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*Articles*

# Role of Electrostatics in the Interaction between Cytochrome *f* and Plastocyanin of the Cyanobacterium *Phormidium laminosum*†

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ABSTRACT: The role of charged residues on the surface of plastocyanin from the cyanobacterium *Phormidium laminosum* in the reaction with soluble cytochrome *f* in vitro was studied using site-directed mutagenesis. The charge on each of five residues on the eastern face of plastocyanin was neutralized and/or inverted, and the effect of the mutation on midpoint potentials was determined. The dependence of the overall rate constant of reaction, $k_2$, on ionic strength was investigated using stopped-flow spectrophotometry. Removing negative charges (D44A or D45A) accelerated the reaction and increased the dependence on ionic strength, whereas removing positive charges slowed it down. Two mutations (K46A, K53A) each almost completely abolished any influence of ionic strength on $k_2$, and three mutations (R93A, R93Q, R93E) each converted electrostatic attraction into repulsion. At low ionic strength, wild type and all mutants showed an inhibition which might be due to changes in the interaction radius as a consequence of ionic strength dependence of the Debye length or to effects on the rate constant of electron transfer, $k_{et}$. The study shows that the electrostatics of the interaction between plastocyanin and cytochrome *f* of *P. laminosum* in vitro are not optimized for $k_2$. Whereas electrostatics are the major contributor to $k_2$ in plants [Kannt, A., et al. (1996) *Biochim. Biophys. Acta 1277*, 115−126], this role is taken by nonpolar interactions in the cyanobacterium, leading to a remarkably high rate at infinite ionic strength ($3.2 \times 10^7$ $M^{-1}$ $s^{-1}$).

Plastocyanin (Pc)[1] and cytochrome *f* are two redox proteins in the electron-transfer chain of oxygenic photosynthesis. Cytochrome *f*, an integral part of the cytochrome *bf* complex, receives electrons from the Rieske FeS protein in the cytochrome *bf* complex. It passes them onto the soluble, lumenal electron carrier Pc, which in turn reduces $P_{700}^+$, the primary electron acceptor of photosystem I. For a high rate of turnover each reaction must be fast and binding of the soluble reaction partner transient.

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[1] Abbreviations: Cyt *f*, soluble redox-active domain of cytochrome *f*; $E_m$, midpoint potential; ESI, electron spray ionization; $k_{obs}$, pseudo-first-order rate constant of the overall reaction; $k_{on}$, rate constant of complex formation; $k_{off}$, rate constant of complex dissociation; $k_2$, bimolecular rate constant of the overall reaction; $k_\infty$, $k_2$ at infinite ionic strength; $KP_i$, potassium phosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; mut, mutant; nd, not determined; NMR, nuclear magnetic resonance; Pc, plastocyanin; wt, wild type.

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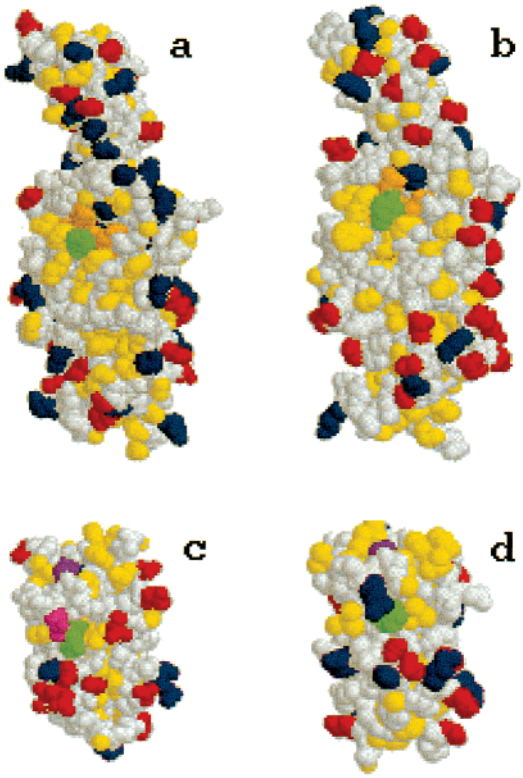


FIGURE 1: Space-filling representations of Cyt *f* and Pc drawn in Rasmol: (a, b) the lumenal domain of Cyt *f* from turnip and *P. laminosum*; (c, d) Pc from pea and *P. laminosum*. Color code for amino acid side chains/heme: yellow, hydrophobic; orange, heme; red, acidic; green, Cyt *f* Y1 (sixth heme ligand), Pc Y83 (pea)/Y88 (*P. laminosum*); blue, basic; dark purple, H87 (pea)/H92 (*P. laminosum*) (Cu ligand); light purple, Q88 (analogous to R93 in *P. laminosum*).

