Hydrophobic Patch Impairs Electron Transfer to Photosystem I

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A number of surface residues of plastocyanin from Prochlorothrix hollandica have been modified by site-directed mutagenesis. Changes have been made in amino acids located in the amino-terminal hydrophobic patch of the copper protein, which presents a variant structure as compared with other plastocyanins. The single mutants Y12G, Y12F, Y12W, P14L, and double mutant Y12G/P14L have been produced. Their reactivity toward photosystem I has been analyzed by laser flash absorption spectroscopy. Plots of the observed rate constant with all mutants versus plastocyanin concentration show a saturation profile similar to that with wild-type plastocyanin, thus suggesting the formation of a plastocyanin-photosystem I transient complex. The mutations do not induce relevant changes in the equilibrium constant for complex formation but induce significant variations in the electron transfer rate constant, mainly with the two mutants at proline 14. Additionally, molecular dynamics calculations indicate that mutations at position 14 yield small changes in the geometry of the copper center. The comparative kinetic analysis of the reactivity of plastocyanin mutants toward photosystem I from different organisms (plants and cyanobacteria) reveals that reversion of the unique proline of Prochlorothrix plastocyanin to the conserved leucine of all other plastocyanins at this position enhances the reactivity of the Prochlorothrix protein.

Plastocyanin (Pc) is a small redox protein (molecular mass, ~10.5 kDa) that functions in photosynthesis as a mobile electron carrier between the two membrane-embedded complexes cytochrome b6f and photosystem I (PSI) (1–3). Whereas higher plants produce just Pc, there is a number of intermediate species, both cyanobacteria and eukaryotic algae, that are able to synthesize cytochrome c6 as an alternative redox carrier under copper deficiency (4). The interaction between these two metalloproteins and PSI has been studied by laser-flash absorption spectroscopy in a wide variety of evolutionarily differentiated organisms, including prokaryotic and eukaryotic systems (5, 6). All these comparative kinetic analyses have allowed us to propose different reaction mechanisms for PSI reduction (7, 8).

Recently, a comparative analysis of the interaction of Pc and cytochrome c6 with PSI from the prochlorophyte Prochlorothrix hollandica has been carried out (9). Prochlorophytes represent a deeply branched group of cyanobacteria containing both chlorophyll a and b (10, 11). These studies have shown that Prochlorothrix Pc reacts with PSI according to a two-step reaction mechanism involving complex formation and electron transfer, the complex being mainly hydrophobic in nature. Cytochrome c6, in its turn, follows a three-step reaction mechanism with rearrangement of redox partners within an intermediate electrostatic complex before electron transfer (9). Such a difference in the kinetic mechanisms reflects interesting differences not only in electrostatic charge surface distribution but also in dynamic properties.

The solution structure of Prochlorothrix Pc has been recently solved by NMR spectroscopy (12). Despite the relatively low number of conserved residues shared with other Pcs, the Prochlorothrix molecule has a similar overall folding pattern, including the classical two-sheet β-barrel tertiary structure. Interestingly, Prochlorothrix Pc has an altered hydrophobic patch, a region that is thought to be crucial in Pc interaction with its redox counterparts. Whereas the backbone and loops at the hydrophobic area of Prochlorothrix Pc are as those of other Pc molecules, the presence of two unique residues (Tyr-12 and Pro-14 in Prochlorothrix Pc versus Gly-10 and Leu-12 in all other Pcs) yields a structurally different hydrophobic surface, with Tyr-12 protruding outwards from this patch (12).

In this paper, we are extending our previous studies of Prochlorothrix PSI reduction by wild-type (WT) Pc to analyze the reactivity of Pc mutants at Tyr-12 and Pro-14. The laser-flash absorption spectroscopy analyses herein reported indicate that the replacement of Pro-14 by leucine, which is the "standard" residue in all other Pcs, makes the copper protein react much more efficiently with PSI.

