Site-directed Mutagenesis of Cytochrome c₆ from *Anabaena* Species PCC 7119

IDENTIFICATION OF SURFACE RESIDUES OF THE HEMEPROTEIN INVOLVED IN PHOTOSYSTEM I REDUCTION

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A number of surface residues of cytochrome c₆ from the cyanobacterium *Anabaena* sp. PCC 7119 have been modified by site-directed mutagenesis. Changes were made in six amino acids, two near the heme group (Val-25 and Lys-29) and four in the positively charged patch (Lys-62, Arg-64, Lys-66, and Asp-72). The reactivity of mutants toward the membrane-anchored complex photosystem I was analyzed by laser flash absorption spectroscopy. The experimental results indicate that cytochrome c₆ possesses two areas involved in the redox interaction with photosystem I: 1) a positively charged patch that may drive its electrostatic attractive movement toward photosystem I to form a transient complex and 2) a hydrophobic region at the edge of the heme pocket that may provide the contact surface for the transfer of electrons to P₇₀₀₀. The isofunctionality of these two areas with those found in plastocyanin (which acts as an alternative electron carrier playing the same role as cytochrome c₆) are evident.

In cyanobacteria and green algae, cytochrome (Cyt)c₆ acts as a soluble redox carrier that can replace plastocyanin in the transport of electrons between the two membrane-embedded complexes Cyt b₅₆₅ and photosystem I (PSI) (see Refs. 1 and 2 for reviews). In higher plants, however, the copper protein is the only electron carrier. The cyanobacterium *Anabaena*, like some other organisms, is able to synthesize either Cyt c₆ or plastocyanin (which both exhibit a basic isoelectric point of ~9) as a function of copper concentration in the growing medium (3).

The structures and functions of these two metalloproteins have been extensively studied in a wide range of organisms (4–11). According to our laser flash-induced kinetic studies, the reaction mechanism of PSI reduction follows three different models: type I, which involves an oriented collision between the two redox partners; type II, which proceeds through the formation of a transient complex prior to electron transfer; and type III, which requires an additional rearrangement step so as to make the redox centers orientate properly within the complex (9). During the evolution of photosynthetic organisms, interaction between a positively charged Cyt c₆ and PSI was first optimized (as is the case with *Anabaena* Cyt c₆, which follows the type III mechanism) and only later in evolution was a more complex kinetic mechanism developed with plastocyanin (2).

The three-dimensional structures of Cyt c₆ from the two eukaryotic green algae *Monoraphidium* (12) and *Chlamydomonas* (13) and from the cyanobacterium *Synechococcus* (14) have been solved. The analysis of the Cyt c₆ molecule compared with the plastocyanin structure allowed us to identify a hydrophobic region around the solvent-exposed heme propionates that resembles the north pole of plastocyanin as well as a negatively charged patch in eukaryotic Cyt c₆ similar to the east face of eukaryotic plastocyanin (12). In *Anabaena*, neither Cyt c₆ nor plastocyanin exhibits the acidic patches at their east face, which is rather positively charged (2).

Extensive mutagenesis studies of plastocyanin have supplied relevant information on the role of specific residues located both in its north and east faces (5, 8, 15–17). Recently, we performed a site-directed mutagenesis analysis of *Synechocystis* Cyt c₆ (18). Aspartates 70 and 72 appear to be located in a negatively charged region of Cyt c₆ that may be isofunctional with the well known “south-east” acidic patch of plastocyanin. In addition, Phe-64 (which is close to the heme group and could be the counterpart of Tyr-83 in plastocyanin (19)) does not appear to be involved in the electron transfer to PSI. In contrast, Arg-67, which is located at the edge of the Cyt c₆ acidic area, seems to be crucial.

This paper reports the kinetic and thermodynamic characterization of PSI reduction by a set of site-directed mutants of *Anabaena* Cyt c₆. The results demonstrate that a single mutation of specific residues in the hydrophobic or positively charged area of Cyt c₆ can promote drastic changes in the reaction mechanism. The two functional areas of Cyt c₆ involved in PSI photoreduction have been identified.

