Identification of the Basic Residues of Cytochrome *f* Responsible for Electrostatic Docking Interactions with Plastocyanin in Vitro: Relevance to the Electron Transfer Reaction in Vivo†

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ABSTRACT: The prominent basic patch seen in the atomic structure of the lumen-side domain of turnip cytochrome *f*, consisting of Arg209 and Lys187, 58, 65, and 66, was proposed to be an electrostatically complementary docking site for its physiological electron acceptor, plastocyanin [Martinez, S. E., Huang, D., Szczepaniak, A., Cramer, W. A., and Smith, J. L. (1994) *Structure 2*, 95−105]. This proposal agrees with solution studies on the cytochrome *f*/plastocyanin electron-transfer reaction that showed a major contribution of electrostatic interactions to the docking, but not with studies on the oxidation rate of cyt *f* in vivo using mutants in which the basic patch of cyt *f* was neutralized. The apparent contradiction might be explained by an unknown electron acceptor protein for cyt *f*. However, (i) flash-induced oxidation of cyt *f* is absent in a PC-deficient mutant. (ii) Lys58, 65, and 66 in the large domain and Lys188 and 189 in the small domain are major contributors to the ionic strength dependence of the electron-transfer reaction in solution. Replacement of Lys58 and 65 by neutral residues and of Lys66 by the acidic residue Glu66 resulted in a >10-fold decrease in the rate of electron transfer in solution and complete loss of its ionic strength dependence. Replacement of Lys188 and Lys189 in the small domain of cyt *f* resulted in a 3−4-fold decrease in the second-order rate constant and a smaller dependence of the overall rate of electron transfer on ionic strength, corresponding to a loss of two positive charges. (iii) Acidification of the thylakoid lumen cannot explain the absence of electrostatic interactions. (iv) Changing the five lysines to acidic residues did not result in any significant retardation of the rate of cyt *f* oxidation in vivo. If the docking of cyt *f* and plastocyanin in vivo is mediated by basic residues of cyt *f*, they are different from those that mediate electron transfer in vitro or that are implicated by simulations of electrostatic interactions of the docking. Alternatively, docking of cyt *f*/PC in vivo is limited by spatial constraints or release of PC from P700 that precludes a rate-limiting mediation of the cyt *f*/PC reaction by specific electrostatic interactions. The cyt *f*/PC system in *Chlamydomonas reinhardtii* is the first electron-transfer couple for which the role of electrostatics in mediating the docking reaction has been studied both in vitro and in vivo.

The physical nature of interprotein interactions in redox proteins has been studied in vitro in several systems including the well-documented cytochrome *c*−cytochrome *c* peroxidase (*2*−*5*), ferredoxin (*6*)−ferredoxin:NADP$^+$ reductase (FNR) (*7*, *8*) and methylamine dehydrogenase-amicyanin (*9*), and the cytochrome c_2 and reaction center in the photosynthetic bacterium *Rhodobacter sphaeroides* (*10*, *11*) for which high-resolution structures of the pairs are known. For these pairs, electrostatic forces have been found to dominate or exert a major influence on the attractive interactions in solution.

The X-ray structure of cocrystals of cytochrome *c* peroxidase and cytochrome *c* from yeast identified a region of acidic residues in CCP[1] within H-bonding distance of basic residues in cyt *c* (*3*). Neutralization of the acidic residues at the cyt *c* docking site on CCP resulted in a 2−3-fold decrease in the equilibrium constant of association (*4*, *12*). Replacement of acidic residues by neutral or positive residues in ferredoxin caused a decrease in the rate of oxidation of ferredoxin by FNR (*13*−*15*). In *Rb. sphaeroides*, soluble cyt c_2 reduces the photooxidized primary donor in the RC. Studies on isolated reaction centers and cyt c_2 show that the cyt c_2−RC docking interaction is dependent on ionic strength (*10*) and that cocrystals of the cyt c_2−reaction center complex have been obtained (*11*) in which complementary interacting positive and negative residues from cyt c_2 and the RC, respectively, have been identified. Previous mutational and chemical modification of the positive residues in cyt c_2 and negative residues on the periplasmic side of the RC have also shown reduced binding of cyt c_2 to the RC (*11*).

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[1] Abbreviations: CCP, cytochrome *c* peroxidase; cyt *f*, cytochrome *f*; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DTT, dithiothreitol; E_{m7}, midpoint potential at pH 7.0; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; HS, high salt; HSA, high salt with acetate; k_2, bimolecular rate constant; k_{obs}, first-order rate constant fit to pseudo-first-order data; MES, 2-(*N*-morpholino)ethanesulfonic acid; PC, plastocyanin; PCR, polymerase chain reaction; PSI, photosystem I; RC, reaction center; TAP, Tris-acetate-phosphate.

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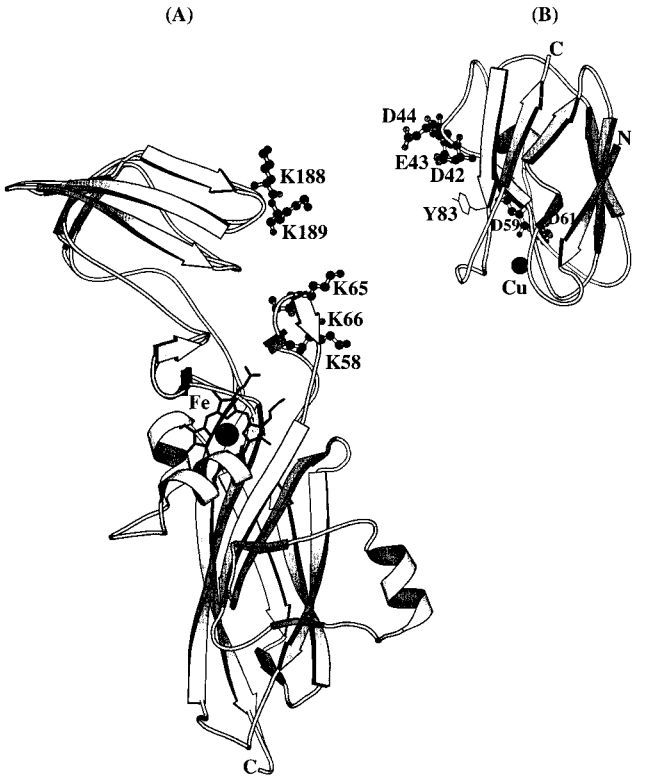

FIGURE 1: Ribbon diagram of cytochrome *f* (A) and PC (B) in a predocking configuration with key residues depicted as "ball-and-stick". This picture was drawn with Molscript (*20*).

