Relation between Interface Properties and Kinetics of Electron Transfer in the Interaction of Cytochrome *f* and Plastocyanin from Plants and the Cyanobacterium *Phormidium laminosum*†

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ABSTRACT: Cytochrome *f* and plastocyanin from the cyanobacterium *Phormidium laminosum* react an order of magnitude faster than their counterparts from chloroplasts when long-range electrostatic interactions have been screened out by high salt concentration [Schlarb-Ridley, B. G., et al. (2002) *Biochemistry 41*, 3279−3285]. To investigate the relative contributions of the reaction partners to these differences, the reactions of turnip cytochrome *f* with *P. laminosum* plastocyanin and *P. laminosum* cytochrome *f* with pea plastocyanin were examined. Exchanging one of the plant reaction partners with the corresponding cyanobacterial protein nearly abolished electron transfer at low ionic strength but increased the rate at high ionic strength. This increase was larger for *P. laminosum* cytochrome *f* than for *P. laminosum* plastocyanin. To identify molecular features of *P. laminosum* cytochrome *f* that contribute to the increase, the effect of mutations in the N-terminal heme-shielding peptide on the reaction with *P. laminosum* plastocyanin was determined. Phenylalanine-3 was converted to valine and tryptophan-4 to phenylalanine or leucine. The mutations lowered the rate constant at 0.1 M ionic strength by factors of 0.71 for F4V, 0.42 for W4F, and 0.63 for W4L while introducing little change in the shape of the ionic strength dependence curve. When the N-terminal tetrapeptide (sequence YPFW) was converted into that found in the chloroplast of *Chlamydomonas reinhardtii* (YPVF), the reaction was slowed further (factor of 0.26). The N-terminal heme-shielding peptide was found to be responsible for 75% of the kinetic differences between cytochrome *f* from chloroplasts and the cyanobacterium when electrostatic interactions were eliminated.

The redox reaction couple cytochrome *f* and plastocyanin (Pc)[1] from the electron transfer chain of oxygenic photosynthesis differs in a number of aspects for chloroplasts and the modern representatives of their evolutionary ancestors, cyanobacteria. Although the overall three-dimensional structures of both proteins are highly conserved among cyanobacteria, algae, and higher plants (*1*−*6*), the surface charge properties vary considerably. Chloroplast Pcs have one or two distinctive "acidic patches" (Figure 1a), which have been shown to interact in vitro with a "basic ridge" on the soluble lumenal domain of cytochrome *f* (Cyt *f*) from chloroplasts [Figure 1c (*7*); for role in vivo see refs *8* and *9*]. The charge distribution on their cyanobacterial counterparts is more varied (*2*, *10*−*13*). In an earlier publication (*14*) we reported on the reaction between Pc and Cyt *f* of the moderately thermophilic cyanobacterium *Phormidium laminosum.* In this case, the area analogous to the acidic patches on chloroplast Pc is occupied by basic and acidic residues whose charges nearly neutralize each other (Figure 1b), and Cyt *f* exhibits, instead of a basic ridge, an area of more diffusely distributed acidic residues (Figure 1d).

We previously concluded (*14*) that the in vitro rate of reaction between Cyt *f* and Pc from the cyanobacterium *P. laminosum* is much less dependent on electrostatic attraction than its counterpart from chloroplasts (*7*) and that at high ionic strength, when long-range electrostatics are screened out, the cyanobacterial proteins react an order of magnitude faster. However, it remained to be clarified what enables the *P. laminosum* proteins to react so fast in the absence of long-range electrostatics. This question was the topic of the present study and was addressed in two ways. First, we investigated what relative contribution the two reaction partners, *P. laminosum* Cyt *f* and *P. laminosum* Pc, make to the fast rate at high ionic strength. Second, we endeavored to identify individual amino acid residues which contribute to the kinetic difference between the chloroplast and *P. laminosum* reaction in the absence of long-range electrostatics, focusing on one of the reaction partners, *P. laminosum* Cyt *f*. A likely

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[1] Abbreviations: Cyt *f*, soluble redox-active domain of cytochrome *f*; E_m, midpoint oxidation−reduction potential; k_2, bimolecular rate constant of the overall reaction; KP_i, potassium phosphate; Pc, plastocyanin.