**EXPERIMENTAL PROCEDURES**

**Purification of Native Cytochrome c₆**—The hemoprotein from *Anabaena* sp. PCC 7119 was purified as described previously (11), but with slight modifications. Cyt c₆ samples were applied to a CM-cellulose column after oxidation with potassium ferricyanide. Elution of the Cyt c₆ concentration was determined spectrophoto-

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* The abbreviations used are: Cyt, cytochrome; PSI, photosystem I; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; WT, wild-type; kₛₒₚ, bimolecular rate constant for the overall reaction; kₛₒₚ, diffusion-limited rate constant.

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Using oligonucleotides of 32 base pairs, 15 ng of DNA templates, and 12 min of extension time, the previously described expression construction for the petD gene from Anabaena (21) was used as a template. The DNA sequencing service Medigenomix carried out the nucleotide sequence analysis. Other molecular biology protocols were standard (22).

Production of Recombinant Proteins and Purification Procedures—Escherichia coli MC1061-transformed cells were grown in M9 medium supplemented with 1 g/liter Tryptone, 6 mg/liter Fe(III) ammonium citrate, and 100 μg/ml ampicillin. Cells from 10-liter microaerobic cultures were collected, and the periplasmic fraction was extracted according to the method of Hoshino and Kageyama (23) as modified by Eftekhar and Schiller (24). The resulting suspension was extensively dialyzed against 2 mM potassium phosphate, pH 7.0. From this point, the purification procedure was that for native Cyt c₆ (see above), except for mutants K66E and D72K, which were eluted with buffer gradients ranging from 1 to 10 mM and from 2 to 120 mM, respectively. In all cases, 50 mM potassium ferricyanide was added to the gradient solutions to keep Cyt c₆ oxidized. Protein concentration was determined as described previously (21).

Redox Potentials—The redox potential value for the heme group in each Cyt c₆ mutant was determined as reported previously (21, 25), for which the differential absorbance changes at 553 minus 570 nm were followed. Errors in the experimental determinations were less than ±0.5 mV.

Preparation of PSI Particles—PSI particles were isolated from Anabaena cells by β-dodecyl maltoside solubilization (26, 27). The chlorophyll/Prap ratio of the resulting PSI preparations was 140:1. The Prap 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 (28). Chlorophyll concentration was determined according to Arnon (29).

Laser Flash Absorption Spectroscopy—The kinetics of flash-induced absorbance changes in PSI were followed at 820 nm as described previously (9, 18). Experimental conditions and the standard reaction mixture were also as reported previously (17); the buffer used throughout this work was 20 mM Tricine/KOH, pH 7.5. Unless otherwise indicated, low and high ionic strengths refer to the absence and addition of 10 mM MgCl₂, respectively. Data collection and kinetic and thermodynamic analyses were carried out as reported by Hervás et al. (9, 10). Apparent thermodynamic parameters were estimated as described Díaz et al. (6) by fitting the experimental data to the Watkins equation (30). The values for the rate constant for electron transfer and Kₛ (see below) were determined according to the formalism by Meyer et al. (31).

Structure Simulation—The structures of WT and mutant Cyt c₆ were modeled using the SYBYL program (Tripos Associates) in an SGI RC10000 workstation. The three-dimensional crystal structure of Cyt c₆ from the green alga Monoraphidium braunii (12) was used as a template. Sequence alignment and subsequent amino acid substitution were performed with the BIOPOLYMER module of SYBYL Version 6.4. Force field parameters for the heme moiety were those in the AMBER package (32). The resulting file was first submitted to energy minimization in vacuo up to a root mean square energy gradient of 0.41 kJ mol⁻¹ Å⁻¹ using the SANDER module of AMBER Version 4.1 (33). During these calculations, the backbone heavy atoms of α-helix regions were restrained at their position by a harmonic force of 62.7 kJ mol⁻¹ Å⁻¹. Then, the whole system was solvated with three-point water molecules using the BLOB option of the EDIT module. Solvent was energy-minimized and submitted to a 9-ps molecular dynamics calculation. The whole system was again energy-minimized and submitted to a 1250-ps molecular dynamics run at 300 K. A total of 10 samples from the last 400 ps of trajectory were quenched by freezing the system in six steps of 1.5 ps. The qualities of the resulting structures were tested using the PROCHECK program (34). Surface electrostatic potentials were estimated using the algorithm of Nicholls and Honig (35), as indicated in the MOLMOL program (36).

