Effect of the Interdomain Basic Region of Cytochrome *f* on Its Redox Reactions *in Vivo*†

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ABSTRACT: The prominent interdomain basic surface region seen in the high-resolution structure of the active lumen-side C-terminal fragment of turnip cytochrome *f*, containing the conserved Lys58,65,66 (large domain) and Lys187 (small domain), has been inferred from *in vitro* studies to be responsible for docking of its physiological oxidant, plastocyanin. The effect of the putative docking region of cyt *f* on its reactivity *in vivo* was tested by site-directed mutagenesis in *Chlamydomonas reinhardtii.* Three charge-neutralizing mutants were constructed involving: (i) the two lysines (Lys188Asn-Lys189Gln) in the small domain, (ii) the three lysines (Lys58Gln-Lys65Ser-Lys66Glu) in the large domain, and (iii) all five of these lysines spanning both domains. All mutants grew phototrophically. The mutants displayed a 20−30% increase in average generation time, and comparable decreases in rates of steady-state oxygen evolution and the slow (millisecond) electrochromic 515 nm band shift. The magnitude of the changes was greatest in the 5-fold Lys-minus mutant (Lys58Gln-Lys65Ser-Lys66Glu-Lys188Asn-Lys189Gln). The mutants showed a small increase (∼25%) in the $t_{1/2}$, from 0.2 to 0.25 ms, of cyt *f* photooxidation, far less than anticipated (ca. 100-fold) from *in vitro* studies of the effect of high ionic strength on the cyt *f*−PC interaction. The $t_{1/2}$ of cyt *f* dark reduction via the Rieske protein increased from 5−6 ms in the wild type to 11−12 ms in the 5-fold Lys-minus mutant. Cells grown phototrophically in the absence of Cu, where cyt c_6 is the electron acceptor of cyt *f*, displayed net rates of cytochrome photooxidation that were slightly faster than those in the presence of Cu, which also decreased by a factor of ≤25% in the Lys-minus mutants. It was concluded that (a) the net effect of electrostatic interaction between cytochrome *f* and its electron acceptor *in vivo* is much smaller than measured *in vitro* and is not rate-limiting. This may be a consequence of a relatively high ionic strength environment and the small diffusional space available for collision and docking in the internal thylakoid lumen of log phase *C. reinhardtii.* (b) The efficiency of electron transfer to cytochrome *f* from the Rieske protein is slightly impaired by the neutralization of the lysine-rich domain.

The atomic structure of the redox-active, 252 residue lumen-side domain of the *trans*-membrane cytochrome *f* from turnip chloroplasts has been solved to a resolution of 1.96 Å (Martinez *et al.*, 1994, 1996). One set of questions to be addressed by this structure is the nature of the docking site and electron transfer pathway between cytochrome (cyt)[1] *f* and its physiological electron acceptors, the copper protein plastocyanin (Figure 1), or the alternative carrier cytochrome c_6 in *Chlamydomonas reinhardtii* (cyt c_{553} in cyanobacteria) grown in a low Cu environment.

Before the cytochrome *f* structure was known, it was inferred from *in vitro* biochemical, spectroscopic, and mutagenesis studies on plastocyanin (Morand *et al.*, 1989; Bagby *et al.*, 1990; Nordling *et al.*, 1990; He *et al.*, 1991; Roberts *et al.*, 1991; Modi *et al.*, 1992a,b; Qin & Kostic, 1992; Gross, 1993) that the cyt *f*−PC docking interaction has a significant electrostatic component. A noncovalent electrostatic complex was inferred to exist at low ionic strength between soluble turnip cytochrome *f* and plastocyanin from the pronounced decrease (10^2-fold for a 10-fold increase in ionic strength) with increasing ionic strength of the rate of PC reduction by ferrocytochrome *f*. It was also inferred that the electrostatic interaction was mediated by local charges on cyt *f*, and that the number of local positive charges on turnip cyt *f* involved in complex formation with plastocyanin is 5 (Qin & Kostic, 1992). A decrease of approximately 10^2-fold in the rate of oxidation of turnip cytochrome *f* by PC was also observed when the ionic strength was increased 10-fold from a value of 0.04 M associated with the maximum electron transfer rate to values near 0.4 M (Meyer *et al.*, 1993), the latter approximately equal to the chloroplast internal ionic strength (Kaiser *et al.*, 1983).

The intracomplex first-order rate constant for electron transfer involving turnip cyt f was measured by flash spectroscopy and found to be 2800 ± 300 s^{-1} in either direction at an ionic strength of 4 mM (Qin & Kostic, 1992). A similar rate constant for electron transfer from spinach cyt *f* to PC was determined, also assayed by flash spectros-

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[1] Abbreviations: Chl, chlorophyll; cyt, cytochrome; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EDC, *N*-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HS, HSA, high salt, high salt−acetate; MES, 2-(*N*-morpholino)ethanesulfonic acid; M_r, relative molecular weight; MW, molecular weight; *p*, electrochemically positive side of the membrane; PC, plastocyanin; PCR, polymerase chain reaction; PQ, plastoquinone; PS, photosystem; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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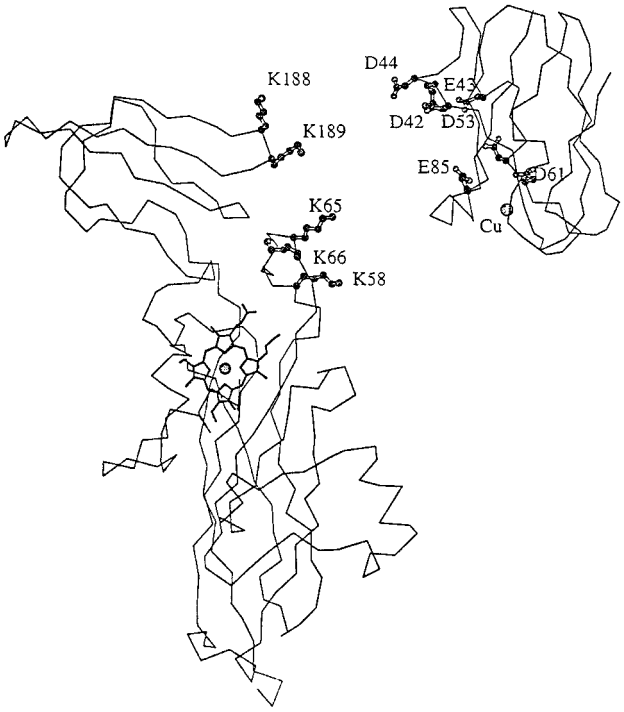