The cytochrome *f*−plastocyanin redox pair has also been described in terms of electrostatic docking interactions. High-resolution structures are known for plastocyanin (*16*−*18*) and the soluble domain of cytochrome *f* (*1*, *19*) that form a bimolecular redox system connecting the b_6f complex with photosystem I in oxygenic photosynthesis. Ribbon diagrams of the two proteins in a predocking configuration are shown (Figure 1).

Cytochrome *f*, consisting of 285 residues in the plant b_6f complex and 286 residues in *Chlamydomonas reinhardtii*, is the largest polypeptide of the cytochrome b_6f complex (*21*). The b_6f complex mediates electron flow from photosystem II to photosystem I, coupling it to proton translocation (*22*). It transfers electrons to PSI through a soluble blue-copper protein plastocyanin which accepts one electron from cytochrome *f*. Relevant to the nature of the interactions between cyt *f* and PC, one of the interesting features revealed by the crystal structure of turnip cyt *f* is the presence of a prominent basic patch at the interface of its small and large domains. This basic patch consists of Lys187 and Arg209 in the small domain and Lys58 and Lys65−66 in the large domain. For *C. reinhardtii* cyt *f*, the basic patch in the small domain consists of Lys188 and 189 (*23*). The crystal structure of plastocyanin (*17*, *18*) also showed a patch of aspartates and glutamates around Tyr83. The presence of a dominant positive region in cyt *f* and a negative region in PC, as revealed by the atomic structures of both proteins, and a high degree of conservation of the charged regions across many plant and algal species (*23*) suggest that the two proteins dock via these charged surfaces. Therefore, from structure considerations, electrostatic docking interactions might be predicted to have a significant effect on the overall rate of electron transfer from cyt *f* to PC. It has also been proposed that, at least in some cyanobacteria, there are partial reciprocal changes of the positive and negative domains in cyt *f* and PC (*24*), further implicating a role of electrostatic docking interactions.

Evidence for electrostatic interactions was obtained from in vitro studies where the rate of cyt *f* oxidation by PC was shown to markedly and systematically decrease with increasing solution ionic strength (*25*−*27*), consistent with increased screening of the charges on the two proteins by the solvent. Modeling of the interaction between the two molecules by molecular dynamics (*28*) and Brownian dynamic simulations (*29*) also predicted docking of cyt *f* and plastocyanin to be mediated by electrostatic interactions.

However, in the cyt *f*−PC redox system, it is important to note that in the electrostatically docked complexes the distance between the cytochrome *f* heme and the plastocyanin Cu is more than 25 Å (*30*, *31*), which would require the intracomplex electron-transfer rate (*32*, *33*) to be much smaller than the rates of 2800 s^{-1} (*25*, *26*) and approximately 3000 s^{-1} (*34*, *35*) obtained, respectively, in vitro and in vivo. Therefore, in order for electron transfer to occur, the proteins must rearrange from the initial electrostatically mediated docking configuration to one with a shorter heme−Cu distance that is favorable for electron transfer. The role of electrostatic interactions is perhaps to provide initial long-distance guidance for docking to form a pre-electron-transfer complex. The solution studies of the cyt $f \rightarrow$ PC electron-transfer rate as a function of ionic strength suggest that the initial electrostatically mediated docking is rate limiting in the overall rate of cyt *f* oxidation by plastocyanin (*27*, *36*).

The question of whether a specific electrostatically docked complex of cyt *f* and platocyanin is required in vivo was addressed by site-directed mutagenesis using the transformable green alga, *C. reinhardtii*, in which the basic residues in the cyt *f* basic patch, five lysine residues in *C. reinhardtii*, were neutralized (*35*). If the docking would be electrostatically limited, neutralization of these highly conserved lysines in the basic patch of cyt *f* would be predicted to result in a significant retardation of the rate of cyt *f* oxidation. However, neutralization of four of the five lysine residues and change of the fifth to a carboxylate resulted in, at most, a 25% retardation in the rate of cytochrome *f* oxidation in vivo by plastocyanin (*35*). The results were unexpected because, as mentioned above, solution studies had already indicated the dominant role of electrostatic interactions. Their apparent disagreement with solution studies led to several questions regarding cyt *f*/PC interactions. (i) Is there an alternate electron acceptor for cyt *f* in *C. reinhardtii* in place of PC or cyt c_6? (ii) Does the *C. reinhardtii* cyt *f*/PC system show the same ionic strength dependence as does turnip cyt *f*/PC? If this dependence would be greatly diminished, it could explain the results in vivo. (iii) Are the Lys residues that have been substituted by neutral or acidic residues those that are responsible for the electrostatic interactions in vitro? (iv) Will more extreme changes of net electrical charge in the basic patch of cytochrome *f* result in the retardation of its oxidation rate in vivo? (v) Can the apparent absence of electrostatic interactions between cyt *f* and PC in vivo be explained by neutralization of the PC acidic residues at the ambient acidic pH of the chloroplast thylakoid lumenal space?