RESULTS

To analyze the role of specific residues of Anabaena Cyt c₆ in the reaction mechanism of PSI reduction, six amino acids (two near the heme group and four in the positively charged end face) were chosen for mutations (Fig. 1). At the edge of the heme crevice, modifications were made at residues 25 and 29: Val-25, which is located near the heme β-meso-position, was substituted by alanine and glutamate; and Lys-29, which exhibits its side chain lying close to propionate 7, was replaced with histidine. Val-25 is located in the middle of the hydrophobic patch, but Lys-29 is not. However, mutation of the latter to histidine was considered to be interesting because the residue at position 29 is lysine in all Cyt c₆ with the exception of that from Monoraphidium, in which it is histidine (actually, the EPR spectra of Monoraphidium Cyt c₆ suggested an unusual histidine-histidine axial coordination for the heme iron, a ligand system that is not possible in the rest of Cyt c₆ with just one histidine residue (37)). In addition, basic residues at positions 28–30 in type I cytochromes have been proposed to con-
trol the redox potential of the heme group through stabilization of propionate 7 (38). On the east face, modifications included the replacement of Lys-62 and Lys-66 by glutamates; Asp-72, which is also located in the middle of the east patch, was changed to lysine. Finally, Arg-64, which is at the edge of the east patch and has been shown to be crucial in *Synechocystis* Cyt *c* 6 (18), was replaced with glutamate.

The EPR and electronic absorption spectra of *Anabaena* Cyt *c* 6 were not changed by the mutations (data not shown), but its midpoint redox potential (\( E_{m}^{c} \)) could be significantly affected (Table I). The \( E_{m}^{c} \) value was unchanged when the residue mutated was at the east face, including Arg-64, but it was −50 mV lower when the mutations were located near the heme group. Only minor differences were observed in the \(^1\)H NMR spectra and nuclear Overhauser effect intensities of heme resonances with protons from other residues.²

The kinetics of PSI reduction by WT Cyt *c* 6 are biphasic, but those with the mutants (with the exception of D72K) are monoeXponential, lacking the fast phase typically observed with the WT species. As shown in Fig. 2, the kinetics corresponding to D72K and WT Cyt *c* 6 exhibited a sharp initial fast phase (with a rate constant that was independent of donor protein concentration) followed by a slower decay. In contrast, the oscilloscope traces with mutants V25A and V25E fit to single exponential curves.

Fig. 3 shows that the observed pseudo first-order rate constant (\( k_{obs} \)) of PSI reduction by any mutant (with the exception of D72K) varied linearly with Cyt *c* 6 concentration. This can be interpreted by assuming that there is no formation of any stable complex between PSI and Cyt *c* 6, and the reaction thus follows a collisional kinetic mechanism (type I). With mutant D72K, however, the protein concentration dependence of \( k_{obs} \) exhibited a saturation profile, which indicates the formation of a bimolecular Cyt *c* 6:PSI complex prior to electron transfer. The extrapolated rate constant at infinite D72K concentration is similar to its electron transfer rate constant, which is obtained directly from the fast kinetic phase, thus suggesting a type II mechanism.

Such a saturation profile with D72K was even more evident at lower ionic strengths, which increase attractive electrostatic interactions. As shown in Fig. 4, the D72K mutant showed efficient complex formation, with an equilibrium constant (\( K_{a} \)) of 1.06 × 10⁵ M⁻¹ at low ionic strength. Extrapolation of the observed rate constant to infinite D72K concentration yielded a value that approached one-half the experimental electron transfer rate constant, which indicates that this mutant may follow a type III mechanism at low ionic strength. This finding also suggests that the rearrangement step (see above) is not limiting at high ionic strength.