EXPERIMENTAL PROCEDURES

Expression and Reconstitution of Wild-type and Mutant Plastocyanine—Mutant and WT P. Hollandica Pc was expressed as inclusion bodies in Escherichia coli BL21(DE3) pLysS (Novagen, Madison, WI) as previously described (13). Specifically, the Prochlorothrix Pc expression plasmid, pVAPC10, was used as a template for polymerase chain reaction-mediated mutagenesis (Stratagene QuikChange kit, La Jolla, CA). Custom mutagenic PCR primers were obtained from Life Technologies.
characterization of reconstituted plastocyanin—refolded Pc preparations were analyzed by absorption spectroscopy as described previously (13). Redox titration of Pc preparations was monitored by absorption spectroscopy at 602 nm in increasing ratios of ferrocyanide/ferricyanide as previously described (14). Alternatively, redox potentials were determined by cyclic voltammetry (15).

Preparation of Photosystem I Particles—PSI particles from Prochlorothrix were obtained by β-dodecyl maltoside solubilization as described by Rögner et al. (16) and modified by Hervás et al. (17). The following modification was indeed made. The preparation of PSI-enriched particles obtained after the discontinuous sucrose gradient was washed with buffer D (20 mM MES, pH 6.5, 10 mM CaCl₂, 10 mM MgCl₂, 0.5 mM N-mannitol, 20% glycerol) to remove sucrose and then diluted 50% with buffer A (20 mM MES, pH 6.5, 10 mM CaCl₂, 10 mM MgCl₂) plus 0.25 mM N-mannitol before being applied to a second continuous sucrose gradient (10–25%). The lower, darker, green band containing PSI was washed and concentrated as previously described (17). The P700 content in PSI samples was calculated from the photoinduced absorbance changes at 820 nm using the absorption coefficient of 6.5 mM⁻¹ cm⁻¹ determined by Mathis and Sétif (18). Chlorophyll concentration was determined according to Arnon (19). The chlorophyll/P700 ratio of the resulting PSI preparations was 150/1. Spinach, Synechocystis and Arabaena PSI were purified as previously described (7).

Laser-flash Absorption Spectroscopy—Kinetics of flash-induced absorbance changes in PSI were followed at 820 nm as described in Hervás et al. (7). 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% β-dodecyl maltoside, 10 mM MgCl₂, an amount of PSI-enriched particles equivalent to 0.35 mg of chlorophyll ml⁻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 (kobs) was less than 10%, based on reproducibility and signal-to-noise ratios.

Kinetic Analyses—Data collection was as previously described (7). Oscilloscope traces were treated as sums of several exponential components. Kinetic analyses were performed using the Marquardt method with the software devised by P. Sétif (Saclay, France). Kinetic analyses were carried out according to the reaction mechanisms previously proposed (7).

Molecular Dynamics Studies—The structures of WT and P14L mutant Prochlorothrix Pcs were analyzed using the SWISS-PDB Viewer. Charges of most atoms were taken from the AMBER 6.0 force field. Charges and other force field parameters for the copper atom and its ligands were obtained from NMR structural data and compared with similar Pc structures in the literature (20, 21). Topology and parameter files for both proteins were generated using the xLeap module of AMBER. MD data were obtained by sampling every 100 fs, yielding 10,000 conformations.

RESULTS AND DISCUSSION

The two variant residues at the hydrophobic patch of Prochlorothrix, namely Tyr-12 and Pro-14 (Fig. 1), were chosen to be mutated to investigate their role in the reactivity of the copper protein toward PSI. By studying the properties of this natural variant of Pc, the minimum structural requirements for complex formation and efficient electron transfer to PSI could be investigated. Tyrosine at position 12 was thus replaced with two different aromatic residues, phenylalanine and tryptophan, as well as with glycine, the latter being a conserved residue at this position in virtually all known sequences of Pc. Additionally, proline at position 14 was replaced with leucine, which is also a conserved residue in all other Pcs. A double mutant with reversion of both tyrosine and proline to the standard residues glycine and leucine, respectively, was likewise constructed.

Description of the Mutant Plastocyanins—Most of the mutations do not significantly alter the midpoint redox potential value of the copper protein, with the exception of the P14L mutant and the Y12G/P14L double mutant, whose redox potentials are ~15 mV lower (Table I). Thus, only those mutations affecting position 14 yield measurable changes in the environment of the copper center, and such changes in redox potential slightly increase the driving force for electron transfer to PSI. Nonetheless, such modifications do not significantly alter the electronic absorption spectra, as all mutant Pcs yield an absorption peak at 602 nm identical to the WT (13).