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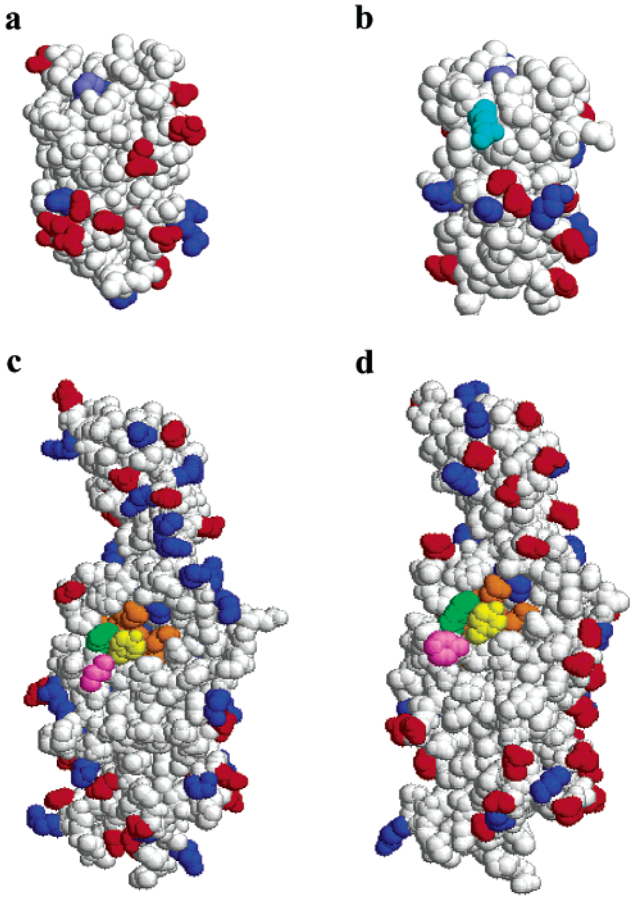

FIGURE 1: Space-filling representations of Pc and Cyt *f* drawn in Rasmol: Pc from pea (a) and *P. laminosum* (b); the lumenal domain of cytochrome *f* from turnip (c) and *P. laminosum* (d). Color code for amino acid side chains/heme: red, acidic; blue, basic; purple, copper ligand His87 (pea Pc)/His92 (*P. laminosum* Pc); cyan, Arg93; yellow, Tyr1; orange, heme; magenta, Ile3 (turnip Cyt *f*)/Phe3 (*P. laminosum* Cyt *f*); green, Phe4 (turnip Cyt *f*)/Trp4 (*P. laminosum* Cyt *f*).

candidate was the N-terminal hydrophobic heme-shielding peptide of Cyt *f*, which differs between plants or algae and cyanobacteria in residues 3 and 4 (shown in magenta and green, respectively, in parts c and d of Figure 1). Residue 3 is either Ile or Val in chloroplasts but invariably Phe in all known cyanobacterial Cyt *f* sequences. Residue 4 is either Phe or Tyr in plants and algae but Trp in all but one known cyanobacterial Cyt *f* sequence (see Table 1). The position of the α-band of the characteristic Cyt *f* spectrum is also shifted to the red by 2 nm for those cyanobacterial cytochromes *f* compared to chloroplasts, and their redox potential is significantly lower (*15*). It has been shown (*15*) that the Trp in position 4 alone determines the spectral shift and accounts for a large proportion of the difference in redox potential, whereas the difference in residue 3 has no significant influence on either spectrum or redox potential. In this study we have investigated the role of both F3 and W4 of *P. laminosum* Cyt *f* in reaction with *P. laminosum* and pea Pc.

MATERIALS AND METHODS

Molecular Biology and Mutagenesis. Molecular biological methods were essentially as described by Sambrook et al. (*16*), and materials were as in Schlarb et al. (*17*). Mutagenesis

Table 1: Alignment[a] of the First 26−27 Amino Acid Residues of Known Sequences of Cytochrome *f* from Plants, Eukaryotic Algae, and Cyanobacteria