In the present work, answers to the above questions are provided. The Lys residues responsible for the ionic strength dependence in vitro are identified. Substitutions for these Lys residues by site-directed mutagenesis, which cause a ≥20-fold decrease in cyt *f* → PC electron-transfer rate in vitro and loss of the ionic strength dependence, have no significant effect on the rate of oxidation of cytochrome *f* in vivo. To our knowledge, the cyt *f*-PC system in *C. reinhardtii* is the first electron-transfer pair for which the role of electrostatics in the docking reaction has been studied both in vitro and in vivo.

METHODS

(1) Growth and Culturing of C. reinhardtii. Wild-type and the K188D−K189E, K58E−K65E−K66E, and K58Q−K65S−K66E mutants of *C. reinhardtii* were grown with minimal media in agar plates (*37*) while mutant *C. reinhardtii*, which makes only soluble cyt *f*, was grown in agar plates using TAP medium (*37*). For isolation of soluble cytochrome *f*, *C. reinhardtii* was inoculated from plates into 300 mL liquid cultures. Acetate was added to the HS growth medium (*37*) for heterotrophic growth. Cells from the initial 300 mL cultures were transferred to 10 L carboys which were bubbled with air and stirred. For isolation of plastocyanin, wild-type *C. reinhardtii* was used.

(2) Mutagenesis and Transformation. Plasmid pADI283ST (*38*), kindly provided by F. A. Wollman and R. Kuras, was used to express wild-type soluble cytochrome *f*. To generate the construct for the soluble K58Q−K65S−K66E mutant form of cytochrome *f*, the plasmid pTP101 with the substitutions, K58Q−K65S−K66E (*35*), was combined with I252stop mutation from plasmid pADI283ST. pADI283ST was cut with *Apa*I and *Afl*II enzymes to generate a 2.3 kb fragment which was purified and digested with *Bst*EII to generate a 0.5 kb fragment carrying the I252stop mutation. The latter was subcloned into pTP101 digested with *Bst*eEII and *Afl*II to yield the pTP101I252stop construct. pTP101 carrying K58Q−K65S−K66E changes was cut with *Xba*I and *Bst*EII, and the resulting 1.2 kb fragment was ligated into the pTP101I252 stop plasmid digested with the same enzymes. The PetA gene with combined mutations was recloned into the pTP103 plasmid for chloroplast transformation. The K188N−K189Q mutant of cyt *f* was made in *Escherichia coli* (see below).

Mutations K188D−K189E and K58E−K65E−K66E were generated on plasmid pTP101 in *E. coli* according to ref *39*. The *Xba*I−*Afl*I fragments carrying mutated sequences were recloned into the pTP103 construct to be used in transformation. A mutant of *C. reinhardtii* from which the cyt *f* gene (petA) had been deleted, kindly provided by R. Malkin, was transformed by the biolistic method (*40*).

(3) Flash Kinetic Measurements of Cytochrome f Oxidation in Vivo. The time course of flash-induced cyt *f* oxidation in intact cells of *C. reinhardtii* was assayed by flash kinetic spectroscopy as described in ref *35*.

(4) (a) Purification of the Lumen-Side Domain of C. reinhardtii cytochrome f. The redox active soluble fragment of cytochrome *f* from wild-type and the K58Q−K65S−K66E mutant was purified from *C. reinhardtii*, as was wild-type PC. For cytochrome *f*, cells from ca. 40 L were grown for 3 days to late logarithmic phase, sedimented at 1500*g*, and washed and stored at −80 °C in 10 mM phosphate buffer, pH 7.5, with 1 mM EDTA added as a protease inhibitor. Cells were broken by subjecting them to two freeze−thaw cyles. After removing cell debris by sedimentation at 8000*g*, cytochrome *f* was precipitated with 60% acetone and the precipitate dissolved in 10 mM phosphate buffer, pH 7.5, and frozen. The thawed solution was centrifuged at 12000*g* (20 min), the precipitate discarded, the solution dialyzed overnight with two changes of 10 mM phosphate buffer, pH 7.5, 1 mM EDTA, and 1 mM DTT to keep the cytochrome reduced, and then passed through a Whatman DE-52 ion-exchange column. The fractions enriched in cytochrome *f* were pooled and then passed through a hydroxyapatite column. The cyt *f*-enriched fractions were pooled again, concentrated to 1 mL, and passed through a G-100 gel filtration column. The final A_{554}/A_{280} for the wild-type and mutant = 0.86.

(b) Expression and Purification of C. reinhardtii Cytochrome f from E. coli. The K188N−K189Q mutant form of soluble cytochrome *f* was expressed heterologously in *E. coli* (strain MV1190). The *C. reinhardtii* petA gene (with the stop codon in place of I252 codon) fused with the sequence containing the *Pel*B leader and the ribosome binding site from expression vector pET25b (Novagen) was cloned into pUC19 vector (New England Biolabs) in the right orientation to the *lac* promoter as described in Ponamarev and Cramer (manuscript in preparation). More than 95% of cytochrome *f* was found in the water fraction of osmotically shocked cells. The yield of holocytochrome in the crude extract was estimated to be 0.4−0.6 mg/L of culture. Purification of cyt *f* from the water fraction was done as in paragraph 4a above except that acetone precipitation was omitted. The final $A_{554}/A_{280} = 0.95$.

(5) Purification of Plastocyanin from C. reinhardtii. Cells were broken using two freeze−thaw cycles, sedimented, ammonium sulfate added to 60% saturation, the solution stirred at 4 °C (ca. 6 h), and centrifuged for 20 min at 6000*g*. Ammonium sulfate was added to the supernatant to 90% saturation to precipitate PC. This solution was stirred overnight at 4 °C, centrifuged, and the precipitated plastocyanin was collected and redissolved in 10 mM Tris-HCl buffer, pH 7.0. Potassium ferricyanide was added to oxidize the plastocyanin, the PC dialyzed overnight against 10 mM Tris-HCl, pH 7.0, with two buffer changes and then passed through a Whatman DE-52 ion-exchange column. All fractions containing PC were pooled, and the solution was concentrated to about 1−2 mL and passed through a Sephadex G-100 gel filtration column. The final value of $A_{597}/A_{280} \approx 0.9$. Excess reductant and oxidant for cytochrome *f* and plastocyanin, respectively, were removed by gel filtration and by washing in Centriprep tubes.