FIGURE 1: Wire diagram of *C. reinhardtii* cytochrome *f* (Martinez *et al.*, 1994) (left) and plastocyanin (Redinbo *et al.*, 1993) (right) showing a predocking state that would precede the mode of electrostatically-driven docking discussed in the present study. The prominent basic patch on cyt *f* includes Lys188-Lys189 in the small domain and Lys58, Lys65, and Lys66 in the large domain. Prominent acidic surface regions on PC that have been implicated in the cyt *f* docking reaction are Asp42-Glu43-Asp44, Asp53, Asp59, Asp61, and Glu85. Because the crystal structure of *C. reinhardtii* cyt *f* has not been separately determined, the structure of turnip cyt *f* was used as a framework in which the surface Lys residues around Lys187 in turnip cyt *f* were replaced by the analogous residues of *C. reinhardtii*. Picture drawn using Molscript (Kraulis, 1991).

copy (Meyer *et al.*, 1993). A 20-fold larger rate constant ($\sim 6 \times 10^4$ s^{-1}, at an ionic strength of 0.1 M corresponding to a $t_{1/2}$ $\sim$10 μs) was calculated from stopped-flow measurements from a small difference on a 10 ms time scale between the time course of absorbance changes associated with binding and with electron transfer (He *et al.*, 1991).

One of the significant features of the cytochrome *f* structure is a predominant basic region of the protein at the interface between its large and small domains, near Lys187 [Lys189 in the green alga *Chlamydomonas reinhardtii* (Gray, 1992)] in the small domain that was shown (Morand *et al.*, 1989) to cross-link to Asp44 in a predominantly acidic region of plastocyanin (Figure 1). The other residues in the basic region of turnip cyt *f* are Lys58, Lys65, Lys66, and Arg209, a total of five potentially positive charges available for interaction with plastocyanin, consistent with the prediction of Qin and Kostic (1992). Except for Arg209, the above residues are conserved in at least nine plant sequences and *C. reinhardtii* (Gray, 1992). Wire diagrams of cyt *f* and PC are shown with residues enumerated that may function in the electrostatic docking interaction (Figure 1). There is an additional lysine at position 188 in *C. reinhardtii* (Gray, 1992).

Along with the above data that imply a major role of electrostatic docking interactions *in vitro* between cyt *f* and plastocyanin, the interaction of plastocyanin with two cyt *c* molecules indicated that the reduction of the plastocyanin Cu can occur through docking to a hydrophobic surface patch at a rate comparable to that via the surface acidic patch (Qin & Kostic, 1996). A role for a hydrophobic docking interaction was also proposed from the biphasic response to ionic strength of the rate of the turnip cyt *f*−PC *in vitro* reaction, the reaction rate increasing as the ionic strength (*I*) was increased from 0 to 0.04 M and decreasing as *I* was increased further. This implied that nonelectrostatic or hydrophobic reactions can dominate for $I < 0.04$ M, a value of the ionic strength that is approximately 10-fold lower than that of the chloroplast interior (Kaiser *et al.*, 1983). From modeling of the docking reaction of cyt *f* and PC based on the known structures (Guss & Freeman, 1992; Martinez *et al.*, 1994), it was proposed that docking of PC with cyt *f* initially utilizes a long-range (ca. 30 Å from the heme at an ionic strength of 0.1 M) attractive electrostatic interaction that is propagated from the interdomain lysine-rich region of cyt *f* (Pearson *et al.*, 1996). The initial electrostatic guidance and contact were proposed to be followed by nonelectrostatic surface interactions and movement of PC on the surface of cyt *f* that would allow the Cu to approach closer to the heme (Pearson *et al*., 1996).

In addition to the cyt *f*−PC interaction, there is a substantial precedent for mediation by electrostatic interactions of the docking and formation of other interprotein electron transfer complexes. The role of such interactions would again be to utilize the relatively long-range physical nature of these interactions for "guidance" of the docking reactant proteins, and to use the complementary basic and acidic amino acid residues on the respective proteins to provide for their orientation in the docking. The additional precedents include some that involve plastocyanin or similar proteins: (i) the electron transfer reaction of plastocyanin with its electron acceptor, the photosystem I complex, via the positively charged *psa*F subunit which is believed to complex with plastocyanin (Farah *et al.*, 1995; Drepper *et al.*, 1996); (ii) the complex of yeast cytochrome *c* peroxidase with cytochrome *c* from (a) horse mitochondria, which has been shown from the crystal structure of the complex to be mainly mediated by electrostatic interactions, and from (b) yeast mitochondria, where these redox proteins associate through hydrophobic and van der Waals forces (Pelletier & Kraut, 1992; Miller *et al.*, 1996). Substitution of a basic for an acidic residue (Asp37) on the surface of cyt *c* peroxidase resulted in a 10-fold decrease in affinity and turnover number for cytochrome *c* (Corin *et al*., 1991). (iii) Amicyanin, which is related in structure to plastocyanin, interacts *in vitro* with cytochrome *c*-551 through polar and charged residues in the ternary methylamine dehydrogenase complex (Chen *et al.*, 1994).

The relevance of the electrostatic interaction between these redox partners *in vivo* may depend upon (i) ambient ionic strength and (ii) spatial constraints. There have been few or no *in vivo* tests of the nature of the requirement for electrostatic interactions between the above pairs of proteins. In the present study, the necessity of electrostatic interactions between cyt *f* and its electron acceptors was tested *in vivo* using site-directed substitution in cyt *f* of the lysines shown in Figure 1, which shows wire diagrams of *C. reinhardtii* plastocyanin (Redinbo *et al.*, 1993) and that predicted for *C. reinhardtii* cytochrome *f* based on the structure of turnip cyt *f* (Martinez *et al.*, 1994).

MATERIALS AND METHODS

(1) Cultivation and Growth of C. reinhardtii. *C. reinhardtii* was grown either phototrophically in liquid cultures in HS medium with 100 nM Cu (Harris, 1989), bubbled with air−5% CO_2, light intensity 90−100 μE/(m^2•s), or heterotrophically in HSA medium, light intensity 5−10 μE/(m^2•s). The cells were then harvested during the later part of their logarithmic growth phase, and sedimented at 500−1000*g*. Cu was omitted in the trace elements of the medium for growth in the absence of Cu (Merchant *et al.*, 1991). Cells were grown through three cycles in the absence of Cu (medium Cu concentration <6 nM). The final concentration of the resuspended cells was equivalent to 1 mg/mL chlorophyll.

Growth was assayed by counting cells (Harris, 1989) with a hemocytometer and a Wild Light Microscope under bright field illumination.