Plants	
Brassica rapa	YP**IF**AQQNYEN-PREATGRIVCANCHL...
Pisum sativum	YP**IF**AQQGYEN-PREATGRIVCANCHL...
Vicia faba	YP**IF**AQQGYEN-PREATGRIVCANCHL...
Glycine max	YP**IF**AQQGYEN-PREATGRIVCANCHL...
Spinacea oleracea	YP**IF**AQQGYEN-PREATGRIVCANCHL...
Nicotiana tabacum	YP**IF**AQQGYEN-PREATGRIVCANCHL...
Oenothera hookeri	YP**IF**AQQGYEN-PREATGRIVCANCHL...
Oryza sativa	YP**IF**AQQGYEN-PREATGRIVCANCHL...
Tricitum aestivum	YP**IF**AQQGYEN-PREATGRIVCANCHL...
Pinus thunbergii	YP**IF**AQQGYEN-PREATGRIVCANCHL...
Picea abies	YP**IF**AQQGYEN-PREATGRIVCANCHL...
Zea mays	YP**IF**AQQGYEN-PREATGRIVCANCHL...
Atropa belladonna	YP**IF**AQQGYEN-PREATGRIVCANCHL...
Lotus japonicus	YP**IF**AQQGYEN-PREATGRIVCANCHL...
Psilotum nudum	YP**IF**AQQSYEN-PREATGRIVCANCHL...
Marchantia polymorpha	FP**IY**AQQGYEN-PREATGRIVCANCHL...
Algae	
Chlamydomonas reinhardtii	YP**VF**AQQNYAN-PREANGRIVCANCHL...
Chlamydomonas subcaudata	YP**IF**AQQNYEN-PREANGRIVCANCHL...
Porphyra purpurea	FP**IY**AQQAYES-PREATGRIVCANCHL...
Cyanophora paradoxa	FP**IY**AQQAYQI-PREATGRIVCANCHL...
Odontella sinensis	YP**VF**AQQGYSN-PRAANGKLACANCHL...
Chlorella vulgaris	YP**IF**AQQNYAN-PREANGRIVCANCHL...
Chaetosphaeridium globosum	FP**IY**AQQNYEN-PREATGRIVCANCHL...
Guillardia theta	FP**VF**AQQAYEN-PREATGRIVCANCHL...
Cyanidium caldarium	YP**IY**AQQTYEN-PRESTGRIVCANCHL...
Nephroselmis olivacea	YP**IY**AQENYAY-PREATGRIVCANCHL...
Mesostigma viride	YP**IF**AQQNYAS-PREATGRIVCANCHL...
Cyanobacteria	
Synechocystis sp.PCC 6803	YP**FW**AQETAPLTPREATGRIVCANCHL...
Synechococcus sp.PCC 7002	YP**FW**AQQTAPETPREATGRIVCANCHL...
Nostoc sp.	YP**FW**AQQTYPETPREPTGRIVCANCHL...
Anabaena variabilis	YP**FW**AQQTYPETRPEPTGRIVCANCHL...
Anabaena sp.PCC 7120	YP**FW**AQQTYPETPREPTGRIVCANCHL...
Arthrospira maxima	YP**FW**AQETAPETPREATGRIVCANCHL...
Phormidium laminosum	YP**FW**AQQNYAN-PREATGRIVCANCHL...
Synechococcus elongatus	YP**FY**AQQGYES-PREATGRIVCANCHL...
	:* :**: ** *:: ******

[a] Invariant residues among 35 known Cyt *f* sequences are marked by an asterisk and highly conserved residues by a colon. Sequences were aligned by Clustal W (*35*); the table was adapted from ref *15*.

of the *petA* gene in vector pUC19li5 (*17*) was carried out according to the QuickChange site-directed mutagenesis method (Stratagene; cf. ref *18*). The following codon changes were introduced to obtain the respective mutations: TTC to GTC for mutant F3V, TGG to CTG for mutant W4L, TTG to TTC for mutant W4F, and TTCTGG to GTCTTC for mutant F3V-W4F. 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 pUCfF3V, pUCfW4L, pUCfW4F, and pUCfF3VW4F, respectively, and transformed into *Escherichia coli* strain W3110 (*19*).

Protein Methods. Expression, purification, and characterization of *P. laminosum* wild-type Pc were carried out as in ref *17* and those of wild-type and all mutant Cyt *f* proteins as in Crowley et al. (*20*). Turnip Cyt *f* was bought from Sigma and used without further purification; its ratio $A_{280}/A_{554.5}$ was 1.4. Pea Pc was expressed in an *E. coli* BL21-(DE3)pLysS expression system analogous to that described for *P. laminosum* Pc in ref *17*. The purification procedure was as for *P. laminosum* Pc in ref *17* except that 10 mM HEPES, pH 7.5, and salt gradients of 0−200 mM NaCl were used. Extinction coefficients used to calculate protein concentrations were 4700 M^{-1} cm^{-1} for *P. laminosum* and pea 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 as in Schlarb-Ridley et al. (*14*), except that the concentration of Cyt *f* in all kinetic experiments was 0.1 μM

and the concentration of Pc in measurements of ionic strength dependence was 2.4 μM.