(6) Stopped-Flow Kinetics of Cytochrome f Oxidation; Determination of Bimolecular Rate Constant. The kinetics of oxidation of wild-type cytochrome *f* by plastocyanin was measured over a pH range of 4.6−7.0 and at 25 °C. Buffers: 2 mM K_2HPO_4/KH_2PO_4, pH 7, or sodium acetate, pH 5.5, 5.0, and 4.6, with 0.2 mM EDTA. The ionic strength was adjusted by addition of NaCl. The cytochrome *f* concentration was 0.25−0.5 μM, while the plastocyanin concentration was 10−20 times that of cyt *f* to obtain pseudo-first-order kinetics for the oxidation of cyt *f* by PC. The kinetics of cyt *f* oxidation by PC in solution were studied

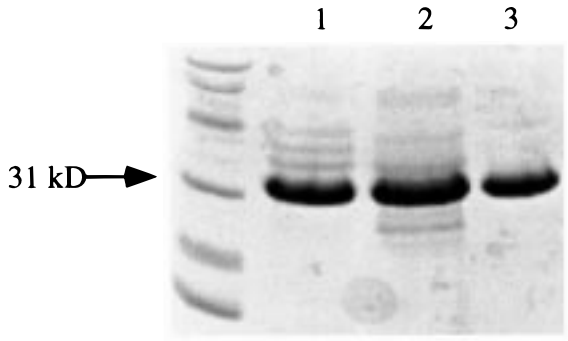

FIGURE 2: SDS−PAGE of *C. reinhardtii* cytochrome *f*. Lanes: (1) wild-type; (2) K58Q−K65S−K66E mutant; (3) K188N−K189Q mutant expressed in *E. coli*.

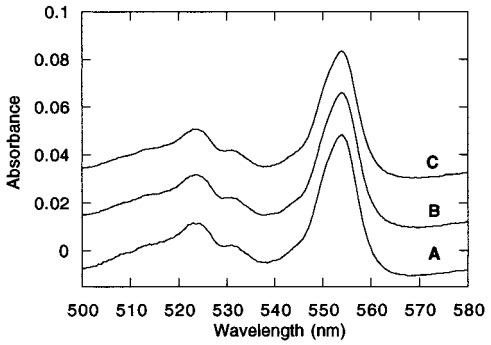

FIGURE 3: Ascorbate-reduced minus ferricyanide-oxidized difference spectra of cytochrome *f*. (A) Wild-type; (B and C) K188N−K189Q and K58Q−K65S−K66E mutants, respectively. [cyt *f*] ≈ 1.5 μM in 50 mM K_2HPO_4/KH_2PO_4, pH 7.0. Peak of α-band, 554 nm, half-bandwidth, 9 nm.

using a stopped-flow spectrophotometer (Applied Photophysics SX.18MV). The decrease in absorbance was monitored at 420 nm, which is the peak of the Soret band of reduced cyt *f*. The pseudo-first-order kinetic data were fit with a single exponential to obtain the first-order rate constant, k_{obs} (s^{-1}). The resulting k_{obs} values were plotted vs PC concentration to obtain the bimolecular rate constant, k_2.

(7) Determination of Redox Potentials of Cytochromes f and Plastocyanin. Midpoint oxidation−reduction potentials at pH 7.0 in 50 mM K_2HPO_4/KH_2PO_4 for wild-type and mutant forms of cyt *f* oxidized by ferricyanide (0.4 mM) were determined by titration with ascorbate as the reductant while monitoring absorbance of cyt *f* at 554 nm. The potential at each point of the titration was measured with a digital multimeter (Fluke 73 Series II). The electrochemical cell consisted of a Pt wire and a Ag/AgCl electrode (MF 2052 microelectrode, Bioanalytical Systems, West Lafayette, IN) as the reference. The potential of the Ag/AgCl electrode was calibrated vs a saturated solution of quinhydrone (*41*). The redox titrations were fit to a one-electron Nernst equation.

RESULTS

(A) Properties of Wild-Type and Mutant Proteins. The 251 residue lumen-side soluble domains of wild-type and mutant (K188N−K189Q, K58Q−K65S−K66E) cyt *f* were obtained in essentially pure form, all of which migrated on SDS−PAGE with $M_r \approx 30\,000$ (Figure 2). Reduced-oxidized chemical difference spectra for the proteins are identical (Figure 3), with $\lambda_{max} = 554$ nm, and a half-bandwidth ~9 nm (*42*). The midpoint potentials for the wild-type and mutants are approximately 355−360 mV (Table 1), hardly shifted from the value for the wild-type protein, as predicted (*30*). It should be noted that the K188N−K189Q mutant expressed in *E. coli* also has a Phe237Leu amino acid change that was introduced by PCR. Phe237, in the protein core of the large domain is conserved in most plant and algal species (*23*). However, this mutation did not cause any significant changes in cyt *f* as indicated by its difference spectrum (Figure 3B) and E_{m7} value (Table 1).

(B) Cytochrome f Oxidation in Vivo. The rate of cytochrome *f* oxidation in vivo induced by a saturating light flash (Figure 4, Table 2) is similar in wild-type ($t_{1/2} \approx 200$ μs) (Figure 4A), and the K188D−K189E ($t_{1/2} = 220$ μs) (Figure 4B) and K58E−K65E−K66E ($t_{1/2} = 185$ μs) (Figure 4C)

Table 1: Summary of Midpoint Potentials[a] at pH 7.0 of Wild-Type and Mutant Cytochromes *f* [b]

cytochrome *f*	midpoint potentials (mV)
wild-type	366 ± 4 ($n = 3$)[c]
K188N−K189Q	358 ± 4 ($n = 4$)
K58Q−K65S−K66E	355 ± 4 ($n = 2$)

[a] vs H_2 electrode. [b] From *C. reinhardtii*. [c] *n*, number of experiments.