(2) Mutagenesis and Transformation. A 2.0 kb region encompassing the *pet*A gene with a 0.8 kb 5′ flanking sequence was amplified by PCR from plasmid P-87c (Duke University *Chlamydomonas* Center) containing the 11.6 kb chloroplast DNA *Bam*HI 7 fragment as an insert. The PCR product was cloned into the *Eco*RV site of the pBluescript II KS^+ vector (Stratagene), with the *pet*A gene start close to the *Eco*RI site of the vector, to generate plasmid pTP101. The *petA* sequence was confirmed by DNA sequencing. The P-87c stock plasmid was cut with *Pst*I, and the resulting fragment of chloroplast DNA was digested with *Hin*dIII. The *Hin*dIII fragment was ligated with *Hin*dIII-digested pTP101, giving plasmid pTP102 with an intact *pet*A gene and 2.5 kb of 3′ flanking region of chloroplast DNA. The *aad*A cassette (Goldschmidt-Clermont, 1991), coding for aminoglycoside-3″-adenyl transferase, was inserted into the *Eco*RV site located 0.3 kb downstream from the *pet*A gene to generate plasmid pTP103.

Site-directed mutagenesis was performed in *Escherichia coli* according to Kunkel (1985). The pTP101 single-strand DNA was annealed with mutagenic oligonucleotide primer, and the second strand DNA synthesis reaction was carried out. The mutagenic primers, with nucleotides differing from wild type written in boldface type, were 5′-CTTTCT-GAGAA**CC**AAGGTGGTTTT-3′, which also introduced a unique *Sty*I site (underlined) to generate the double mutant Lys188Asn-Lys189Gln, and 5′-TAAACAAGTT**C**AA-CAAGTGCTAGCTAATGGTA**GCG**AAGGT-GACTTAAACG-3′ (the interjected unique and silent *Nhe*I site is underlined) to generate the triple mutant Lys58Gln-Lys65Ser-Lys66Glu. Screening of candidate colonies was carried out using the unique restriction sites for each mutation. The mutated sequence was confirmed by DNA sequencing. (iii) The 5-fold mutant was generated using the internal *petA Bst*EII site, unique in the pTP101 plasmid, to combine the double and triple Lys^- mutations. The *Xba*I−*Afl*II fragments carrying mutated sequences were recloned into the pTP103 construct to replace the wild-type *pet*A sequence.

Chloroplast transformation was carried out with a *C. reinhardtii* Δ*pet*A strain (Zhou *et al.*, 1996). The Δ*pet*A strain showed no PCR product of the cytochrome *f* gene, showed no light-induced cyt *f* response, and did not grow phototrophically [30−40 μE/(m^2•s) illumination] on HS plates. The "wild type" used in all experiments was made by transformation of the Δ*pet*A strain with a plasmid containing the wild-type *pet*A gene. Transformation was performed using a biolistic gun (Boynton & Gillham, 1993). Cells on the HSA plates were bombarded with tungsten microprojectiles, coated with plasmid DNA (approximately 5 μg). Spectinomycin-resistant transformants were selected on HSA agar in the presence of 100 μg of spectinomycin. Cells from single spectinomycin-resistant colonies were propagated and probed for expression of cytochrome *f* by Western blot or heme stain. Strains showing cyt *f* expression were stored on HSA agar in the presence of 70 μg of spectinomycin. Phototrophic cultures were started by inoculating cells from stock HSA agar plates. The mutant cell strains were always maintained on acetate-containing plates in dim [5 μE/(m^2•s)] light. Phototrophically grown cells were screened by isolating whole cell DNA, amplifying the *pet*A gene fragment by PCR. The PCR fragments were subjected to *Nhe*I (triple Lys^-), *Sty*I (double Lys^-), or *Nhe*I, *Sty*I (quintuple mutant) restriction digestion. The demonstration of the unique restriction site (sites) indicated the presence of the mutation. The mutation was also confirmed by sequencing after the PCR product was cloned into the pGEM-T vector (Promega). The triple Lys^- mutant was rescreened by sequencing over 60 bp on either side of the original mutations after storage for 1 year on HS plates to document that the mutation was preserved. In addition, to search for the possibility of intragenic second-site revertants, the entire cytochrome *f* gene, amplified from phototrophically-grown pentuple Lys mutant cells, was sequenced except for the first 60 bp (20 residues). The sequence was found to be unchanged from that of the pentuple mutant except for a silent substitution, CCG→CCA, in the codon for Pro86, which could be attributed to an error of the Taq enzyme in the amplification reaction.

(3) SDS−PAGE. *C. reinhardtii* cells, at a concentration equivalent to approximately 1 mg/mL Chl, were frozen and thawed in 10 mM Tris-HCl, pH 7.0. Proteins from the whole cell extract or the soluble fraction of broken cells were separated on SDS−PAGE, using the gel system of Schägger and von Jagow (1987) with a 9.9−16.5% (w/w) acrylamide concentration gradient [acrylamide:(bisacrylamide) ratio = 8.25:1].

(4) Heme Staining of Gels. Samples were dissolved immediately before electrophoresis in 2% SDS, 50 mM Tris, pH 7.5, and 10% glycerol at 90 °C for 40 s. Staining of heme proteins was performed according to Thomas *et al.* (1976).

(5) Oxygen Evolution. Rates of oxygen evolution of whole cells were measured at room temperature with a Clark-type oxygen electrode, as described previously (Tae & Cramer, 1994). The reaction medium consisted of 40 mM HEPES buffer, pH 7.5, 10 mM bicarbonate, and *C. reinhardtii* cells at a Chl-equivalent concentration of 10 μg/mL.

(6) Cytochrome f Oxidation. Cytochrome *f* oxidation in whole cells was induced by a short, saturating flash from a xenon lamp with a Corning 2-58 filter to select light of wavelength greater than 600 nm. The time course of the oxidation and re-reduction of cyt *f* was measured with a weak [0.1−0.2 μE/(m^2•s)] beam from a 150 W Oriel tungsten lamp, incident on the sample 110 ms before the flash and for a total of 150 ms. The reaction medium in the cuvette volume of 2 mL consisted of 20 mM MES/NaOH, pH 7.0, 10% Ficoll, 10 μM DCMU/1 mM hydroxylamine to inhibit

Table 1: Comparison of (A) Growth Rates, Rates of O_2 Evolution, and (C) Half-Times of the Slow Electrochromic Band Shift for Phototrophically-Grown Wild-Type and Lysine$^-$ Mutants of *Chlamydomonas reinhardtii*[a]

	wild type	K188N-K189Q	K58Q-K65S-K66E	K188N-K189Q-K58Q-K65S-K66E
A	8.7 ± 1.1[b]	9.5 ± 1.8	10.4 ± 1	11.9 ± 1.1
B	157 ± 20[c] ($n = 3$)[d]	114 ± 22 ($n = 5$)	106 ± 13 ($n = 3$)	81 ± 28 ($n = 3$)
C	1.9 ± 0.2[e] ($n = 3$)	2.4 ± 0.2 ($n = 2$)	3.6 ± 0.6 ($n = 2$)	3.5 ± 0.2 ($n = 3$)

[a] Growth in the presence of Cu (see Materials and Methods); rates are expressed as the time (hour) for doubling of the cell population. [b] Approximately 10 points on each growth curve; standard deviations include all differences between consecutive points on growth curves. [c] Micromoles of O_2 per hour per milligram of Chl, light intensity, 2000 μE/(m^2·s). [d] *n*, number of trials. [e] Half-time of rise of slow electrochromic band shift, in milliseconds.