Cytochrome *f* is anchored in the thylakoid membrane with a C-terminal transmembrane helix. The redox-active 28 kDa N-terminal domain (abbreviated as Cyt *f*) protrudes into the lumen. This soluble domain, which has been crystallized (*1*, *2*), was used in this study. It is an unusual *c*-type cytochrome: it is elongated, mainly $\beta$-sheet, and its sixth heme ligand is the N-terminus, Tyr-1. The overall structure is highly conserved between higher plants and cyanobacteria (*2*). The main difference lies in the charge properties. Whereas both proteins are acidic [p*I* of parsley Cyt *f*, 4.7 (*3*); p*I* of *Phormidium laminosum* Cyt *f*, 4.2 (*4*)], in higher plants and green algae Cyt *f* possesses a prominent basic ridge which has been shown to interact with acidic patches on Pc (*5*−*8*). In *P. laminosum*, the basic ridge is replaced by a less prominent acidic ridge (Figure 1 a,b).

Pc is a type I copper protein of ca. 11 kDa. Again, its overall structure of an antiparallel $\beta$-barrel is conserved among cyanobacteria and higher plants (*9*−*12*), whereas the charge properties differ considerably. In higher plants, two prominent acidic patches have been shown to interact with the basic ridge of Cyt *f* (*5*, *6*, *13*). The p*I* ranges from 4.0 to 4.2 (*14*). In cyanobacteria, the p*I* range is much larger [5.2 for *P. laminosum* (*15*) to 8.4 for *Anabaena variabilis* (*16*)]. Figure 1d shows that, in *P. laminosum*, the acidic patches of higher plant Pc (Figure 1c) are replaced by a cluster of positive and negative charges.

For spinach Pc and turnip Cyt *f*, a structure of the transient complex in vitro has been obtained by paramagnetic NMR and restrained rigid body molecular dynamics (*17*). On the basis of this structure and the crystal structures of Pc and Cyt *f* from *P. laminosum* (*2*, *18*), a homology model of the analogous complex for the cyanobacterial proteins was obtained, which enabled identification of residues most likely to be involved in the electrostatic interaction. Site-directed mutagenesis of these residues for kinetic studies on their role in the interaction is possible since expression systems for both Pc and Cyt *f* have been established (*4*). This study presents the analysis of wt and seven charge mutants of Pc with Cyt *f* from *P. laminosum*.

Scheme 1

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} (AB) \underset{k_{-et}}{\overset{k_{et}}{\rightleftharpoons}} (A^{-}B^{+}) \longrightarrow A^{-} + B^{+}$$

The kinetic model used in this study for the interaction between the Cyt *f* and Pc in solution is given in Scheme 1, and eq 1 is the formula derived from it assuming steady-

$$k_2 = k_{on}k_f/(k_{off} + k_f) \quad (1)$$

state conditions [for a detailed description, see Bendall (*19*, *20*); $k_2$ = the second-order rate constant of the overall reaction; $k_{on}$ = the rate constant of complex formation; $k_{off}$ = the rate constant of dissociation before electron transfer has taken place; $k_f$ is related to $k_{et}$, the intrinsic rate constant of electron transfer, and would become equal to $k_{et}$ if the driving force were large enough]. Whereas the overall rate constant of reaction, $k_2$, can be measured in a stopped-flow spectrophotometer, possible methods to determine $k_{et}$ and the binding constant, $K_A$ ($=k_{on}/k_{off}$), are still under investigation. Determination of redox potentials, however, enables calculation of the driving force $\Delta G$, one of the three variables determining $k_{et}$ at a given temperature. The overall rate constant, $k_2$, can also be segregated into electrostatic and nonelectrostatic contributions, i.e., electrostatic binding (effects on $k_{on}$) on one hand and nonelectrostatic binding and electron transfer on the other hand. Ionic strength dependence curves of $k_2$ provide information about both aspects: the shape of the curve relates to the role of electrostatic interactions, and the extrapolated endpoint, i.e., the rate constant at infinite ionic strength, $k_\infty$, represents the rate constant at which all electrostatic contributions to $k_{on}$ are abolished. In this study, the role of electrostatics in the transient interaction of Cyt *f* and Pc from the cyanobacterium *P. laminosum* is elucidated using stopped-flow spectrophotometry and redox potentiometry.

## MATERIALS AND METHODS

*Molecular Biology and Mutagenesis.* Molecular biological methods were essentially as described by Sambrook et al. (*21*), and materials were as in Schlarb et al. (*4*). Mutagenesis of the *petE* gene in vector pET11PC (*4*) was carried out according to the QuickChange site-directed mutagenesis method (Stratagene; cf. ref *22*). The following codon changes were introduced to obtain the respective mutations: GAT to GCT for mutant D44A, GAC to GCC for mutant D45A, AAG to GCG for mutant K46A, AAA to GCA for mutant K53A, and CGG to GCG for mutant R93A. Incorporation

of the correct mutation and absence of undesired mutations were checked by sequencing of the mutated constructs. The plasmids with the correct mutations were named pETPcD44A, pETPcD45A, pETPcK46A, pETPcK53A, and pETPcR93A, respectively, and transformed into *Escherichia coli* strain BL21(DE3)pLysS or BL21(DE3). *E. coli* strain BL21(DE3)-pLysS carrying the pET11PC vector with the mutations R93Q and R93E (named pETPcR93Q and pETPcR93E) was received as a gift from M. J. Wagner.