Electron Transfer Kinetics to Prochlorothrix Photosystem I—The laser-flash-induced kinetic traces of PSI reduction by WT and mutated Pcs are monoexponential, even at high donor protein concentration (not shown). The dependence of the observed pseudo first-order rate constant (kobs) upon donor protein concentration shows a saturation profile (Fig. 2). This finding suggests the formation of a bimolecular transient Pc-PSI complex before electron transfer, as previously described for the WT system (9) according to the following minimal two-step reaction mechanism.

\[
Pc_{red} + PSI_{ox} \rightarrow [Pc_{red} \cdot PSI_{ox}] \rightarrow Pc_{ox} + PSI_{red}
\]  

where \( K_A \) stands for the equilibrium constant of complex formation, and \( k_{eq} \), which can be experimentally inferred from the limiting \( k_{eq} \) at infinite Pc concentration, denotes the subsequent intracomplex electron transfer first-order rate constant. Table I shows the values for \( K_A \) and \( k_{eq} \) with WT and mutant Pcs at pH 7.5 as calculated from experimental data in
Proline Impairs Electron Transfer in Plastocyanin

TABLE I
Midpoint redox potentials ($E_m$) of wild-type and mutant plastocyanins as well as association rate constants ($K_A$) and electron transfer rate constants ($k_{et}$) for photosystem I reduction by the different copper proteins

| Protein | $E_m$ (mV) | $K_A$ ($M^{-1}$) | $K_A$ (+0.1 M NaCl) ($M^{-1}$) | $k_{et}$ ($s^{-1}$) | $k_{et}$ (+0.1 M NaCl) ($s^{-1}$) |
|---------|------------|------------------|--------------------------------|---------------------|----------------------------------|
| WT      | 370        | $1.4 \times 10^9$ | $1.6 \times 10^4$              | 1390                | 1450                             |
| Y12G    | 367        | $2.7 \times 10^9$ | $2.5 \times 10^4$              | 1000                | 1150                             |
| Y12F    | 358        | $1.5 \times 10^9$ | $2.0 \times 10^4$              | 1500                | 1200                             |
| Y12W    | ND*        | $2.8 \times 10^9$ | $2.0 \times 10^4$              | 1160                | 1300                             |
| P14L    | 355        | $0.9 \times 10^9$ | $0.6 \times 10^4$              | 3900                | 4050                             |
| Y12G/P14L | 356     | $0.6 \times 10^9$ | $0.8 \times 10^4$              | 2600                | 2400                             |

* ND, not determined.

Fig. 2. Dependence of the observed rate constant ($k_{obs}$) for Prochlorothrix photosystem I reduction by wild-type and mutated plastocyanins upon donor protein concentration. The continuous line corresponds to the theoretical fitting to the two-step formalism described in Meyer et al. (22). Other conditions were as described under “Experimental Procedures.”

Fig. 3. Comparison of the structures of the wild-type and P14L mutant hydrophobic patches. Structures were modeled from averaging the MD simulation described under “Experimental Procedures.” The positions of Pro-14, Leu-14, and His-85 are indicated by arrows.

Concerning the electron transfer step, Table I shows that none of the mutations at position 12 significantly alters the efficiency of Pc in donating electrons to PSI, with the exception of the replacement of tyrosine by glycine, which induces a decrease of about 30% in $k_{et}$ with the exception of individual mutations. In fact, this mutant yields a $k_{et}$ value that doubles that with WT and is 67% that with the single mutant P14L, a decrease that compares fairly well with that observed with the single mutant Y12G with respect to WT (see above).

Electron Transfer Kinetics to Heterospecific Photosystem I—It has been previously reported that Prochlorothrix WT Pc does not form any electron transfer complex with PSI from either spinach or the cyanobacteria Anabaena and Synechocystis.
The copper protein does exhibit a very low reactivity in all cross-reactions (9). Because the mutants constructed in this study are aimed to revert the “exclusive” hydrophobic patch of Prochlorothrix to the standard configuration, we have also checked the reactivity of mutants toward heterospecific PSI. In all cases, linear dependences were observed when plotting the observed rate constants versus protein concentration, as shown in Fig. 5 for Synechocystis PSI. Because no complex formation was observed, the bimolecular rate constants ($k_2$) for PSI reduction were calculated (Table III). Replacement of Pro-14 with leucine makes the bimolecular rate constant of PSI reduction by Prochlorothrix wild-type and mutant plastocyanins.