PSII, 40 μM FCCP, and *C. reinhardtii* cells at a chlorophyll concentration of 30 μg/mL. The sample was preilluminated with saturating light for about 1 min and then incubated in the dark for about 15 min before data acquisition was started. The absorbance changes were measured in the presence of DCMU/NH_2OH to minimize reduction by PSII, and the protonophoric uncoupler FCCP to minimize the contribution of the electrochromic band shift. Using cells of *C. reinhardtii*, the uncoupler does not completely suppress the residual contribution of the electrochromic band shift at 554 nm. Therefore, the measured absorbance change at 554 nm was corrected at all measured time points for residual electrochromic band shift and nonspecific absorbance changes by subtraction of weighted absorbance changes at reference wavelengths at 545 and 572 nm and a ramp correction method (Joliot & Joliot, 1984; Kuras *et al.*, 1995). The corrected change in absorbance of cytochrome *f* at 554 nm, Abs(cyt *f*), was derived from the measured absorbance changes, $A(\lambda)$, at wavelength λ by using the formula: Abs(cyt *f*) = $A(554) - {}^1/_3[2A(545) + A(572)]$.

The detector was a UV444BQ photodiode protected from the effects of the Xenon flash by a Corning 4−96 filter and a Corion LS-600 filter. The data were acquired using Labview 3.1 software from National Instruments, Inc.

(7) Electrochromic Band Shift. The slow electrochromic band shift (Joliot & Delosme, 1974) induced by a saturating light flash was measured at 515 nm. Reaction conditions are as described above for cytochrome *f* oxidation, except for the omission of DCMU, hydroxylamine, and FCCP.

RESULTS

(1) Mutants of Cytochrome f. The hypothesis that the patch of basic residues that bridges the large and small domains of cytochrome *f* is involved in the docking with plastocyanin (Figure 1) was tested by substitution of the Lys residues in this patch through site-directed mutagenesis and transformation of a cytochrome *f* deletion mutant (Zhou *et al.*, 1996) of *Chlamydomonas reinhardtii* as described under Materials and Methods. The physiological consequences, and the kinetics and amplitude of cyt *f* oxidation, were studied in whole cells of wild-type and mutant strains of *C. reinhardtii* in which (i) Lys188 and -189, (ii) Lys58, -65, and -66, and (iii) all five lysines were replaced by neutral or acidic (K66E) residues in (i) a double (K188N-K189Q), (ii) a triple (K58Q-K65S-K66E), and (iii) a quintuple Lys$^-$ mutant (K188N-K189Q-K58Q-K65S-K66E) that combined the mutations in (i) and (ii).

(2) Physiological Properties of Mutants. The rates of growth (Table 1A) and oxygen evolution (Table 1B) of the transformed mutants under phototrophic conditions were slightly decreased relative to the wild type. The decrease in growth and oxygen evolution rates of (a) the double and triple mutants was 15−30%, and (b) of the quintuple mutant was 30−50% (Table 1A,B), for cells grown in the presence of Cu, for which plastocyanin is the electron acceptor (Merchant *et al.*, 1991).

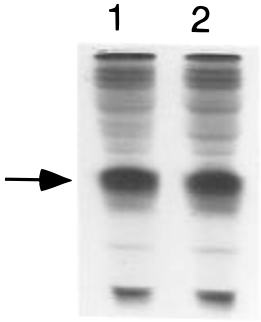

FIGURE 2: Comparison of level of cytochrome *f* expression of phototrophically-grown wild-type and triple Lys$^-$ mutant. Whole cell protein extracts, 20 μg of Chl per lane; cyt *f* levels assayed by heme stain (Thomas *et al.*, 1976). Arrow points to cyt *f*.

(3) Expression of Cytochrome f Mutants. The expression of mutant cytochrome *f* was compared to that of wild type (lane 2 vs 1 in Figure 2), and was shown for the triple Lys$^-$ mutant to be approximately the same as that of wild type in phototrophically-grown cells as shown by heme stain (Figure 2). The levels of expression of the double and quintuple Lys$^-$ mutants were not checked in this way because there was no indication from the flash spectroscopic assay (see below, Figures 5 and 6) of any significant deficiency in the phototrophically-grown mutants.

(4) Electron Acceptor of Cytochrome f in Wild-Type and Lys$^-$ Mutants in the Presence and Absence of Cu. The electron acceptor of cyt *f* in *C. reinhardtii* has been shown to change from Cu-containing plastocyanin to cytochrome c_6 when the growth medium is depleted of Cu (Merchant *et al.*, 1991). This transition occurred after three culture cycles of growth of the wild type (Figure 3) in the absence of Cu ($\leq$6 nM Cu). Cyt c_6 is found in the wild type grown in the absence (Figure 3, lane 2) but not in the presence (lane 1) of Cu, and also in the double (lane 3), triple (lane 4), and quintuple (lane 5) mutants grown for three culture cycles in the absence of Cu. The transition from plastocyanin to cyt c_6 was not reproducible if the cells were grown for fewer than three cycles in the Cu-deficient medium.