The interpretation of the results used the same kinetic model for the interaction as described in refs *14* and *21*. The measured second-order rate constant, k_2, is defined as

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

where k_{on} is the rate of association of the reaction partners, k_{off} is the rate of dissociation before reaction has occurred, and k_f is related to k_{et}, the rate of electron transfer in the complex, and would become equal to k_{et} if the driving force were large enough (*21*).

Depending on the relative magnitude of k_f and k_{off}, the above equation (eq 1) can be simplified in two ways. (a) Activation control: this applies when $k_{off} \gg k_f$, then $k_2 = k_{on}k_f/k_{off}$. (b) Diffusion control: this applies when $k_f \gg k_{off}$, then $k_2 = k_{on}$.

Viscosity measurements (unpublished data) have shown that the reaction between Pc and Cyt *f* of both plants and the cyanobacterium *P. laminosum* is mainly diffusion controlled. Hence the measured bimolecular rate constant k_2 can be seen as an approximation of k_{on}.

Redox Potentiometry. The determination of the midpoint potential of all proteins was performed essentially as in Schlarb-Ridley et al. (*14*). The values for wild-type *P. laminosum* Pc and Cyt *f* are slightly higher than those reported in Schlarb-Ridley et al. (*14*); we attribute this to new equipment providing better anaerobic conditions. Titrations for all wild-type proteins were also performed in 20 mM MES, pH 6, containing 3 M KCl. No calibration method for the Ag/AgCl-in-polymer reference electrode filled with 3 M KCl (type KCMMPtBL, Russell) was available for this salt concentration; hence at 3 M KCl only the values of driving forces are given. The experimental conditions (a flow of argon through the solution at 300 K for several hours) led to partial precipitation of protein during titrations at 3 M KCl, thus increasing the experimental error.

Electrostatic Potentials. Electrostatic potentials of reduced wild-type *P. laminosum* Cyt *f* were calculated at 0.04, 0.2, 1.0, and 3.0 M ionic strength by a finite difference solution of the Poisson−Boltzmann equation with DelPhi II (*22*, *23*). The Swiss PbdViewer was used to add polar and aromatic ring hydrogens to PDB file 1ci3. Atomic radii and partial charges were assigned from the PARSE list of Sitkoff et al. (*24*), and the protein dielectric constant was set to 4.

RESULTS

Choice of Proteins. The first aim of this study was to determine the relative contributions of *P. laminosum* Pc and *P. laminosum* Cyt *f* to the observed increase in rate constant at high ionic strength compared to the reaction of the chloroplast proteins (*14*; see also Figure 2a). This can be achieved by mixing the reaction partners from chloroplasts and the cyanobacterium, i.e., by letting a chloroplast Cyt *f* react with *P. laminosum* Pc and a chloroplast Pc react with *P. laminosum* Cyt *f*, and comparing the rate constants obtained at high ionic strength with those of the homologous chloroplast and *P. laminosum* reactions. The three-dimensional structures and p*I*s of chloroplast cytochromes *f* and Pcs, respectively, are well conserved, so that it is less important than in the cyanobacterial case to work with