Table 2: Summary of Rates of Flash-Induced Oxidation of Wild-Type and Mutant Cytochromes *f* [a]

strains	half-times (μs)
wild-type	202 ± 11 ($n = 7$)[c]
K188N−K189Q[b]	226 ± 40 ($n = 7$)
K58Q−K65S−K66E[b]	233 ± 51 ($n = 5$)
K58Q−K65S−K66E−K188N−K189Q[b]	253 ± 23 ($n = 4$)
K188D−K189E	220 ± 33 ($n = 6$)
K58E−65E−66E	184 ± 33 ($n = 7$)

[a] From *C. reinhardtii*. [b] From ref 35. [c] *n*, number of experiments.

mutants. These mutations are more drastic in terms of changes in charge than those tested previously, K188N−189Q and K58Q−K65S−K66E (*35*), which also did not cause a decrease in the rate of cytochrome *f* oxidation. The question then arises as to whether an alternate electron acceptor in place of PC or cyt c_6 in *C. reinhardtii* (*43*) could dock at a site on the surface of cytochrome *f* that does not include its basic patch. This could explain the lack of effect of the lysine mutations on cyt *f* oxidation, although this unknown alternate acceptor would have to react as efficiently as PC or cyt c_6. However, flash-kinetic experiments on a deletion mutant of plastocyanin [strain *ac*208 (*44*, *45*)] showed no observable cyt *f* oxidation (Figure 4D), indicating that, in the absence of PC or cyt c6, there is no alternative active electron acceptor for cytochrome *f*.

(C) Ionic Strength Dependence of the Reaction of cyt f and PC in Solution. The effect of ionic strength on the bimolecular rate constant, k_2, for the oxidation of wild-type cyt *f* from *C. reinhardtii* by PC, first shown by Niwa et al. (*46*) with proteins from *Brassica komatsuna*, is shown (Figure 5). The reaction of cytochrome *f* and plastocyanin in solution is known to have a marked dependence on ionic strength (Figure 5, open triangles; see also refs *25* and *26*). A similar behavior was also displayed by the reaction between the cytochrome *f* mutant, K188N−K189Q, and PC (Figure 5, ×), although the ionic strength dependence in this

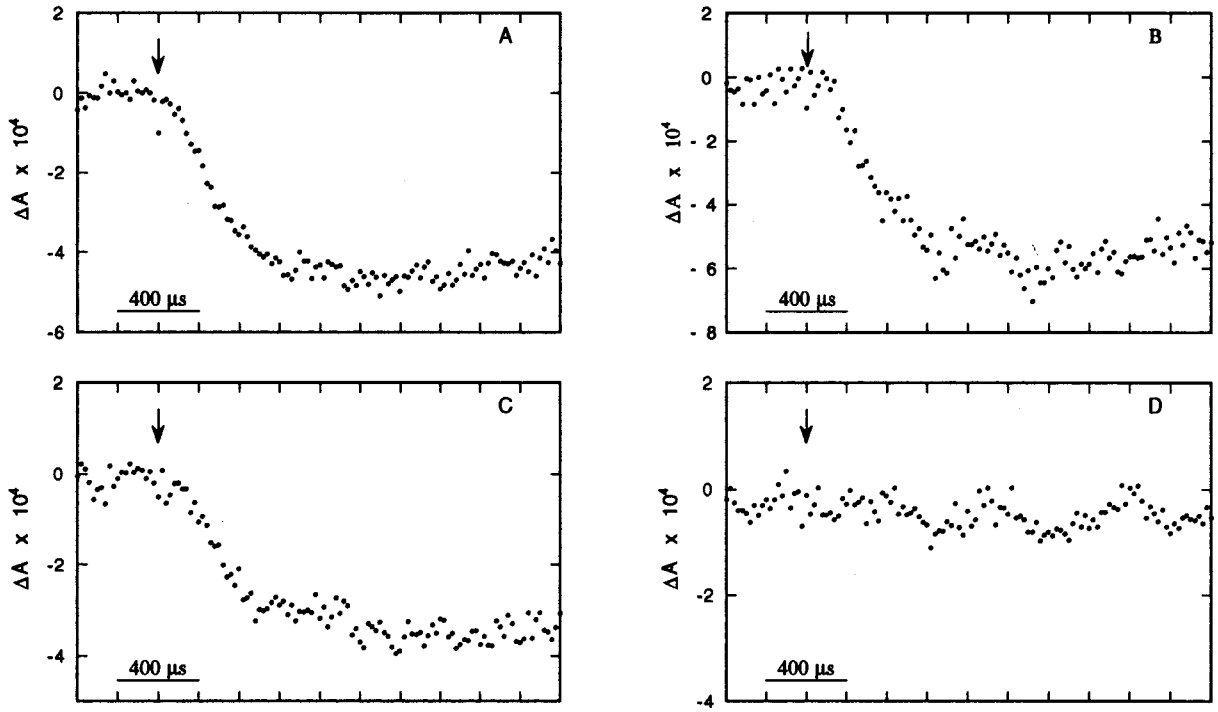

FIGURE 4: Flash-induced oxidation of cytochrome *f* in vivo. (A) Wild-type; (B and C) K188D−K189E and K58E−K65E−K66E mutants, respectively, and (D) PC deletion mutant. Cells A−C were grown phototrophically in HS medium while D was grown heterotrophically in HSA medium (*37*). Reaction medium: 20 mM MES/NaOH, pH 7.0, 40 μM FCCP, 10 μM DCMU, 1 mM hydroxylamine, and *C. reinhardtii* with [Chl] = 30 μg/mL. Arrows indicate start of flash.