(5) Oxygen Evolution of Wild-Type and Lys$^-$ Mutants. The steady-state rates of oxygen evolution in the double and triple Lys$^-$ mutants are ${}^3/_4$ and ${}^2/_3$, and that of the quintuple Lys$^-$ mutant somewhat more than ${}^1/_2$, that of the wild type (Table 1B). All cell cultures for these measurements, as well as the flash experiments described below, were harvested in late-logarithmic phase. Within experimental error, there is

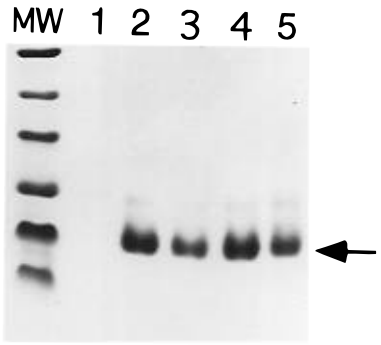

FIGURE 3: Detection of cytochrome c_6 expression in wild-type and Lys$^-$ mutants by heme staining. Protein in soluble fraction derived from broken cells equivalent to 10 μg of Chl was added to each lane. Lanes: (1) control, wild type, "plus Cu"; (2) wild-type, "minus Cu"; (3−5) double Lys$^-$ mutant, K188N-K189Q, triple mutant, K58Q-K65S-K66E, and quintuple mutant, K188N-K189Q-K58Q-K65S-K66E, respectively, grown in the absence of Cu. MW, molecular mass markers (kDa), 43, 29, 18.4, 14.3, 6.2, 2.3 and 3.4. Arrow points to cyt c_6.

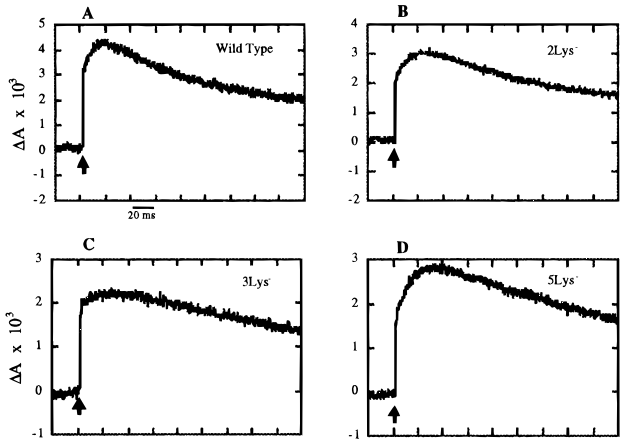

FIGURE 4: Flash-induced electrochromic band shift *in vivo* in phototrophically-grown *C. reinhardtii*: wild type (A), double Lys$^-$ mutant K188N-K189Q (B), triple mutant K58Q-K65S-K66E (C), and quintuple mutant K188N-K189Q-K58Q-K65S-K66E (D), grown in the presence of Cu. Reaction medium: 20 mM MES/NaOH/10% Ficoll, pH 7.0. Each time course is an average of 10 flashes with 5 s of darkness between sweeps. Arrow indicates imposition of light flash. Time scale the same for all panels.

a pattern of a decrease in these rates, as well as the growth rates, with increasing number of mutated lysines.

(6) Flash-Induced Electrochromic Band Shift of Wild-Type and Lys$^-$ Mutants. The carotenoid electrochromic band shift of wild-type *C. reinhardtii* contains a slow (millisecond) component (Figure 4) that arises from *trans*-membrane charge separation in the cytochrome b_6f complex (Joliot & Delosme, 1974l; Velthuys, 1978). In the present experiments, the ratio of fast and slow components is characterized by an approximate 2:1 ratio of fast ($t_{1/2}$ not resolved) and slow components. The time course of the band shift of all the Lys$^-$ mutants is similar to that of the wild-type (Figure 4A) and the double (Figure 4B), triple (Figure 4C), and quintuple (Figure 4D) Lys$^-$ mutants in the presence of Cu. As was found for the growth rates and oxygen evolution discussed above (Table 1A,B), the rate of the slow electrochromic phase, characterized by the $t_{1/2}$ of an approximately monoexponential process, decreases with increasing number of mutated lysines (Table 1C). The maximum decrease of 2-fold in the $t_{1/2}$ of the pentuple mutant (Table 1C) is similar to the changes of the O_2 evolution rates (Table 1B). Single

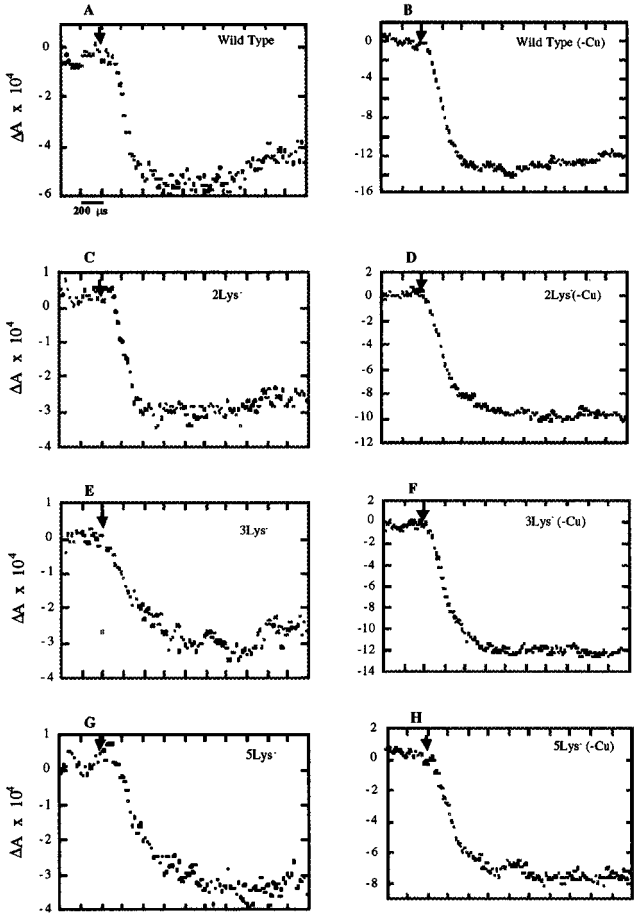

FIGURE 5: Flash-induced oxidation of cytochrome *f in vivo* using phototrophically-grown *C. reinhardtii*: wild type (A, B), double Lys$^-$ mutant K188N-K189Q (C, D), triple mutant K58Q-K65S-K66E (E, F), and quintuple mutant K188N-K189Q-K58Q-K65S-K66E, grown in the presence (A, C, E, G) and absence (B, D, F, H) of Cu. Reaction medium: 20 mM MES/NaOH/10% Ficoll, pH 7.0, 10 μM DCMU, 1 mM hydroxylamine, and 40 μM FCCP and cells with [Chl] = 30 μg/mL. Each time course is an average of 36 flashes with 5 s of darkness between sweeps. Arrow indicates imposition of light flash. Time scale the same for all panels.

measurements of the $t_{1/2}$ of the electrochromic band shift of the wild type and each mutant grown in the absence of Cu showed very similar $t_{1/2}$ values (data not shown).