*Protein Methods.* Expression, purification, and characterization of wild-type Pc and Cyt *f* as well as all Pc mutant proteins were carried out as in Schlarb et al. (*4*). Extinction coefficients used to calculate protein concentrations were 4700 $M^{-1}$ $cm^{-1}$ for *P. laminosum* and spinach Pc and 31500 $M^{-1}$ $cm^{-1}$ for *P. laminosum* and turnip Cyt *f*.

*Kinetic Analysis.* Measurements of the second-order rate constant, $k_2$, and its ionic strength dependence were performed at 300 K and pH 6.0 in an Applied Photophysics stopped-flow spectrophotometer (SF.17MV). Conditions and experimental procedures were essentially as in Kannt et al. (*5*) except that the concentration of Cyt *f* in the reaction mixture was held constant at 0.0825 $\mu$M instead of 0.1 $\mu$M and that, for ionic strength dependence measurements, 20 mM MES, pH 6.0, was used as buffer instead of 10 mM $KP_i$, pH 6.0. The ionic strength of the zwitterionic buffer at this pH was assumed to be 10 mM. The concentration of Pc in the reaction mixture was held constant during each ionic strength dependence measurement. It was 1.6 $\mu$M for wt, R93Q, and R93E, 1.2 $\mu$M for D44A, D45A, and K53A, and 1.08 $\mu$M for K46A and R93A.

*Redox Potentiometry.* The determination of the midpoint potentials of *P. laminosum* Pc and Cyt *f* was performed as in Wagner et al. (*23*), except that the reference electrode was an Ag/AgCl in polymer electrode (type KCMMPTRL, Russell) filled with 3 M KCl, resulting in a half-cell potential of 199 ± 1 mV. The electrode was calibrated at 289 K with 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide in 10 mM $KP_i$, pH 7.0, taking a value of $E_m$ = 407.5 mV (*24*). The titrations were carried out at 300 K in 10 mM $KP_i$ containing 0, 40, or 90 mM KCl and 50 $\mu$M potassium ferricyanide, pH 6.0, or in 20 mM MES, containing 900 mM KCl and 50 $\mu$M potassium ferricyanide, pH 6.0. The difference between the absorbances at 598 and 680 nm for Pc and at 555.6 and 620 nm for Cyt *f* were taken as a measure of partial reduction. The potential was adjusted by addition of 0.1−1 $\mu$L aliquots of freshly prepared 10 mM sodium ascorbate solution.

## RESULTS

In the homology model of the transient complex between Pc and Cyt *f* from *P. laminosum*, five charged residues on Pc most likely to be involved in the electrostatic interaction were identified: D44, D45, K46, K53, and R93 (Figure 2).

To investigate the influence of these residues on the overall rate of reaction, their charge was neutralized by replacement with the small hydrophobic amino acid alanine. The use of alanine substitution as a reference has been discussed by Wells (*25*). For R93, mutations to Q and E were also investigated to elucidate the influence of an uncharged, but hydrophilic residue and of charge inversion in this position. This Arg residue is conserved among all cyanobacteria but

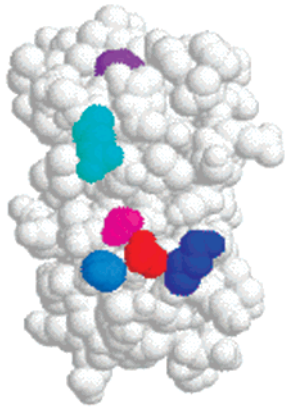

FIGURE 2: Space-filling representation of *P. laminosum* Pc drawn in Rasmol. Color code for side chains: purple, H92 (Cu ligand); pink, D44; red, D45; light blue, K46; blue, K53; cyan, R93.

Table 1: Kinetic Data and Parameters of the Watkins Equation for the Interaction of wt and Mutant *P. laminosum* Pc with wt *P. laminosum* Cyt *f* and of wt Spinach Pc with wt Turnip Cyt *f*[a]

