10). However, the P14L mutant, designed to revert the "exclusive" proline in the hydrophobic patch of *Prochlorothrix* to the standard leucine, showed a general enhanced reactivity toward PSI from different sources (11). Here we have also checked the reactivity of mutants toward heterospecific PSI (Table II). In all cases, linear dependences were observed when plotting *k*_obs versus protein concentration (not shown), thus allowing the estimation of *k*_2 for the different Pc/PSI systems (Table II). None of the mutants show reactivity with spinach PSI, as deduced by the low *k*_2 values shown in Table II for this photosystem, and *k*_2 values for spinach Pc could not be calcu-
labeled because this Pc follows a three-step reaction mechanism with its PSI (6). When studying cyanobacterial PSI, replacement of Met-33 by the standard asparagine, either in the single mutant or in the M33N/P14L, promotes significant increases in the bimolecular rate constant of PSI reduction, both with *Synechocystis* and *Anabaena* PSI. Whereas the R86Q mutant does not significantly change its reactivity against cyanobacterial PSI, the R86E mutant shows a lower efficiency with both cyanobacterial photosystems (Table II). Regarding mutations in position 53, both mutants show a significantly higher efficiency with both cyanobacterial photosystems, rendering similar values to those obtained with the M33N Pc (Table II).

**Molecular Dynamics Studies**—To determine structural changes in the protein introduced by these mutations and observe their dynamic behavior, we have run 1 ns molecular dynamics (MD) simulations for WT and the P14L, M33N, and P53E mutants. The stability of the simulation was checked by molecular dynamics (MD) simulations for WT and the P14L, M33N, and P53E mutant Pc. The calculated average root mean square deviation (r.m.s.d.) for all atoms was less than 2 Å (Table III), suggesting that proteins are able to maintain their global structure. The introduction of the protein of non-native amino acids leads to the rearrangement of protein structure, which can be monitored through the changes in the r.m.s.d. In case of Pc, the copper center determines its function, thus the alteration of the copper center geometry by mutations can be assessed by r.m.s.d. of its ligands. The calculated data are presented in Table III. It can be seen that in the P14L mutant there is slight displacement of all copper ligands, suggesting that this mutant has a slightly altered geometry, which in its turn affects midpoint redox potential of the protein as reported previously (Table I and Ref. 11). On the contrary, in the M33N mutant the three ligands have the same position except for His-39, which is spatially adjacent to residue 33. The higher r.m.s.d. for His-39 in WT is a result of fluctuations of the Met-33 side chain. Hydrogen bond analysis suggests that there is a hydrogen bond from sulfur to the backbone HN hydrogen of the Met-39 residue (Table IV and Fig. 4). In the case of asparagine, which has a shorter side chain and smaller radius of oxygen compared with that of sulfur, there is a change in H-bonding pattern around the copper center. Lastly, MD analysis of the P53E mutation shows that Glu-53 yields alterations in the protein backbone of the short α-helix proposed to interact with the Psaa protein of the PSI core (Table III) (13). The WT and other mutants adopt similar conformations in this helical region (Table III).

**TABLE II**

|           | *Spinach* PSI | *Synechocystis* PSI | *Anabaena* PSI |
|-----------|---------------|---------------------|----------------|
| **Plastocyanin** | **k<sub>s</sub> × 10<sup>-5</sup>** |                     |                |
| WT        | 2.0           | 12.0                | 28.0           |
| P14L<sup>a</sup> | 18.0          | 130.0               | 150.0          |
| M33N      | 1.8           | 45.0                | 180.0          |
| M33N/P14L | 4.0           | 170.0               | 58.0           |
| R86Q      | 0.4           | 10.0                | 40.0           |
| R86E      | 1.7           | 4.0                 | 10.0           |
| P53A      | 0.7           | 58.0                | 220.0          |
| P53E      | 1.7           | 51.0                | 220.0          |
| *Synechocystis* Pc | ND<sup>b</sup> | 90.0                | ND             |
| *Anabaena* Pc | ND<sup>b</sup> | ND                 | 700.0          |

<sup>a</sup> Values from Ref. 11.
<sup>b</sup> ND, not determined.

**TABLE III**

|                | WT | P14L | M33N | P53E |
|----------------|----|------|------|------|
| **Values**     |    |      |      |      |
| **All atoms**  | 1.59 | 1.79 | 1.59 | 1.80 |
| **Cytochrome** | 0.21 | 0.15 | 0.15 | 0.16 |
| **His-39**     | 0.17 | 0.12 | 0.17 | 0.14 |
| **His-85**     | 0.15 | 0.22 | 0.15 | 0.20 |
| **Met-90**     | 0.24 | 0.35 | 0.24 | 0.22 |
| **Residues 51–55** | 0.36 | 0.32 | 0.33 | 0.46 |
| **Pc**         |    |      |      |      |
| **Values**     |    |      |      |      |
| **All atoms**  |    |      |      |      |
| **Cytochrome** |    |      |      |      |
| **His-39**     |    |      |      |      |
| **His-85**     |    |      |      |      |
| **Met-90**     |    |      |      |      |
| **Residues 51–55** |    |      |      |      |