(7) Flash-Induced Oxidation and Reduction of Cytochrome f in Wild-Type and Lys$^-$ Mutants. The rate of cytochrome *f* oxidation upon imposition of a light flash (Figure 5) and its subsequent re-reduction (Figure 6) were measured at 554 nm, the peak of its reduced − oxidized difference spectrum. The absorbance changes were measured in the presence of (a) DCMU/NH_2OH, to minimize reduction by PSII, and (b) the protonophoric uncoupler FCCP, to minimize the contribution of the electrochromic band shift. Using cells of *C. reinhardtii*, the uncoupler does not completely suppress the contribution of the electrochromic band shift at 554 nm. Therefore, the measured absorbance change at 554 nm was corrected for the residual electrochromic band shift and nonspecific absorbance changes at all time points as described under Materials and Methods.

The corrected absorbance change at 554 nm arising from cyt *f* oxidation in wild-type phototrophic cells in the presence of Cu was 4×10^{-4} (Figures 5A and 6A). Using a differential extinction coefficient at 554 nm of 25−26 mM^{-1} cm^{-1} (Metzger *et al.*, 1996) and taking into account the

Table 2: Half-Times for Oxidation and Re-reduction of Cytochrome *f* in Wild-Type and Mutant *Chlamydomonas reinhardtii*, in the Presence (A) and Absence (B) of Cu

	wild-type and mutant strains	$t_{1/2}$(oxidation),[a] μs	$t_{1/2}$(rereduction),[b] ms
(A)	wild type	202 ± 11 ($n = 5$)[c]	5.4 ± 2.6 ($n = 6$)
	K188N-K189Q	226 ± 40 ($n = 7$)	6.7 ± 1.6 ($n = 7$)
	K58Q-K65S-K66E	233 ± 51 ($n = 4$)	6.8 ± 2.0 ($n = 4$)
	K188N-K189Q-K58Q-K65S-K66E	253 ± 23 ($n = 6$)	11.1 ± 3.7 ($n = 4$)
(B)	wild type	146 ± 11 ($n = 3$)[c]	5.4 ± 2.0 ($n = 3$)
	K188N-K189Q	183 ± 13 ($n = 3$)	6.9 ± 0.8 ($n = 3$)
	K58Q-K65S-K66E	165 ± 16 ($n = 5$)	7.9 ± 2.6 ($n = 5$)
	K188N-K189Q-K58Q-K65S-K66E	180 ± 24 ($n = 4$)	7.3 ± 0.4 ($n = 4$)

[a] $t_{1/2}$, half-time for oxidation of cytochrome *f*, in microsecond. [b] Half-time for re-reduction of cyt *f*, in milliseconds. [c] *n*, number of trials.

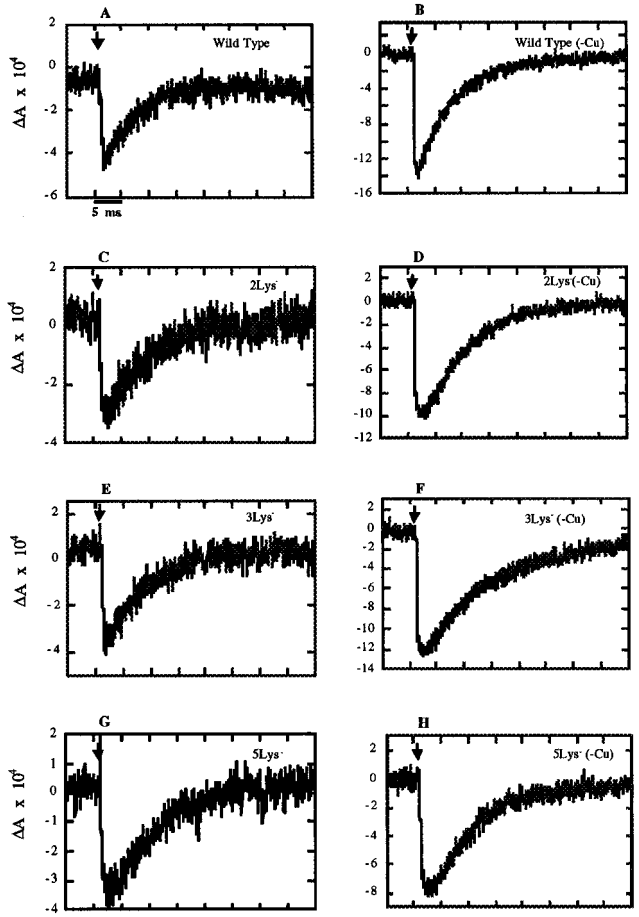

FIGURE 6: Flash-induced oxidation of cytochrome *f in vivo* using phototrophically-grown *C. reinhardtii*: wild type (A, B), double Lys⁻ mutant K188N-K189Q (C, D), triple mutant K58Q-K65S-K66E (E, F), and quintuple mutant K188N-K189Q-K58Q-K65S-K66E (G, H), grown in the presence (A, C, E, G) and absence (B, D, F, H) of Cu. Experiments as in Figure 5 except for display on a slower time scale. Arrow indicates imposition of light flash. Time scale the same for all panels.

chlorophyll concentration, this absorbance change corresponds to about 1 cyt *f* molecule per 2000 chlorophyll molecules, corresponding to oxidation of approximately 35−40% of the cyt *f*. The amplitude of the flash-induced absorbance change of cytochrome *f* in the presence of Cu was the same in the double (Figures 5C and 6C), triple (Figures 5E and 6E), and quintuple mutants (Figures 5G and 6G), consistent with the presence in these mutants of the same amount of assembled cytochrome *f* as in wild type, as shown above for the triple mutant by heme stain (Figure 2A).