| Pc | $k_2$ ($10^7$ $M^{-1}$ $s^{-1}$) | $k_\infty$ ($10^7$ $M^{-1}$ $s^{-1}$) ± fitting error | $V_{ii}$ ± fitting error | $\rho$ (Å) ± fitting error | $C_\alpha$−Cu distance (Å) |
|---|---|---|---|---|---|
| wild type | 4.7 | 3.22 ± 0.07 | −0.96 ± 0.08 | 5.6 ± 1.2 | |
| D44A | 6.0 | 3.98 ± 0.11 | −2.95 ± 0.15 | 8.9 ± 0.7 | 15.14 |
| D45A | 5.9 | 3.34 ± 0.08 | −2.60 ± 0.10 | 8.0 ± 0.5 | 18.48 |
| K46A | 3.1 | 3.21 ± 0.04 | 0.56 ± 0.02 | 17.0 ± 1.7 | 20.43 |
| K53A | 3.3 | 3.55 ± 0.03 | 0.34 ± 0.03 | 24.4 ± 5.3 | 21.33 |
| R93A | 1.9 | 2.69 ± 0.12 | 0.73 ± 0.03 | 3.8 ± 0.8 | 7.17 |
| R93Q | 2.1 | 2.84 ± 0.11 | 0.85 ± 0.06 | 6.7 ± 1.4 | 7.17 |
| R93E | 1.0 | 2.07 ± 0.17 | 2.07 ± 0.05 | 5.5 ± 0.8 | 7.17 |
| wild type higher plants | 15.1 | 0.4 ± 0.12 | −13.1 ± 3.2 | 6.9 ± 1.5 | |

[a] The data for higher plants were taken from Kannt et al. (*5*). Individual errors given in the table refer to the curve fitting. The overall error of $k_2$ is estimated to be ≥5% of the given values and that of the parameters of the Watkins equation ca. 10%. Watkins equation: $\ln k_2 = \ln k_\infty - V_{ii}e^{-\kappa\rho}/(1 + \kappa\rho)$, with $k_\infty = k_2$ at infinite ionic strength ($M^{-1}s^{-1}$), $V_{ii}$ = electrostatic attraction term, $\kappa = 0.3277\sqrt{I}$ (Debye−Hückel parameter at 300 K), and $\rho$ = radius of active site (Å).

is either Q or A in all known higher plant sequences. Of the residues mutated in this study, it is closest to the site of electron transfer (Table 1). The analogous residue in Pc from *Anabaena* PCC 7119, R88, has recently been shown to have a specific effect in reaction with *Anabaena* PSI (*26*). Molecular masses of purified proteins as determined by ESI−mass spectrometry were in agreement with the values predicted from the translated DNA sequence. The results of isoelectric focusing (data not shown) were as expected, in that the p*I* of D44A and D45A increased and that of the other mutants decreased relative to the wild-type protein. The visible spectra of the mutants were essentially identical to those of the wild type (data not shown). These results indicate that the mutant proteins were expressed correctly. Structural analysis by 2D $^1$H NMR was not carried out, as Kannt et al. (*5*) have shown that mutations of surface charges do not significantly alter the overall structure of spinach Pc. The kinetic behavior of wild-type and mutant Pc with wild-type Cyt *f* was investigated at 300 K and pH 6.0 by stopped-flow spectrophotometry. Midpoint potentials were determined by redox potentiometry under the same conditions.

*Second-Order Rate Constant.* Values for the second-order rate constant, $k_2$, under standard ionic strength conditions

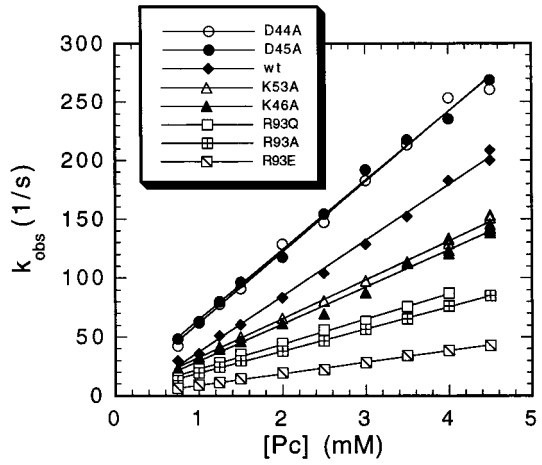


FIGURE 3: Concentration dependence of $k_{obs}$: wt and mutant *P. laminosum* Pc with wt *P. laminosum* Cyt *f*. The solid lines represent linear regressions, and their slopes represent $k_2$. Experimental conditions: 300 K, pH 6.0; concentration of Cyt *f*, 0.0825 $\mu$M.