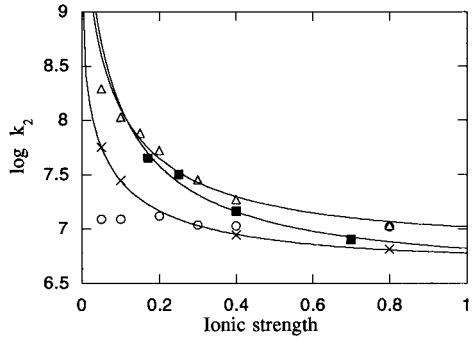

FIGURE 5: Dependence of second-order rate constant k_2 on ionic strength for the oxidation of cytochrome *f* by plastocyanin in solution. (■) Turnip; (△, ×, and ○) wild-type, K188N−K189Q, and K58Q−K65S−K66E mutants, respectively from *C. reinhardtii*. The solid lines for turnip, *C. reinhardtii* wild-type and K188N-K189Q mutant cyt *f* are results of fits to eq 1 in the text. Values for Z_1Z_2 and k_∞ (M^{-1} s^{-1}) obtained from fit to eq 1 for (a) wild-type, -15.8 ± 1.2 and 7.7×10^6; (b) K188N−K189Q, -9.7 ± 0.1 and 5.0×10^6, and (c) turnip cyt *f*, 18.9 ± 2.0 and 4.6×10^6.

case is less pronounced. However, there was no observable dependence on ionic strength for the interaction between the cyt *f* mutant K58Q−K65S−K66E and PC (Figure 5, open circles). At the smallest ionic strength that was used (50 mM), the calculated second-order rate constant k_2 for wild-type cyt *f*/PC was at least twice that for the K188N−K189Q mutant and at least an order of magnitude higher than that for the K58Q−K65S−K66E mutant.

For wild-type cyt *f*/PC and K188N−K189Q−cyt *f*/PC, k_2 can be seen to decrease with increasing ionic strength, while k_2 is independent of ionic strength for the reaction of K58Q−K65S−K66E−cyt *f* with PC. The relationship between the bimolecular rate constant, k_2, and ionic strength can be described by the so-called "parallel-plate" model that treats the local charges on a molecule as a single charged domain (*47*):

$$\ln k_2 = \ln k_\infty - Vf(I) \tag{1}$$

with

$$V = \alpha Z_1 Z_2 r_{12}/\rho^2 D_e \tag{2a}$$

$$\alpha = 89.2 \text{ Å} \tag{2b}$$

$$f(I) = \exp(-\kappa\rho)/(1 + \kappa\rho) \tag{3}$$

$$\kappa \text{ (Å}^{-1}) = 0.3295(I)^{1/2}$$

where k_∞ is the rate constant at infinite ionic strength when the screening of the reactant charges by the solvent is maximum. *V*, which is related to the interaction energy between reactant, depends linearly on the product, Z_1Z_2, of the local charges of the reactants and the distance r_{12} between the two reactants. ln k_2 depends inversely upon D_e, the dielectric constant at the interface of the reaction complex, and on ρ^2, the square of the cross-sectional dimension of the charged domain of plastocyanin. Since *V* is a unitless quantity, the interaction energy is obtained by multiplying *V* by *RT*. The ionic strength dependence of ln k_2 is expressed in the function *f*(*I*), to which it is linearly related (eq 1, above), and through the dependence of the Debye−Hückel parameter κ on the square root of the ionic strength.

For fitting to eq 1, the values for r_{12}, ρ, and D_e were 3.5 Å, 7 Å, and 10 (*26*). Results obtained from the fit were the product Z_1Z_2 and ln k_∞. The dependence of ln k_2 vs ionic strength for wild-type cyt *f* yielded a value of $Z_1Z_2 \approx -15.8 \pm 1.2$ and an interaction energy of −6.0 kcal/mol. A reasonable interpretation of this result is that there are +5

and −3 charges in wild-type cyt *f* and PC, respectively, that are involved in the formation of the reactive complex. A similar result was obtained with turnip cytochrome *f* and *C. reinhardtii* PC (Figure 5) with Z_1Z_2 equal to -18.9 ± 2.0 and an interaction energy of −7.0 kcal/mol. For the reaction between K188N−K189Q cyt *f* mutant and wild-type PC, a graph of ln k_2 vs ionic strength gives a value of ∼9.7 for Z_1Z_2 and an interaction energy of −3.6 kcal/mol after fitting to eq 1. This is consistent with neutralization of two of the five positive charges in the prominent basic patch of cyt *f*. The second-order rate constants at infinite ionic strength for wild-type, K188N−K189Q, and turnip cyt *f* obtained from curve-fitting are (M^{-1} s^{-1}) 7.7×10^6, 5.0×10^6, and 4.6×10^6, respectively.

As seen in eqs 1 and 2, Z_1Z_2 and V are very sensitive to changes in the value of ρ. The results of the fit are also subject to errors in the determination of k_2 from pseudo-first-order rate constants. Thus, although the Watkins model may be used to qualitatively describe effects of electrostatics on the reaction of two proteins, the number of local charges actually involved and the interaction energy obtained through the model may be approximations of the true values.

The dependence of ln k_2 on ionic strength for the K58Q−K65S−K66E cyt *f* mutant is very small. The loss of ionic strength dependence for K58Q−K65S−K66E cyt *f* mutant was surprising, because with these amino acid changes, the local charge in the 5 Lys region of cyt *f* should be equal to +1. A possible explanation for the disappearance of the ionic strength dependence is that the Lys66Glu mutation has a greater effect on the interaction of cyt *f* and PC than mutations at the other lysines. Removal of the basic patch in the large domain of cytochrome *f* resulted not only in a decrease in the bimolecular rate constant k_2 for cyt *f*/PC, but also in the loss of an observable rate dependence on the ionic strength of the solution. Although mutants E59K−E60Q−E43N of spinach plastocyanin caused a ∼30-fold decrease in reaction with cytochrome *f*, there was still an indication of ionic strength dependence as shown by a 10-fold difference in k_2 for ionic strength values of 0.03 and 0.3 M (*27*).