The mean half-times ($t_{1/2}$) for the oxidation and reduction of wild-type cyt *f* in the presence of Cu were 0.20 and 5.4 ms, respectively (Figures 5A and 6A; Table 2A). An anticipated time-lag after the flash (Whitmarsh & Cramer, 1979) of approximately 50 μs is observed before the onset of a detectable oxidative absorbance change. The range of $t_{1/2}$ measured previously for the photooxidation of cyt *f in vivo* was estimated to be 0.11−0.35 ms (Delosme, 1991). The half-times of oxidation (Figure 5C,E,G) and re-reduction (Figure 6C,E,G) in the presence of Cu of the double (Figures 5C and 6C), triple (Figures 5E and 6E), and quintuple (Figures 5G and 6G) Lys⁻ mutants are 0.23 and 6.7 ms for oxidation and reduction of the double mutant, 0.23 and 6.8 ms (triple mutant), and 0.25 and 11.1 ms (quintuple mutant) (Table 2A). Thus, the oxidation rates are 15−25% slower for the mutants compared to wild type. Heterotrophic growth of the quintuple Lys⁻ mutant had no significant effect on the rates of cyt *f* oxidation and reduction (data not shown). However, there is no clear correlation between decreased rate and extent of the Lys⁻ mutations, and the effect on the oxidation rate is much smaller than would be expected from removal of an obligatory electrostatic binding step (Qin & Kostic, 1992; Meyer *et al*., 1993). Unexpectedly, the rate of reduction of cytochrome *f*, which is correlated with the rate-limiting step of photosynthetic electron transport, was also decreased in the mutants. The probability (Lewis, 1966) that the re-reduction half-time for the 5-fold Lys⁻ mutant (11.1 ± 3.7 ms) is different from that of the wild type (5.4 ± 2.6 ms) is 0.98. While the half-times for the double and triple Lys⁻ mutants are not significantly different from that of the wild type, the ratios of the rates of cyt *f* reduction in the different mutants relative to the wild type are similar to the respective ratios of the rates of O_2 evolution (Table 1B). This correlation is expected if the hierarchy of rates is correct and if changes in the rate of reduction of cytochrome *f* correlate with the rate-limiting step of photosynthetic electron transport (Whitmarsh & Cramer, 1979). It may be noted that in the context of the Q-cycle model for electron transfer in the b_6f complex, the half-times for the slow electrochromic band shift (Table 1C) appear to be appreciably smaller than those for cyt *f* reduction (Tables 1C and 2A), a disparity noted previously (Selak & Whitmarsh, 1982). In the present case, a possible explanation of this discrepancy is that the measurements of the reductive half-times were made in the presence of DCMU and NH_2OH, which inhibit generation of reductant in PSII.

For cells grown in the absence of Cu, the amplitudes of the oxidative absorbance changes measured at 554 nm are larger by a factor of 2−3 for the wild type and mutants (Figures 5B,D,F,H; 6B,D,F,H). The $t_{1/2}$ values for oxidation are ca. 25% smaller (Table 2B), and the lag time for oxidation after the flash is smaller, than for the "plus-Cu"

cells (Figure 5). The light-oxidized minus dark-reduced difference spectrum of the "minus-Cu" cells has a peak at 554 nm and a bandwidth at half-height of approximately 8 nm (data not shown). The larger amplitude is attributed to the contribution of cyt c_6, whose reduced minus oxidized α-band absorbance maximum at 552 nm is close to that of cyt *f* and well within the half-height bandwidth (ca. 8−10 nm) of both cytochromes. This increase in amplitude, ∼12 × 10^{-4} vs ∼4 × 10^{-4} absorbance units, arising from the contribution of oxidized cyt c_6, has been previously observed in the light minus dark spectrum of the cyanobacterium *Synechocystis* 6803 (Zhang *et al*., 1992) grown in the absence of Cu. Because the extinction coefficient of cyt c_6 is probably smaller than that of cyt *f* (Metzger *et al.*, 1996), it is not clear why the amplitude of the absorbance change at 554 nm is increased by more than a factor of 2 in the "minus-Cu" cells. Either (i) cyt c_6 oxidizes cyt *f* more efficiently than does PC, or (ii) two molecules of cyt c_6 mediate the oxidation of cyt *f*. It is of interest in this respect that cyt c_6 crystallizes as a dimer (Kerfeld *et al.*, 1995). The difference between the $t_{1/2}$ for re-reduction of cyt *f* in the quintuple Lys^- mutant compared to wild type in the "minus-Cu" cells, 7.3 ms vs 5.4 ms (Table 2B), is smaller than in the "plus-Cu" cells. Taking the standard deviations and number of trials into account, the probability (Lewis, 1966) that the dark reduction rate of cyt *f* for the quintuple Lys^- mutants is different from the wild type is 0.89.

The probability that the small effect on cyt *f* oxidation could arise from compensatory second site mutations is regarded as small because of (a) the techniques used for maintaining strains and growing cultures, (b) the absence of intragenic changes in the complete sequence of cyt *f* [(a,b); Materials and Methods], and (c) the pattern of small but systematic effects in the three mutants, in proportion to the number of lysines changed, in both the presence and absence of Cu.

DISCUSSION

(1) Small Effect of Electrostatic Interaction between Cytochrome f and Plastocyanin in Vivo. In vitro interprotein electron transfer data form the experimental precedent for the hypothesis summarized most recently in Pearson *et al.* (1996) that the interaction of PC with cyt *f* is mediated by electrostatic interactions between (a) the prominent basic domain of plant chloroplast cyt *f* at the interface of its large and small domains and (b) one to two prominent acidic patches on the surface of plant plastocyanins. This electrostatic interaction should be substantially decreased by neutralization/acidification of the lysines either in the cyt *f* small domain, in its large domain, or in both domains. Such a modification should have the same effect as increasing the ionic strength of the *in vitro* reaction to very high values. The *in vitro* electron transfer rate was decreased by a factor of approximately 100 when the ionic strength was increased by a factor of 10, from 0.1 to 1.0 M (Qin & Kostic, 1992), or decreased by a factor of 15 when the ionic strength was increased from 0.04 to 0.4 M (Meyer *et al.*, 1993). In the present studies, the absence of an observable effect larger than a factor of 1.25 on the $t_{1/2}$ for cyt *f* oxidation by plastocyanin (Figure 5A,C,E,G), after elimination of any electrostatic effect associated with the positive charges of the interdomain lysines, implies that either (i) long-range electrostatic guidance is not required *in vivo* in logarithmic phase cells for interaction of PC with cyt *f* or (ii) the five (Qin & Kostic, 1992) basic residues on cyt *f* responsible for the electrostatic interaction with PC are in a region distinct from that occupied by the interdomain lysines implicated by *in vitro* cross-linking experiments (Morand *et al.*, 1989). The latter possibility seems less likely because (a) there are only 12 additional Lys or Arg, including Arg250, in the whole protein that are conserved in the plant and algal sequences (Gray, 1992) and (b) mapping of the cyt *f* potential field indicates that it is only the 5 closely spaced interdomain lysines that provide a significant source of positive surface potential (Pearson *et al.*, 1996).

(2) Explanation for the Small Effect of Electrostatic Interaction between Cyt f and PC. The simplest explanation for the lack of a large effect of an electrostatic interaction between cyt *f* and PC *in vivo* would be that the effect exists, but is masked by a slower step, which could be the release of PC from P700. The $t_{1/2}$ for release of oxidized PC is 50−100 μs (Drepper *et al.*, 1996), and the intrinsic time for cyt *f* oxidation would then have to be ≲10 μs. However, the maximum pseudo-first-order rate constants observed by flash spectroscopy for electron transfer between cyt *f* and PC are equivalent to $t_{1/2}$ values of approximately 250 μs (Qin & Kostic, 1992; Meyer *et al.*, 1993), close to the values for oxidation of cyt *f in vivo* measured in the present and other studies. The 20-fold larger *in vitro* rate constant for the reaction discussed above (He *et al.*, 1991), that was calculated from small differences in the time course of absorbance changes associated with binding and electron transfer in stopped-flow measurements, is presently discrepant.