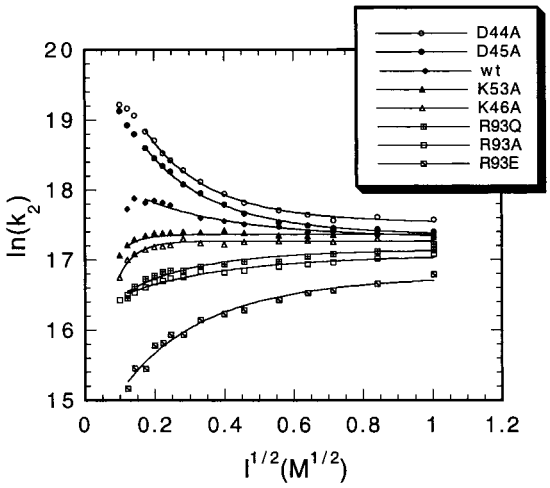


FIGURE 4: Ionic strength dependence of $k_2$: wt and mutant *P. laminosum* Pc reacting with wt *P. laminosum* Cyt *f*. All measured data points are shown; for the fits to the Watkins equation the first data points were excluded (up to 30 mM ionic strength for wild type and the Asp mutants, up to 15 mM ionic strength for the Lys and Arg mutants). Fitting parameters are summarized in Table 1. Experimental conditions: 300 K, pH 6.0; concentration of Cyt *f*, 0.0825 $\mu$M; concentration of Pc, 1.6 $\mu$M for wt, R93Q, and R93E, 1.2 $\mu$M for D44A, D45A, and K53A, and 1.08 $\mu$M for K46A and R93A.

(10 mM $KP_i$, 90 mM NaCl) were calculated from the slopes of the observed pseudo-first-order rate constant against Pc concentration (Figure 3, Table 1). Neutralizing acidic residues ($\Delta$charge $= +1$) increased $k_2$, whereas neutralizing basic residues ($\Delta$charge $= -1$) or introducing an additional acidic side chain ($\Delta$charge $= -2$) decreased $k_2$.

The effect of ionic strength on the second-order rate constant was also determined at 300 K and pH 6.0. Results are shown in Figure 4. The data were fitted to the equation of Watkins [$\ln k_2 = \ln k_\infty - V_{ii}e^{-\kappa\rho}/(1 + \kappa\rho)$], where $k_\infty$ equals $k_2$ at infinite ionic strength, $V_{ii}$ describes the electrostatic attraction energy in units of $k_BT$, $\kappa$, the Debye−Hückel parameter, is $0.3277\sqrt{I}$ at 300 K, and $\rho$ is the radius of the active site in angstroms (*27*). This equation is based on the so-called parallel plate model (*27*) and interprets charge−charge interactions as dominated by local charges on the interactive sites. Here, and in what follows, we use the term

Table 2: Midpoint Potentials ($E_m$ in mV) for wt and Mutant *P. laminosum* Pc and wt *P. laminosum* Cyt *f* at Varying Salt Concentrations[a]

| | $E_m$ (mV) at | | | |
|---|---|---|---|---|
| protein | 10 mM $KP_i$ | 10 mM $KP_i$, 40 mM KCl | 10 mM $KP_i$, 90 mM KCl | 20 mM MES, 900 mM KCl |
| wt Pc | 333 ± 3 | 335 ± 3 | 332 ± 2 | 322 ± 2 |
| D44A | nd | nd | 343 ± 3 | 332 ± 2 |
| D45A | nd | nd | 341 ± 2 | 332 ± 2 |
| K46A | nd | nd | 333 ± 2 | 323 ± 2 |
| K53A | nd | nd | 336 ± 3 | 323 ± 3 |
| R93A | nd | nd | 331 ± 3 | 325 ± 2 |
| R93Q | nd | nd | 330 ± 3 | 324 ± 2 |
| R93E | nd | nd | 326 ± 2 | 320 ± 2 |
| wt Cyt *f* | 315 ± 3 | 321 ± 2 | 323 ± 3 | 322 ± 2 |

[a] All measurements were done at pH 6.0 and 300 K in the buffer described in each column using 50 $\mu$M potassium ferricyanide as a redox buffer.

"interactive site/surface/area" to refer to the region of the surface that influences $k_{on}$, which may be larger than the interface of the final complex. Data points at 10 mM ionic strength, and in addition at 15 and 20 mM for wild type, D44A, and D45A Pc, were excluded to obtain a better fit. The fitting parameters of the Watkins equation are listed in Table 1. The most reliable parameter of the curve fit is $k_\infty$, the extrapolated rate constant at infinite ionic strength. It represents rate constants of binding and electron transfer in the absence of any long-range charge−charge interaction. With increasing ionic strength the curves of wild type and all mutants converge. The comparatively small kinetic differences between wild-type Pc and most of the mutants which remain at infinite ionic strength ($k_\infty$; Table 1) are within the overall error of 10% and not considered significant. The exception is R93E, but if the difference in this case is real, its origin is unknown.

It can be seen that the electrostatics of the wild-type reaction are attractive, since $k_2$ decreases with increasing ionic strength, i.e., with increased shielding of the (favorable) charges. This is mirrored in a negative electrostatic attraction term $V_{ii}$. The dependence on ionic strength increases for D44A and D45A ($V_{ii}$ more negative), implying that the electrostatic interaction is more favorable in these mutants. For K53A and K46A, however, the influence of the charges on the interaction is abolished apart from an inhibition effect at low ionic strength that is also visible in all other curves (in the R93 mutants superimposed with the overall repulsion). If the data points for the K53A mutant at 10, 15, and 20 mM ionic strength are excluded, the Watkins fit becomes a straight line ($V_{ii}$ zero); for the K46A mutant, only a negligible curvature remains. Neutralizing a single positive charge closer to the Cu (R93A, R93Q) not only abolishes the attraction between the two proteins but even converts it into repulsion (positive $V_{ii}$). A change in net charge of −2 leads to a more pronounced repulsion.