Under conditions where the rate constant for electron transfer is orders of magnitude larger than the rate constant for complex dissociation, the reaction is in the diffusion-limited regime (*48*). This is probably true for cyt *f*−PC in solution, and thus, k_2 is also a measure of the association of the two proteins. In previous experiments with cyt *f* and mutant forms of PC, no significant change in the intrinsic rate of electron transfer in solution was observed (*27*, *36*). The differences among the rate constants for the reactions of wild-type cytochrome *f* with mutant forms of plastocyanin, e.g., 2-, 3-, and 30-fold in mutants D42N, E43N, and E59K−E60Q−E43N, respectively, were attributed to variations in the values of the association constant. The equilibrium constant K_D for binding of wild-type cytochrome *f* and plastocyanin was determined at 5 mM ionic strength to be 0.2 ± 0.5 μM (Zhang, H., unpublished results), approximately 3 orders of magnitude smaller than the ∼140 μM obtained at 100 mM from NMR line-broadening experiments (*27*). Determination of the K_D was performed by measuring the enhancement of the Soret band at 410 nm of oxidized cytochrome *f* upon binding to oxidized plastocyanin as described in He et al. (*49*). The K_D for the binding of PC to the K58Q−K65S−K66E mutant of cytochrome *f* could not be determined accurately because of the extremely small absorbance changes at 410 nm. This implies that the binding of PC to the cytochrome *f* missing the lysine residue in the basic patch of the large domain is much weaker relative to wild-type, consistent with the inference of a weaker interaction from data on cyt *f* oxidation rates (Figure 5).

(D) pH Dependence of the Bimolecular Rate Constant. The possibility that an acidic pH in the chloroplast lumen in the energized state that would neutralize PC carboxylates could be responsible for the loss of electrostatic interaction between cyt *f* and PC was considered. This is unlikely to be the case because an excess of the protonophore FCCP was added to the reaction medium during measurements of flash-induced cyt *f* oxidation to eliminate the contribution of the carotenoid band shift to the measured absorbance changes. FCCP will collapse any existing transmembrane pH gradient so that the lumenal pH should be equilibrated with the other cellular compartments. In the present study, the pH of the suspending medium for cells of *C. reinhardtii* is 7.0, and so, under the conditions of the experiment, the pH of the thylakoid lumen should be close to neutrality. The pH dependence of the bimolecular rate constant, k_2, for oxidation of cyt *f* from wild-type *C. reinhardtii* by PC from the same source, in 0.10 M ionic strength, is ($M^{-1}s^{-1}$) 107 ± 5, 96 ± 13, 63 ± 2, and 50 ± 4 at pH 7.0, 5.5, 5.0, and 4.6, respectively. These experiments at acidic pH were done to mimic extreme pH values of the thylakoid lumen that might prevail under high light intensities. The decrease in cytochrome *f* oxidation rate at pH 4.6−5.0 could be attributed to the masking of an electrostatically mediated association or docking reaction involving basic residues other than those of cytochrome *f*. Alternatively, the decreased rate at pH 4.6−5.0 might be the result of a structural change in plastocyanin (*50*).

DISCUSSION

(1) True Value of k_{et} for Oxidation of Cytochrome f by Plastocyanin. The rate of intracomplex electron transfer, k_{et}, obtained by direct measurement was found to be 2800 s^{-1} in two independent studies (*25*, *26*). This rate is very similar to that which corresponds to the first-order oxidation of cyt *f* in vivo with a $t_{1/2} = 220-250$ μs. It is possible that this correspondence is fortuitous and the true rate constant for electron transfer from cyt *f* to PC is much faster. This is implied by calculations of a minimum value, 2.6×10^4 s^{-1}, of the rate constant for wild-type proteins from line broadening of the ^{1}H resonances of plastocyanin (*27*) in the presence of cytochrome *f*, using concentrations of both proteins 10^2-10^3 greater than what was used in the electron-transfer studies. Whether or not the oxidation rate measured in vivo represents the true k_{et}, the rate is independent of the presence of the cyt *f* lysine residues that are necessary for fast rates of cyt *f* oxidation in solution.

(2) Identification of Basic Residues of Cytochrome f Responsible for Initial Docking to PC in Solution. This study and others (*24*−*27*) have shown that although both cytochrome *f* and plastocyanin are negatively charged, the oxidation of cytochrome *f* by PC in solution is rate limited by long-range attractive electrostatic interactions involving local clusters of positively and negatively charged residues

in cyt *f* and PC, respectively. Mutations that removed some of the acidic residues of plastocyanin caused a decreased rate of electron transfer in solution by lowering the affinity between the two proteins (*27, 36*). However, the intrinsic rate of intraprotein electron transfer was not affected significantly.

The basic residues in cytochrome *f* that have been the subject of mutagenesis in the present study and in ref *35*, Lys 188 and 189 in the small domain and Lys 58, 65, and 66 in the large domain (Figures 1 and 5), have been shown in the present work to be responsible for the ionic-strength dependence of the cyt *f*/PC reaction in solution. Neutralization of Lys188 and 189 caused a decrease in the ionic strength dependence that could be attributed to a loss of two positive charges. Changing the net charge of Lys58 and 65−66 from +3 to −1 resulted in a decrease in the rate constant of cytochrome *f* oxidation by plastocyanin of at least 10-fold, and complete loss of the ionic strength dependence. This lysine-rich region includes Lys187 (residue 188 in *C. reinhardtii*) that was found from cross-linking experiments to dock to Asp44 of PC (*51*).