There appear to be two critical differences between (a) the *in vitro* conditions that allow involvement of a long-range electrostatic interaction and maximum rates of electron transfer between cyt *f* and PC and (b) their *in vivo* reaction environment. These are (i) the small size of their reaction volume in the thylakoid lumenal space and (ii) an ambient ionic strength *in vivo* of 0.3−0.4 M (Kaiser *et al.*, 1983). Regarding (i), the cross-sectional width of the chloroplast internal lumen space is 40−90 Å, the smaller and larger dimensions existing in the light and dark, respectively (Haehnel, 1984; Whitmarsh, 1986). The small value of this dimension is illustrated by the 75 Å length of cyt *f* that protrudes into the lumenal space (Martinez *et al*., 1994), but can only do so if its main axis is oriented at an angle of ca. 30° relative to the membrane plane (Cramer *et al*., 1994). Similarly, the motion of a plastocyanin molecule with dimensions of 28 Å × 32 Å × 40 Å will be constrained within this space (Haehnel, 1984). The small diffusional and reaction volume was also implied by the sensitivity to osmotic strength of the PC donor reactions (D. Kramer, personal communication). The small volume of the chloroplast lumen (Lavergne & Joliot, 1991) might obviate the requirement for long-range attractive forces that are needed in a test tube experiment. The kinetic sufficiency of the natural collision frequency in this small volume would be more easily attained if there is no precise requirement for the surface geometry of PC relative to cyt *f* and for the position of the plastocyanin Cu relative to the cyt *f* heme (Meyer *et al.*, 1993). Given the ubiquity of long-range electron transfer in proteins (Beratan *et al*., 1991; Langen *et al*., 1995), electron transfer between PC and cyt *f* could occur when the Cu and heme edge or histidine-25 ligand are separated by as much as 17−18 Å, assuming an average or

conservative rate−distance dependence of electron transfer (Gray & Winkler, 1996). The edge−edge distance between the PC Cu and chlorophyll of P700 has been estimated to be 20−23 Å (Hippler *et al.*, 1995). There may be many surface orientations of the 28 Å × 32 Å × 40 Å PC bound on the cyt *f* surface that would fall within this constraint, including the nonpolar or hydrophobic surface domain of PC that has been shown to interact with cyt *c* (Qin & Kostic, 1996). Two surface conformations of PC bound nonelectrostatically to cyt *f* have been proposed that allowed the plastocyanin Cu to be near the tyrosine heme ligand (Pearson *et al*., 1996).

(ii) The high internal ionic strength of the chloroplast, ca. 0.35 M (Kaiser *et al.*, 1983), might suggest that the influence of electrostatic interactions between cyt *f* and PC would be small, because the rate of electron transfer *in vitro* between cyt *f* and PC is smaller by ca. 10^2-fold at this ionic strength compared to that at low ionic strength (Meyer *et al.*, 1993). The *caveat* to this conclusion is that, although the thylakoid membrane is known to be leaky to ions, the actual ionic strength of the internal lumen space is not known.

(3) Cytochrome c_6. The studies shown above (Figures 5 and 6B,D,F,H) with *C. reinhardtii* cells grown in the absence of Cu (Figure 3, lanes 2−5), to our knowledge, provide the first analysis of the *in vivo* or *in situ* kinetic consequences for cyt *f* oxidation of substituting cyt c_6 for PC in *C. reinhardtii*. The kinetics of flash-induced oxidation of both cyt *f* and cyt c_{553}, which is synthesized under low Cu concentrations in the cyanobacterium *Synechocystis* 6803 (Zhang *et al.*, 1992), have the same profile as that of *C. reinhardtii*. It was thought that the docking site for cyt c_6 on cyt *f* might differ from that of PC because the cyt c_6 does not contain broad regions of anionic surface potential as does PC (Kerfeld *et al.*, 1995). However, this argument about the nature of the docking sites loses strength because of the lack of evidence for a role of electrostatic interactions between cyt *f* and its electron acceptors *in vivo*. The only indication of a difference between the docking sites on cyt *f* for cyt c_6 and PC is the larger effect of the removal of five lysines on the rate of cyt *f* dark-reduction for cells grown in the presence (electron acceptor, PC) compared to the absence of Cu (electron acceptor, cyt c_6) (Table 2A vs 2B).

(4) Function of Conserved Interdomain Lysines of Cytochrome f. The present studies show that the conserved interdomain lysines of cyt *f* are not required to catalyze oxidation by its redox partners *in vivo*. It is important to note that the *in vivo* measurements were all made in late log phase cells of *C. reinhardtii*, in order to ensure reproducibility and a yield of cells sufficient for the experiments. The nature of the positive interdomain basic region of cyt *f*, and the *in vitro* studies discussed above, is suggestive of a functional role for the lysine-rich region. It is suggested that long-range electrostatic guidance between cyt *f* and its soluble redox partners would be important during the development and biogenesis of the *C. reinhardtii* chloroplast when the lumen space is larger or not yet enclosed. Thus, experiments with broken or leaky thylakoids or with the isolated PSI complex and plastocyanin (e.g., Drepper *et al*., 1996), or b_6f complex−PC, would be expected to show a larger contribution of the long-range electrostatic interaction between these components.

(5) Significance of the Small Effect of Interdomain Lysines and Cyt f Electrostatic Interactions in Vivo. Even though the net effects of lysine removal from the interdomain region of cyt *f* are smaller than anticipated, they can still be very significant in growth and selection. A 1−3 h difference in growth rate (Table 1A) will exert a large selective pressure on the cell population.

(6) Consequences for Docking of the Rieske Protein to Cyt f. The systematic decrease with increasing extent of Lys neutralization on the rate of cyt *f* reduction is surprising because of: (i) the large distance (ca. 60−70 Å) of the interdomain basic region of cyt *f* from its C-terminus that enters the membrane surface (Martinez *et al.*, 1994), and (ii) the likelihood, based on the structure of the bovine mitochondrial Rieske fragment (Iwata *et al*., 1996), that the [2Fe-2S] center of the *p*-side extrinsic Rieske domain is located at the membrane interface near the bound quinol hydrogen donor. An alternative possibility is that extensive substitution of the interdomain lysines results in significant allosteric changes at a site used for docking of the Rieske protein.

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