*Redox Potentials.* To shed light on the contribution of driving force to the kinetic changes, midpoint potentials were determined at 300 K and pH 6.0 under standard ionic strength conditions (10 mM $KP_i$, 90 mM KCl) or in 900 mM KCl and 20 mM MES as an approximation to the $E_m$ values at infinite ionic strength. KCl rather than NaCl was used in the buffer as the reference electrode was filled with KCl. Results are summarized in Table 2. The midpoint potential

at standard ionic strength conditions increased by ~10 mV for Δcharge = +1 (D44A, D45A), remained essentially the same as wild type for Δcharge = −1 (K46A, K53A, R93A, R93Q), and decreased by 6 mV for Δcharge = −2 (R93E). At high salt concentration, the midpoint potentials for all Pcs were ~10 (±4) mV lower, whereas the value for Cyt *f* was essentially the same as that under standard conditions. Hence the driving force decreased whether the rate constant increased or decreased with ionic strength. This implies that the changes in driving force had little influence on the observed changes in rate constant. It is surprising that mutations D44A and D45A, with a Δcharge of 1, lead to a larger change in redox potential than R93E with a Δcharge of 2. This change is therefore not solely due to the altered charge, which is confirmed by the fact that it appears to be independent of ionic strength (Table 2). Thus some subtle structural change influencing the redox potential seems to be introduced in D44A and D45A but not in the other mutants.

The midpoint potential of wild-type *P. laminosum* Pc is significantly lower than previously reported (*4*, *23*), because determination of the potential of the reference electrode as in Materials and Methods gave a value which differed appreciably from that previously assumed (*23*). The corrected value (322 mV; cf. Table 2) is substantially lower than commonly found for higher plant Pcs (345−396 mV; *28*) but is similar to that reported for another cyanobacterial Pc from *A. variabilis* (*29*, *30*). Determination of the midpoint potential of *P. laminosum* Cyt *f* under identical conditions (cf. Table 2) showed that the driving force of the reaction studied in this paper is approximately zero.

For wild-type Pc and Cyt *f*, midpoint potentials were also determined in 10 mM $KP_i$ and in 50 mM KCl and 10 mM $KP_i$ (Table 2). For Pc, the potential did not change significantly, whereas it dropped by ca. 8 mV from 100 to 10 mM salt for Cyt *f*. The driving force thus increased at low salt concentrations and cannot explain the inhibition effect observed for $k_2$ at low ionic strength.

It is clear that the electrostatic interaction between Pc and Cyt *f* from *P. laminosum* in vitro is based on a fine balance of the charges on the interactive surface and is not optimized for $k_2$. Considerably faster rate constants can be obtained in vitro by single point mutations (D44A/D45A).

## DISCUSSION

Results presented here show that all five mutated residues influence the rate of reaction between Cyt *f* and Pc of *P. laminosum*. Only for the Asp mutants could the change in driving force account for the change in rate constant at 100 mM ionic strength [using Marcus theory prediction as described by Rich and Bendall (*31*)] and then only if one assumed full activation control. Hence the observed kinetic effects for all mutants can mainly be attributed to $k_{on}$ or binding. This is surprising in light of recent NMR results obtained by Crowley et al. (*32*) on the *P. laminosum* complex, which suggest that the hydrophobic northern end of Pc is the sole area of contact in the complex in solution, in contrast to the homology model on which the choice of mutants used in this study was based. However, the complex observed by NMR represents the conformation(s) present during the majority of the lifetime of the complex. More transient conformations, which may involve electrostatics, remain invisible by NMR since they do not result in observable chemical shift changes of Pc nuclei. They may, however, affect the number of productive collisions and thus $k_{on}$. The apparent discrepancy between the kinetic and NMR data is solved if the reaction is diffusion limited. Then $k_{on}$ is the main determinant of $k_2$, and the changes observed for the charge mutants reflect effects on $k_{on}$. The conclusion under this assumption is that although the charged residues do not make contact with Cyt *f* in the complex, they serve to increase $k_{on}$. Hence the results presented here are a strong indication that the reaction is indeed diffusion controlled. Gong et al. (*6*) came to a similar conclusion for the higher plant system. Temperature dependence measurements which will further investigate diffusion control are under way.