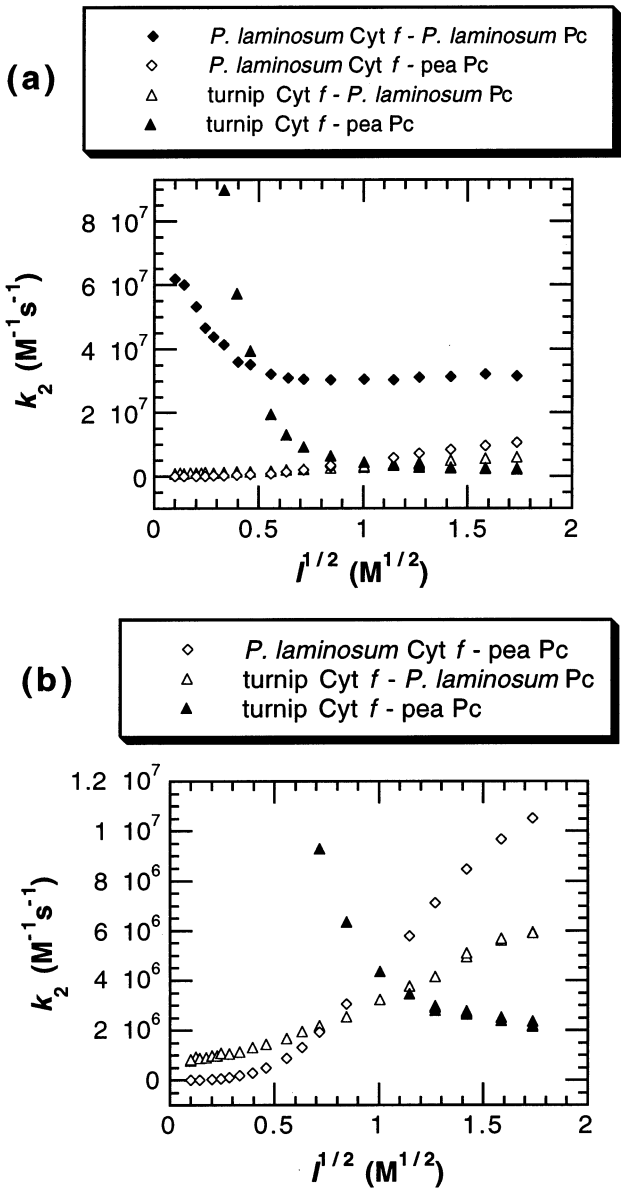

FIGURE 2: Ionic strength dependence of k_2: (a) *P. laminosum* Cyt *f* reacting with *P. laminosum* or pea Pc and turnip Cyt *f* reacting with *P. laminosum* or pea Pc; (b) enlargement of the slow region of (a). Experimental conditions: 300 K, pH 6.0; concentration of Cyt *f*, 0.1 μM; concentration of Pc, 2.4 μM.

reaction partners from the same organism. We used Cyt *f* from turnip and Pc from pea and treated their reaction as being homologous. This is justified by the fact that at 100 mM salt the reaction of turnip Cyt *f* with pea Pc has a rate constant of reaction k_2 which is almost identical to that of turnip Cyt *f* with turnip Pc [1.5×10^8 M^{-1} s^{-1} (*7*) and 1.4×10^8 M^{-1} s^{-1} (N. Fisher, personal communication), respectively].

Homologous and Mixed Wild-Type Reactions. To determine the relative contribution of *P. laminosum* Pc and *P. laminosum* Cyt *f* to the fast rate at high salt concentrations, the ionic strength dependence of the second-order rate constant (k_2) of the (mixed) reactions *P. laminosum* Cyt *f*−pea Pc and turnip Cyt *f*−*P. laminosum* Pc was measured and compared with that of the homologous reactions (Figure 2). The k_2 value of turnip Cyt *f* reacting with pea Pc decreased rapidly with increasing ionic strength. The k_2 value

Table 2: Kinetic Data and Midpoint Potentials for Wild-Type and Mutant *P. laminosum* or Turnip Cyt *f* and *P. laminosum* or Pea Pc[a]

protein		k_2 (10^7 M^{-1} s^{-1})	E_m (mV)	ΔE_m (mV) (3 M KCl)	
				P.l. Pc	pea Pc
P.l. Cyt *f*	wild type	4.4 ± 0.05	331 ± 4	−12 ± 12	43 ± 13
	F3V	3.2 ± 0.05	314 ± 3		
	W4L	2.8 ± 0.06	328 ± 3		
	W4F	1.9 ± 0.06	354 ± 2		
	F3V-W4F	1.1 ± 0.02	343 ± 3		
turnip Cyt *f*			355 ± 5	−37 ± 15	18 ± 16
P.l. Pc			339 ± 3		
pea Pc			370 ± 3		