**DISCUSSION**

**Mutations in Met-33**—Reverting the exclusive methionine at position 33 in *Prochlorothrix* Pc to the standard asparagine in other Pcs yields enhanced *k<sub>et</sub>* while maintaining unaltered the association constant for (Pc-PSI) complex formation. This effect is qualitatively similar to that previously observed for mutations reverting the unique proline of *Prochlorothrix* Pc to the standard leucine (11), although about two-times higher *k<sub>et</sub>* values are reported here (Table I). Similarly, the increased efficiency of the P14L mutant in electron transfer toward heterospecific PSI (11) is also seen in Met-33 mutants toward cyanobacterial PSI (Table II). Taken together, these results indicate that reverting the peculiar hydrophobic patch of *Prochlorothrix* Pc to the standard configuration promotes significantly increased electron transfer efficiencies, in agreement with the proposed role of this area as providing an adequate hydrophobic surface for the electron transfer step (3).

We have carried out a MD study in order to better explain the greater reactivity of the M33N mutant as compared with WT Pc (Table III). The residue at position 33 is adjacent to the copper-ligand His-39. As a result, there is a slight displacement of His-39 (r.m.s.d. 0.217) in WT compared with M33N (0.147) because of the bigger side chain of methionine. Also, there is a hydrogen bond from S.D. of Met-33 to the backbone N of His-39,
which is absent in Asn-33 because of its small side chain and smaller oxygen radius (Fig. 4). Replacement of methionine by asparagine rearranges the H-bonding network within the protein by formation of novel bonds (Table IV). Thus, the MD studies indicate that when Met-33 is changed to the canonical asparagine, the internal organization (H-bond network) changes around the Cu site. Such changes are not reflected in significantly altered redox potential, so the increases in rates are probably because of decreased donor-acceptor distance, yielding subtle changes in conformation with a better fit to PSI. The similar kinetic behavior of M33N and M33N/P14L mutants can be explained by assuming that the optimization of the PSI-Pc interaction promoted by the M33N mutation cannot be improved by additional mutation of Pro-14.

Mutations in Pro-53—A GRAMM (30) computational simulation suggested an interaction between the Prochlorothrix Pc eastern face and a short β-sheet in the PsaA subunit of PSI. Pro-53 is located in the middle of a short α-helix in Pc (13). In fact, altering Pc at this position abolishes complex formation with PSI in both P53A and P53E mutants, which surprisingly also leads to an increased electron transfer efficiency. Thus, it is clear that Pro-53 is important in the Pc-PSI interaction, acting by fixing the electron transfer complex in a non-optimized configuration. Replacement of this proline group appears to promote the breakdown of specific interactions with PSI, allowing enhanced electron transfer. This seems to be a general feature of Prochlorothrix Pc, because replacement of Pro-53 also promotes increased electron transfer efficiencies toward other cyanobacterial PSIs, although not toward the spinach photosystem. MD analyses reveal that the P53E mutation affects the geometry of the protein backbone within the interacting Pc α-helix (residues 51–55, Table IV), likely yielding a more flexible, less hydrophobic structure that may not interact with the PsaA β-sheet. Breaking this interaction site may result in collisional interactions that minimize donor-acceptor distance and hence improve reactivity.

Mutations in Arg-86—Cyanobacterial Pscs contain just one arginine residue (numbered Arg-88 in spinach) proposed to be crucial in PSI interaction because its substitution makes Anabaena Pc unable to reduce PSI (5, 14). Mutating the equivalent Arg-86 in Prochlorothrix promotes a decrease in $K_e$ but to a lesser extent increases $k_{av}$, the P53E mutant yielding a more pronounced effect. Although ionic strength effects are not observed in both mutants, overall these data suggest a role for electrostatics in helping to establish a docked conformation. The effects are not as pronounced as when such mutations are introduced into Anabaena, showing that in the peculiar Prochlorothrix Pc the role played by Arg-86 is not essential for the interaction with PSI. Furthermore, weakening specific interactions of this arginine with PSI groups by its substitution actually optimizes the electron transfer process. On the other hand, introducing a negative charge at position 86 has a negative effect on the bimolecular rate constants with heterospecific PSI, yielding the lower $k_2$ values of all the Prochlorothrix Pc mutants tested until now (Table II) (11). These data confirm a role for this arginine in helping to establish an efficient Pc-PSI interaction in other cyanobacteria distinct from Prochlorothrix.

Concluding Remarks—The data presented here confirm previous investigations indicating that Prochlorothrix Pc presents an unique electron donating mechanism to PSI. This process is based in the formation of an electron transfer complex mainly stabilized by hydrophobic interactions of PSI with an altered site 1 in Pc. However, the electron transfer complex is not optimized in a productive configuration; mutations in the hydrophobic patch promote increased electron transfer rates without altering Pc-PSI association constants, and mutations in the east face decrease $K_e$ while increasing $k_{av}$. Thus, by reverting the Pc hydrophobic patch to the standard configuration, and/or weakening complex formation, the reactivity of Pc toward its PSI is enhanced.

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