*Ionic Strength Dependence.* The results also show that the electrostatic interaction between Pc and Cyt *f* from *P. laminosum* is dominated by the basic properties of the interactive area of Pc even though overall the protein is acidic. The analysis of the ionic strength dependence is therefore based on an equation of Watkins et al. (*27*) which only takes the charge of the so-called interaction site into consideration ("parallel plate model"; monopole−monopole term of the whole equation). The approach of van Leeuwen (*33*), which considers both the net charge and overall dipole moment of electrostatically interacting proteins, should in theory give more accurate results. However, the number of fitting parameters in this approach makes their interpretation less obvious. The Watkins equation used here has proved suitable for comparative mutagenesis studies. Its limitations are discussed below.

The overall dependence on ionic strength of the wild-type interaction is weak and not optimized for a fast $k_2$. Neutralization of a single positive charge turns attraction into repulsion. The extent of the effect is larger for mutations of the residue closer to the copper (R93A/R93Q). These findings are in stark contrast to the behavior of the analogous higher plant proteins. In 1996, Kannt et al. (*5*) published the ionic strength dependence of the reaction of wild type and acidic patch mutant spinach Pc with wild-type turnip Cyt *f*. Up to four negative charges of the large and small acidic patches were deleted in six different mutants. A 2−3-fold decrease in $k_2$ was observed for each negative charge abolished, regardless of its position. The mutations led to a decreased dependence on ionic strength compared to the wild-type reaction, but not even a change in net charge of +4 turned the attraction into repulsion. When an additional negative charge was introduced in position 88 (Q88E; this position corresponds to R93 in *P. laminosum*), the increase in rate was smaller than predicted. Q88 in higher plants thus seems to be of less relative importance than the acidic patches, whereas the corresponding residue in cyanobacteria, R93, is of greater importance than the cyanobacterial charge cluster corresponding to the acidic patches of higher plants. This Arg residue has also been shown to be important in the interaction of cyanobacterial Pcs with their homologous photosystems I (*26*).

All proteins, wild type as well as mutants, show an inhibition effect at low ionic strength (for the R93 mutants it cannot be distinguished from the overall repulsion). This effect has also been observed by Meyer et al. (*34*) and Kannt et al. (*5*) and was interpreted using a widely accepted

rearrangement model (cf. ref *35*). The optimum in $k_2$ is explained by an equilibrium between an initial electrostatic binding complex which is unfavorable for electron transfer and a tight electron-transfer complex. In this view, the electronic coupling matrix element decreases at low ionic strength as the complex is trapped in the electrostatic binding complex with nonoptimal distance between the redox centers; hence $k_{et}$ becomes rate limiting (decrease of $k_2$ with decreasing salt concentration). At higher salt concentrations the ionic strength increasingly shields the positive electrostatic attraction and lowers the rate of binding, $k_{on}$, and hence $k_2$. However, the curves obtained in this study for the K53A and K46A Pc mutants are more difficult to explain with this model. These curves show virtually no ionic strength dependence apart from the inhibition at low salt concentrations (cf. Figure 4). In this case $k_{et}$ cannot become limiting by formation of an electrostatically favorable, but electronically unfavorable complex. The inhibition must have a different origin, which is likely to be present also in those proteins where attraction was observed. We propose two possible alternatives to the rearrangement model:

(1) The limiting factor becomes $k_{et}$ at low salt concentrations through an effect of ionic strength on the reorganization energy $\lambda$ rather than on $V_{ii}$ (*36*, *37*). This would have to overcompensate the small effect on $\Delta G$ going in the opposite direction; see Table 2.

(2) At low ionic strength the rate constant of binding $k_{on}$ becomes limiting through an increase in the Debye length allowing more charged groups of opposite sign to be incorporated in the interactive radius (M. Ubbink, personal communication; *20*). The Debye length or screening distance is defined as the distance over which the electrostatic field around an ion is reduced to a value of 1/e of what it would have been in the absence of screening. It is 10 Å for an ionic strength of 100 mM, 14 Å for 50 mM, and 22 Å for 20 mM (*20*). The approximate dimensions of a Pc molecule are 40 Å × 32 Å × 28 Å (*38*), those of the interface in the *P. laminosum* homology model complex are approximately 22 Å × 33 Å. The radius of the proposed contact site is hence about 14 Å and equals the Debye length at 50 mM ionic strength. At salt concentrations below 50 mM, charges outside the contact site fall into the Debye length. The interactive site itself appears attractive for wt and the D → A mutants, neutral for the K → A mutants, and repulsive for the R93 mutants. However, the p*I* of both reaction partners is negative for wt and all mutants. As the Debye length increases, the overall charge becomes increasingly influential and will result in a diminished $k_{on}$ and repulsion.