(3) Elimination of Electrostatically Mediated Docking as the Rate-Limiting Step in Vivo. The conclusion in this study that the initial recognition of diffusible plastocyanin by cytochrome *f* through electrostatic interactions does not limit the reaction rate in the thylakoid lumen could be explained by the following:

(a) Limited Diffusional Space in the Thylakoid Lumen. Conditions that are favorable in solution for electrostatic interactions, e.g., large diffusional space and low ionic strength, may not exist in the limited space of the thylakoid lumen. Within the thylakoid lumen, cytochrome *f* and plastocyanin interact in a very small space with an intermembrane dimension of 40−90 Å (*52, 53*). The problem may be not how the two proteins find each other, but rather how they avoid each other. Given the small intralumen diffusional volume and the tendency of plastocyanin to bind or adsorb to the membrane (*54*), the ability of plastocyanin and cytochrome *f* to search phase space for free-energy minima in their interaction configuration may be precluded or far more restricted than in solution. In this situation, the formation of predocking complexes would no longer be rate limiting. In addition, (i) cytochrome *f* is anchored to the thylakoid membrane and (ii) is associated closely with its electron donor, the Rieske [2Fe-2S] protein. The latter may dock with cytochrome *f* partly through the lysine residues, as suggested by a slower rate of cyt *f* reduction in a mutant in which all the lysine residues are altered (*35*). The lysine residues of cytochrome *f* that have been implicated in solution studies as docking sites for plastocyanin may be partly shielded in vivo by the Rieske protein and perhaps not fully available for docking to plastocyanin. (iii) The freedom of PC to diffuse may also be constrained in situ by its tendency to bind or adsorb to the membrane.

It was previously noted that the constrained geometry of the chloroplast lumen may be characteristic of late logarithmic phase *C. reinhardtii*, but not necessarily of all phases and conditions of culture growth. If the lumenal space would be increased by certain culture conditions or by environmental stress or membrane damage, there would be a need under such conditons for electrostatically mediated long-range attractive interactions. This has been demonstrated by a requirement for the interdomain cyt *f* lysines for a maximum rate of cytochrome *f* oxidation in permeabilized cells of *C. reinhardtii* (*55*).

(b). An effect of electrostatic interactions between cyt *f* and PC could also be hidden by a slow release of PC from P700. However, the time estimated for this dissociation, 50−100 μs (*54*), would by itself not account for a 200 μs half-time for cyt *f* oxidation.

(4) Structurally Defined Docked State of Cytochrome f and Plastocyanin. How is molecular recognition achieved between cytochrome *f* and PC in solution and what are the possible binding sites of PC on cytochrome *f* in order to obtain rate constants of 2800 s^{-1} for the electron transfer from cytochrome *f* to plastocyanin? The edge−Cu distance from the cyt *f* heme should be approximately 15 Å, assuming a reorganization energy of 1.0 eV (*33*). Studies of cyt *f*/PC docking based on their atomic structures suggest possible docking sites for PC, which do not involve charged residues, that bring the heme and Cu to a favorable electron transfer distance (*30*). Solution NMR studies (*56*) show that the cyt *f*/PC complex uses both hydrophobic and acidic patches on cyt *f*, and that there is only one complex in which the chemical shifts of PC are independent of ionic strength. In this configuration, the closest approach of cytochrome *f* to PC occurs between His87Nϵ2 of PC and Tyr1Cδ2 of cyt *f*, for which the Fe−Cu distance is about 11 Å. In this state, Lys 58, 65, and 66, which mediate the initial docking, are significantly displaced from PC carboxylate residues. Thus, the structurally defined docking intermediate must result following a rearrangement from the initial electrostatically mediated docked state. It has been proposed that while the electrostatic complexes are not themselves electron-transfer active, the complexes can rearrange into ones that are favorable for electron transfer (*28, 57*). Thus, the question arises as to whether the structurally defined cyt *f*/PC docked state will occur under the constrained spatial conditions in vivo.

(5) Small Compartment for Electron Transfer in Other Membrane Systems. Electron transfer from the cytochrome bc_1 complex via cyt c_1 to cyt *c* in the intermembrane space of mitochondria (*58*) and from cyt c_1 to cyt *c* in (*59*) and from cyt c_2 to the RC (*11*) in the periplasmic space of *Rb. sphaeroides* has been shown in solution to be governed by electrostatic forces. The effective volume of these intermembrane spaces that is available for free diffusion of cytochrome *c* is not known. It would be possible to test the role of electrostatic interactions on electron transfer in vivo in photosynthetic bacteria by the same experimental logic used in ref *35* and the present study.

(6) Reciprocity of Conserved Basic and Acidic Residues of Cytochrome f and Plastocyanin. A role for the interdomain charged residues of cytochrome *f* in electrostatic docking would seem to be implied by (i) the high degree of conservation of these residues (*23*) and (ii) the reciprocal exchange of the acidic character of plastocyanin and the small domain of cytochrome *f* the cyanobacteria *Nostoc PCC 7906* and *Phormidium laminosum* (*23, 24, 60*). The caveat to argument (i) is that the entire cytochrome *f* amino acid sequence is very highly conserved, 64% in 11 plant sequences. Thus, it is difficult to use sequence identity as a strong argument in this case. Argument (ii) is weakened by the fact that it holds only for some cyanobacteria and

particularly by the facts: (a) the "basic patch" is almost completely neutral in the cyanobacterium *Synechocystis* PCC sp 6803 (*23*); (b) although the regions of basic and acidic regions of cyt *f* and PC in the putative docking domain are, respectively, less basic and less acidic in the cyanobacterial sequences, there is no conserved reciprocal exchange in these regions of basic and acidic residues. There are two positions in the cyt *f* sequences, residue 63 in the large domain and residue 184 in the small domain, that contain acidic residues only in the four cyanobacterial sequences. Lys66 is conserved in all sequences. However, it seems unlikely that only one acidic residue in each domain of cyt *f* supports electrostatic docking, especially since one cannot see a pattern of reciprocal changes in the available cyanobacterial PC sequences.

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