Fig. 4 also shows that the V25A mutant was likewise able to form a transient complex with PSI at low ionic strength, even though its kinetic profiles of PSI reduction were monophasic at any ionic strength. The \( K_{a} \) value for complex formation between V25A and PSI is 1.07 × 10⁴ M⁻¹, which is 10 times lower than that with D72K. These data indicate that V25A follows a type II mechanism in the absence of MgCl₂ and a type I mechanism when 10 mM MgCl₂ is added.

The bimolecular rate constant for the overall reaction (\( k_{bim} \)) of PSI reduction, which can be calculated from the linear plots in Fig. 3, is smaller with any mutant than with WT Cyt *c* 6 (Table I). In the hydrophobic patch, replacement of Val-25 with alanine or glutamate promoted a decrease in \( k_{bim} \) of −2 and 60

### Table I

| Cytochrome *c* 6 | \( E_{m}^{c} \) pH 7.5 | \( k_{lim} \) | \( k_{lim} \) s⁻¹ | \( k_{lim} \) s⁻¹ | FP | Type of mechanism |
|------------------|----------------|-------------|----------------|----------------|----|-----------------|
| WT               | 337            | 11.3 × 10⁷  | 11.4           | 1.7            | 35 | III             |
| V25A             | 286            | 6.9 × 10⁷   | 6.4            | I              |
| V25E             | 287            | 0.2 × 10⁷   | 0.2            | I              |
| K29H             | 272            | 6.2 × 10⁷   | 11.0           | I              |
| K62E             | 340            | 4.9 × 10⁷   | 9.4            | I              |
| R64E             | 320            | 2.2 × 10⁷   | 2.6            | I              |
| K66E             | 335            | 5.7 × 10⁷   | 11.7           | I              |
| D72K             | 338            | 1.5 × 10⁻⁵  | 11.2           | 1.5            | 30 | II              |

¹ \( k_{obs} \), electron transfer rate constant; FP, fast phase.
² This value stands for the rate constant of association between D72K and PSI.

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² A. Díaz-Quintana, M. Hervás, J. A. Navarro, and M. A. De la Rosa, unpublished data.
times, respectively, whereas mutation of Lys-29 to histidine involved a parallel decrease of ~50% as compared with WT Cyt c₆. Substitution of Arg-64, in its turn, by glutamate induced the rate constant to decrease by a factor of 5. In the east face, replacement of Lys-62 and Lys-66 with glutamate made the kₙₐ₅ value decrease to half that of WT Cyt c₆. In the case of D72K, it should be noted that the bimolecular rate constant in Table I refers to its association constant, which is kinetically different from the other kₙₐ₅ values in the table. Its electron transfer rate constant, calculated directly from the fast phase, compares well with that of WT Cyt c₆, as does the maximum percentage of fast phase (Table I).

Taking into account the electrostatic nature of the interaction of Cyt c₆ with PSI, a detailed analysis of the effect of ionic strength on kₙₐ₅ was performed. Fig. 5 shows that the kₙₐ₅ values with WT Cyt c₆ monotonically diminished with increasing NaCl concentration, thereby indicating the existence of attractive electrostatic interactions between the reaction partners as described previously (10, 11). A similar effect of ionic strength on kₙₐ₅ was observed with all mutants, but some differences could be found among them. Actually, the kₙₐ₅ values with mutants V25A and D72K are significantly higher than that with WT Cyt c₆ at low ionic strength, but they decreased drastically upon small additions of NaCl. Mutant K29H showed an ionic strength dependence similar to that of WT Cyt c₆, although its electron transfer efficiency was lower in the whole range analyzed. The kₙₐ₅ values with all the other mutants are lower than that with WT Cyt c₆, indicating that the changes in net electrostatic charge alter the attractive interactions between PSI and mutant proteins.