[a] All measurements were done at pH 6.0 and 300 K in the following buffers: 10 mM KP_i and 90 mM KCl, or 20 mM MES and 3 M KCl for the redox titrations, and 10 mM KP_i and 90 mM NaCl for the kinetic measurements. k_2 (the rate constant of reaction with *P. laminosum* Pc) has been determined by linear regression of a k_{obs} vs [Pc] plot. The table shows the fitting errors of k_2; the overall errors of k_2 are estimated to be ≤5% of the given values. Errors given for E_m are estimates of the standard error based on the fitting error of each titration and on the standard error determined for *P. laminosum* Cyt *f*. For the errors of ΔE_m see Materials and Methods. *P.l.* = *P. laminosum.*

of *P. laminosum* Cyt *f* reacting with *P. laminosum* Pc decreased slightly with increasing salt concentration. Both homologous reactions exhibited electrostatic attraction, although to a markedly different degree. The rate of both mixed reactions increased with increasing ionic strength, implying electrostatic repulsion (Figure 2b). For turnip Cyt *f* and *P. laminosum* Pc, which are both mildly acidic (*25*, *26*), the curve had the form of a shallow S. The reaction between the more strongly acidic proteins *P. laminosum* Cyt *f* and pea Pc (*17*, *27*) remained extremely slow below 100 mM NaCl but increased faster at higher salt concentrations.

At high ionic strength, when long-range electrostatics were largely eliminated, both *P. laminosum* proteins led to higher rate constants with the respective redox partners from plants compared to the homologous plant reaction. Hence, nonelectrostatic contributions of both Pc and Cyt *f* from *P. laminosum* appeared to be more favorable than those of the plant proteins. The enhancement effect for *P. laminosum* Cyt *f* was considerably larger than that for *P. laminosum* Pc (Figure 2b): compared to the reaction turnip Cyt *f*−pea Pc at 3 M KCl, the reaction *P. laminosum* Cyt *f*−pea Pc was faster by a factor of 4.8, whereas the reaction turnip Cyt *f*−*P. laminosum* Pc was faster by a factor of 2.7 only.

Viscosity dependence measurements at 90 mM and 2.5 M NaCl have indicated that both the reaction between the *P. laminosum* proteins and that between the plant proteins are mainly diffusion controlled across the range of salt concentrations used (unpublished data). In diffusion-controlled reactions k_2 equals k_{on} (*28*; cf. Materials and Methods), and changes in k_{et} do not contribute to k_2. Hence the differences in driving force between the four reactions (Table 2), although they will influence k_{et}, are unlikely to have caused the kinetic differences observed. One can conclude that nonelectrostatic contributions to k_{on} are more favorable for a fast reaction in the couple *P. laminosum* Cyt *f*−pea Pc than in the couple turnip Cyt *f*−*P. laminosum* Pc, and both in turn are more favorable than in the homologous plant reaction.

Mutants of the N-Terminal Heme-Shielding Region of P. laminosum Cyt f. To investigate the structural basis for the nonelectrostatic differences between *P. laminosum* Cyt *f* and Cyt *f* from chloroplasts, the overall rate constant of reaction, k_2, at 100 mM salt and its ionic strength dependence were measured for a number of N-terminal mutants of *P. laminosum* Cyt *f*. The mutants, which apart from F3V have been described in Ponamarev et al. (*15*), were designed to change the hydrophobicity of the heme-shielding region and to mimic successively the N-terminal sequence of a chloroplast Cyt *f* (for example, that of *Chlamydomonas reinhartii*). The mutants F3V, W4F, W4L, and F3V-W4F were generated, expressed, purified, and characterized as described in Materials and Methods and in Ponamarev et al. (*15*). Molecular masses as determined by ESI−mass spectrometry were in agreement with the values predicted from the translated DNA sequence, indicating that the correct mutations had occurred and the proteins had not been modified. The optical spectrum of the F3V mutant was not changed significantly compared to wild-type *P. laminosum* Cyt *f* (the position of the α-band was 555.5 ± 0.2 nm and that of the reduced Soret band 421.7 ± 0.2 nm). The spectra of wild type and the other mutants have been described and discussed in Ponamarev et al. (*15*).

Midpoint potentials for these mutants are reported in Table 2. The mutation F3V lowered the midpoint potential by 17 ± 7 mV (Table 2). Mutation W4L had no significant influence on the midpoint potential, whereas mutation W4F increased it by 23 ± 6 mV and so did the double mutation F3V-W4F, although to a lesser extent (12 ± 7 mV). The midpoint potentials of mutants W4L, W4F, and F3V-W4F, although measured at a different pH and ionic strength, have already been reported and discussed in Ponamarev et al. (*15*). Although the absolute values we report here (Table 2) for pH 6.0 and 100 mM ionic strength are higher than those in Ponamarev et al. (*15*), the ΔE_m values are the same, and the discussion of the redox shifts in Ponamarev et al. (*15*) applies. The values published in Ponamarev et al. (*15*) were measured with a fault in the reference potential, leading to correct relative but lower absolute values. The molecular basis of the redox shifts for all mutants but F3V has been discussed in Ponamarev et al. (*15*). The decrease in midpoint potential observed for mutant F3V can be explained by increased solvent accessibility of the heme through replacing the larger and well-shielding aromatic side chain Phe with the smaller aliphatic residue Val. As the reaction with *P. laminosum* Pc is largely diffusion controlled, changes in driving force and hence in k_{et} brought about by alterations in midpoint potentials will have little effect on k_2.