Relative small increases in reactivity are also observed with the mutant Y12W. These results with mutants at position 12 are in clear contrast with the previously proposed requirement for a flat surface in the area of Gly-12 at the hydrophobic patch of Pc to ensure efficient electron transfer to PSI (24). In case of cross-reactions with Synechocystis PSI, the mutant P14L was shown to be even more reactive than the Synechocystis WT Pc (Fig. 5 and Table III). Overall, these observations provide some agreement with the suggestion made by Sigfrids-son and co-workers (25), who demonstrated that replacement of leucine with alanine decreased $k_2$ despite a modest increase in the driving force (0.024 eV) for electron transfer to P700. These authors suggested that a bulky hydrophobic residue might yield a better fit to PSI (25). The fact that WT Pc from Synechocystis 6803 PSI and some of the mutant plastocyanins possess a residue that is impairing its redox interaction with its physiological electron acceptor may indicate that this organism is using a divergent protein that appeared before evolution selected for leucine at position 14. We can thus say that reversion of Pro-14 to the standard glycine does not provide some agreement with the suggestion made by Sigfrids-son and co-workers (25), who demonstrated that replacement of leucine in higher plant Pc with the less bulky alanine decreased $k_2$ despite a modest increase in the driving force (0.024 eV) for electron transfer to P700. These authors suggested that a bulky hydrophobic residue might yield a better fit to PSI (25).

**Summary of the structural statistics for selected copper center and hydrophobic patch residues of the wild type and P14L mutant plastocyanins**

| Residue | Average r.m.s.d. | Deviation | Average r.m.s.d. | Deviation |
|---------|-----------------|-----------|-----------------|-----------|
| Tyr-12  | 0.477           | 0.177     | 0.690           | 0.140     |
| Met-33  | 0.827           | 0.060     | 0.479           | 0.083     |
| Val-36  | 0.247           | 0.081     | 0.362           | 0.082     |
| Pro-38  | 0.201           | 0.116     | 0.15            | 0.058     |
| His-39  | 0.295           | 0.056     | 0.261           | 0.045     |
| Cys-82  | 0.273           | 0.071     | 0.485           | 0.071     |
| His-85  | 0.206           | 0.050     | 0.211           | 0.044     |
| Ala-88  | 0.293           | 0.132     | 0.419           | 0.036     |
| Met-90  | 0.347           | 0.024     | 0.195           | 0.039     |

**TABLE III**

| PSI/protein | $k_2$ (μM)$^{-1}$ s$^{-1}$ |
|-------------|---------------------------|
| Spinach PSI |                           |
| WT          | $2.0 \times 10^6$         |
| P14L        | $1.8 \times 10^6$         |
| Y12G/P14L   | $6.5 \times 10^6$         |
| Y12W        | $3.6 \times 10^6$         |
| Y12G        | $2.7 \times 10^6$         |
| Y12F        | $2.2 \times 10^6$         |
| Spinach Pc  |                           |
|             |                           |
| Synechocystis 6803 PSI |     |
| WT          | $1.2 \times 10^3$         |
| P14L        | $1.3 \times 10^3$         |
| Y12G/P14L   | $3.0 \times 10^5$         |
| Y12W        | $2.8 \times 10^5$         |
| Y12G        | $2.2 \times 10^5$         |
| Y12F        | $1.7 \times 10^6$         |
| Synechocystis Pc |   |
| Anabaena 7119 PSI |   |
| WT          | $2.8 \times 10^6$         |
| P14L        | $1.5 \times 10^5$         |
| Y12G/P14L   | $3.3 \times 10^6$         |
| Y12W        | $5.7 \times 10^6$         |
| Y12G        | $1.8 \times 10^6$         |
| Y12F        | $3.6 \times 10^6$         |
| Anabaena Pc | $7.0 \times 10^5$         |

$^a$ $k_2$ could not be calculated because spinach plastocyanin follows a three-step reaction mechanism (7).
enhanced reactivity observed with the P14L mutant should be addressed. Given the fact that the driving force in the mutant increased by 0.015 eV, $k_{cat}$ would be expected to increase slightly (~30%) (26) via this parameter alone. However, the large (10-fold) increases in $k_2$ seen in reactions with spinach and *Synechocystis* PSI suggest that a decrease in distance to the acceptor in the mutant plays an important role in enhancing electron transport.

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