Using the Watkins equation (30), the bimolecular rate constant extrapolated to infinite ionic strength (kₙₐ₅) (which facilitates the analysis of the intrinsic reactivity of redox partners in the absence of electrostatic interactions) can be calculated from the experimental data. As shown in Table I, the kₙₐ₅ values with Cyt c₆ mutated at the positively charged patch are very similar to that with WT Cyt c₆, a fact that can be explained by assuming that the changes in reactivity induced by these mutations are mainly due to electrostatic, and not structural, effects. The only exception is R64E, for which the kₙₐ₅ value is 4–5 times lower than that with WT Cyt c₆, a finding suggesting that the change in electrostatic charge is not the only factor affecting its reactivity toward PSI, as reported previously for *Synechocystis* Cyt c₆ (18). The effect of the K29H mutation seems to be merely electrostatic in nature, as kₙₐ₅ is similar to that with WT Cyt c₆. The two mutations at position 25 involve modifications that make kₙₐ₅ lower (the V25E mutant, in particular, exhibits a kₙₐ₅ value that is 70 times lower than that with WT Cyt c₆).

To gain further insights into the nature of the interactions between PSI and Cyt c₆, a thermodynamic analysis of PSI reduction by the Cyt c₆ mutants was performed. In all cases, the temperature dependence of the observed rate constant (kₙₐ₅) yielded linear Eyring plots with no breakpoints, from which the values for the apparent activation enthalpy (ΔH°), entropy (ΔS°) and free energy (ΔG°) of the overall reaction could be calculated. For comparative purposes, Table II shows the differences in such activation parameters between WT Cyt c₆ and every mutant. The greatest difference was observed with V25E, whose free energy change is 9.41 kJ mol⁻¹ higher than that of WT Cyt c₆, as expected from its inefficient interaction with PSI. This difference is due mainly to a decrease in the entropic term by −28 J mol⁻¹ K⁻¹. Also interesting is mutant R64E, whose free energy change is 4.15 kJ mol⁻¹ higher than that of WT Cyt c₆, a fact that is due to changes in both the enthalpic and entropic terms. Mutant V25A behaved differently than any other mutant; in fact, the free energy term of its reaction is similar to that of WT Cyt c₆, despite the fact that both entropy and enthalpy show dramatic changes as compared with the thermodynamic parameters of WT Cyt c₆.

To check whether the differences in ΔG° between WT and mutant Cyt c₆ (ΔΔG°) were due to electrostatic interactions, the experimental data were fitted to the Watkins equation (30). As shown in Fig. 6, the ΔΔG° values with D72K and V25A perfectly fit the Watkins equation, and those of K29H, K62E, and K66E roughly fit it. Mutants V25E and R64E showed a linear NaCl dependence of ΔΔG° at high ionic strength that caused the data to deviate from the Watkins equation.

**DISCUSSION**

Up to now, the only site-directed mutational analysis of any Cyt c₆ was recently reported by De la Cerda *et al.* (18) in the cyanobacterium *Synechocystis*. This hemeprotein, which is almost neutral, reacts with PSI according to a simple collisional model (type I). On the contrary, *Anabaena* Cyt c₆ is a positively charged protein that exhibits strong electrostatic attractions toward PSI (9, 11) and that reacts with the photosystem following a more complex three-step mechanism (type III). The
goal of this study was to elucidate the role played by some specific amino acids of Anabaena Cyt c₆ in such an attractive interaction with PSI and to investigate the possible involvement of its hydrophobic north area in the type III reaction mechanism.

| Cytochrome c₆ | ΔDG° | ΔDH° | ΔDS° |
|---------------|------|------|------|
|               | kJ mol⁻¹ | kJ mol⁻¹ | J mol⁻¹ K⁻¹ |
| V25A          | 1.11  | -9.14 | -34.57 |
| V25E          | 9.41  | 1.08  | -27.93 |
| K29H          | 1.48  | 2.45  | 3.26  |
| K62E          | 2.10  | 2.16  | 0.21  |
| R64E          | 4.15  | -1.72 | -19.69 |
| K66E          | 1.69  | 2.04  | 1.20  |
| D72K          | 1.33  |       |       |