The notion of changes in Debye length casts a different light on the use of the Watkins equation. Watkins et al. (*27*) discussed the spinach Cyt *f*−Pc curve of Meyer et al. (*34*) with its optimum of $k_{obs}$ at 40 mM ionic strength. They fitted a curve to the data using the monopole−monopole and monopole−dipole terms of the equation; however, the two parameters $V_{ii}$ and $V_{id}$ were of opposite sign. Their physical interpretation was that unfavorable dipolar interactions are balanced by favorable monopolar interaction, which they considered unreasonable. Hence they advised fitting of the high ionic strength portion of the curve only with a monopole−monopole fit. This has been done in our study for wt and the Asp mutants. However, the formalism of Watkins is based on treating the interactive site as a uniformly charged plate of fixed radius and net charge. If the inhibition at low ionic strength is due to changes in Debye length, the Watkins equation is not applicable, because neither radius nor net charge is fixed. In this case, the fit can only be used to extrapolate to $k_\infty$ and has no meaning in itself.

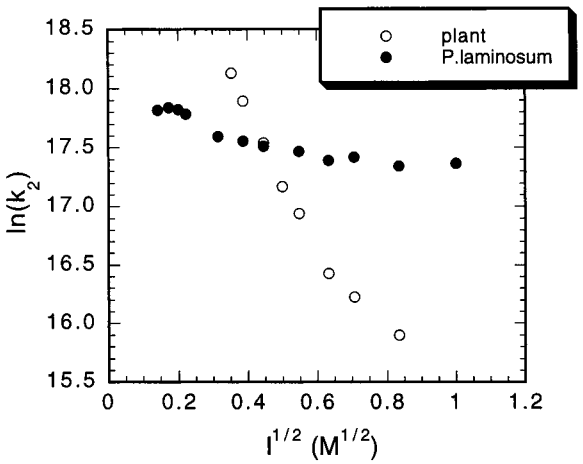


FIGURE 5: Ionic strength dependence of $k_2$ at 300 K and pH 6.0: wt *P. laminosum* Pc with wt *P. laminosum* Cyt *f* (for experimental conditions cf. Figure 4) and wt spinach Pc with wt turnip Cyt *f* [data taken from Kannt et al. (*5*)].

*Comparison of Cyanobacterial and Plant Systems.* Figure 5 compares the ionic strength dependence of the wild-type reactions of *P. laminosum* and higher plants at 300 K. The plant data were taken from Kannt et al. (*5*). The curves intersect at ~200 mM salt. If one corrects for the high growth temperature of *P. laminosum*, the point of intersection moves to lower ionic strength. For example, if one uses a temperature dependence equal to that of the reaction between Pc and PSI (unpublished results) to calculate the *P. laminosum* rate at the temperature at which it is cultured (45 °C), the intersection point moves to ~100 mM salt. To our knowledge, the ionic strength of the lumen of cyanobacteria has not been measured. Published values of the ionic strength in the stroma of chloroplasts vary from 130 to 200 mM (*39*), and it seems reasonable to assume that the lumenal ionic strength lies within a similar range. Hence the *P. laminosum* system reacts at least as fast as that of plants under physiological salt concentrations and temperatures.

Figure 5 and Table 1 also show that once the electrostatic interactions are eliminated, the rate constant ($k_\infty$), which is a minimum estimate for $k_{on}$, is almost 10 times higher for the cyanobacterium than for plants. Whereas electrostatics are the major contributor to $k_2$ in plants, this role is taken by nonpolar interactions in the cyanobacterium, leading to a remarkably high rate at infinite ionic strength ($3.2 \times 10^7$ $M^{-1}$ $s^{-1}$). The NMR results of Crowley et al. (*32*) confirm this feature. According to Gabdoulline and Wade (*40*) and Northrup and Erickson (*41*), diffusion-controlled association rates between proteins without attractive electrostatics are typically of the order of $10^6$ $M^{-1}$ $s^{-1}$. A plausible explanation for the 10-fold higher rate in *P. laminosum*, in terms of the model of Northrup and Erickson (*41*), would be that a number of conformations of the final complex are effective in electron transfer. Such an explanation would be consistent with the NMR model of Crowley et al. (*32*). The general conclusion of this comparison of the plant and cyanobacterial

systems, when examined in vitro, is that both reach similar rates of reaction under physiological salt concentrations and temperatures, but by very different means. Although it is clear that a strong electrostatic interaction is essential for the reaction between the plant proteins in vitro, Soriano et al. (*7*, *42*) have shown that the rate of reoxidation of Cyt *f* in vivo is unaffected by neutralization of the basic ridge of Cyt *f*, at least in *Chlamydomonas reinhardtii* growing under favorable conditions. This indicates that the reaction between Cyt *f* and Pc is not rate limiting in vivo and does not become rate limiting even if favorable electrostatic interactions are eliminated. The role of electrostatics in the reaction of Pc with PSI and the overall evolutionary significance of both reactions will be discussed in another publication.

In this study, we have found striking differences in the kinetics of the in vitro interaction between higher plant and cyanobacterial Pc and Cyt *f* and have discussed their in vivo relevance. We have identified charged residues that have a direct influence on $k_2$ and have found a mutant for which the only effect of changes in salt concentration on $k_2$ is inhibition at low ionic strength. Alternative explanations for this well-known phenomenon have been suggested.

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