*This value stands for ΔDG° at infinite ionic strength.*

Mutations of Val-25 indicate that this residue may contribute to the specific topology of the north hydrophobic area of Cyt c₆ in its interaction with PSI. Similar conclusions were inferred from mutants at the north pole of eukaryotic plastocyanin (5, 8), in which the fast phase of electron transfer to PSI cannot be detected. This suggests that the mutant proteins (both Cyt c₆ and plastocyanin) are unable to reach the optimal orientation required for their redox centers to transfer electrons to PSI. It is interesting to compare the drastic effect induced by mutation of Val-25 to glutamate with mutation to alanine; the severe kinetic phenotype of V25E can be attributed to the presence of a negative charge in the middle of a normally hydrophobic region.

The K29H mutant possesses a redox potential value that is 65 mV more negative than that of WT Cyt c₆. This is probably due to the proximity of Lys-29 to heme propionate 7, in agreement with previous reports (38). Even though the driving force of electron transfer from K29H to PSI is higher, the kinetic profile of PSI reduction by the mutant loses the first fast phase, and its kₖᵢₜ value is about half that of WT Cyt c₆. This can be ascribed in part to electrostatic effects, as the dependence of ΔDG° on ionic strength fits the Watkins equation (30), and the value for kᵢₜ compares well with that of WT Cyt c₆. The change in redox potential induced by the mutation clearly does not affect reactivity with PSI, suggesting that complex formation is the rate-limiting step of the overall reaction.

Mutations of Lys-62 and Lys-66 at the positively charged patch of Cyt c₆ demonstrate that this region is responsible for the attractive electrostatic interactions with PSI. In Anabaena, the PsaF subunit does not seem to be directly involved in the interaction of PSI with its donor proteins, as is the case in Synechocystis (16, 39). Hence, the positive charges of Anabaena Cyt c₆ may interact with certain negatively charged areas in the PsaAPsaB heterodimer of PSI to form a transient electrostatic complex. The surface electrostatic potential of WT and mutant Cyt c₆ was then calculated. Fig. 7 shows that the WT molecule has an extensive area of positive potential at its west face, i.e. close to the hydrophobic patch at the edge of the heme.
cleft. Mutants K66E, K62E, and R64E present a reduction in charge of the positive patch, although the change in the orientation of the dipole moment is $<10^\circ$. This is consistent with the kinetic behavior exhibited by these three mutants, in which the ionic strength dependence of the interaction with PSI is not so much evident.

It is noteworthy that the effect of such mutations on the reaction rates can be closely correlated to changes in the positive electrostatic potential at the edge of the hydrophobic area. In fact, replacement of Arg-64 (which is located in the basic patch, adjacent to the hydrophobic region) by an acidic residue promotes a drastic reduction in size of the positive patch. Mutation of Lys-66 to glutamate likewise induces a diminution of the basic patch, but it does not alter the electrostatic surface potential at the edge of the hydrophobic area.

Mutant D72K is of special relevance as it clearly supports our proposal that the positively charged patch of Cyt $c_6$ is responsible for the interaction with PSI. Replacement of Asp-72 by a positive residue like lysine makes the positive potential region spread out (Fig. 7), thus favoring the attractive interactions between the donor proteins toward PSI. The kinetic behavior of V25A suggests that the solvent molecules are tuning the reactivity of the interaction interphase with PSI allowing electrons to go transient complex with the photosystem; and (ii) a hydrophobic area, responsible for the electrostatic interactions forming the non-covalent interaction of the contact interphase with PSI allowing electrons to go from the heme pocket, responsible for the formation of the contact interphase with PSI allowing electrons to go from the heme iron to $P_{700}^\pm$. The main difference with respect to Synechnocystis Cyt $c_6$ (18) is found at the electrostatically charged patch, which explains the differences in their respective reaction mechanisms of PSI photoreduction (10).

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