The rate constants k_2 for each overall bimolecular reaction under standard ionic strength conditions (10 mM KP_i, 90 mM NaCl) at pH 6.0 and 300 K were calculated from the slopes of the observed pseudo-first-order rate constant against Pc concentration; results are summarized in Table 2. The smallest effect was observed for the single mutation Phe3 to Val, a reduction in k_2 by a factor of 0.72 only relative to the wild-type reaction. When Trp4 was replaced by the smaller aromatic residue Phe, the amino acid occupying this position in most chloroplast cytochromes *f*, the rate constant was reduced by a factor of 0.43. When it was replaced by the nonaromatic, hydrophobic amino acid Leu, k_2 dropped by a factor of 0.63 only. The largest effect, a decrease in k_2 by a factor of 0.25, was observed for the double mutant F3V-W4F, which mimics, among others, the sequence of the alga *C. reinhardtii*.

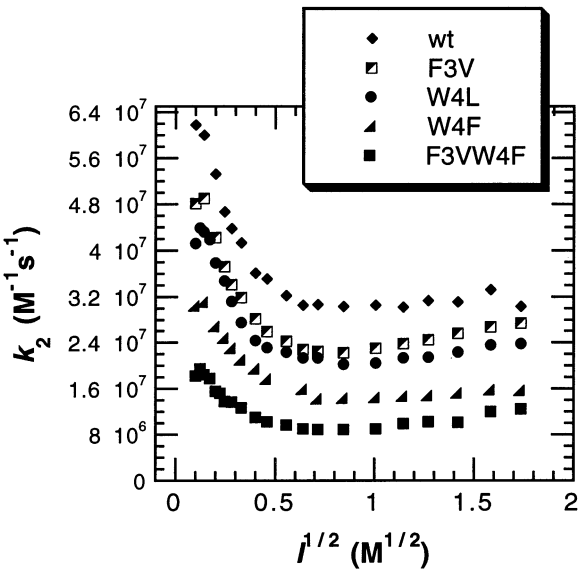

FIGURE 3: Ionic strength dependence of k_2: wild-type or mutant *P. laminosum* Cyt *f* reacting with *P. laminosum* Pc. For experimental conditions see legend of Figure 2.

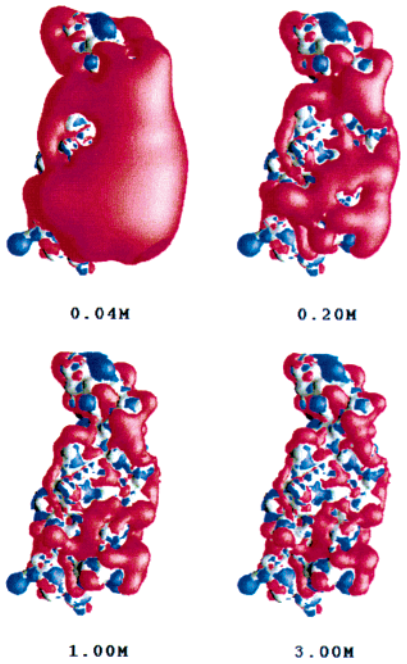

FIGURE 4: Electrostatic potential surface of wild-type *P. laminosum* Cyt *f* at different ionic strengths (0.04, 0.2, 1.0, and 3.0 M), calculated using the program DelPhi II (*22*, *23*) and displayed using the program GRASP (*36*).

The ionic strength dependence of k_2 for wild-type and mutant *P. laminosum* Cyt *f* with *P. laminosum* Pc is depicted in Figure 3. The shape of the curves differed very little between wild type and mutants, indicating that, as expected, the changes in the electrostatic interaction are small. Surprisingly, above 0.7 M NaCl, the rate began to increase slightly, most notably for F3V-W4F, F3V, and W4L. The effect was reproducible and is not considered to be an artifact of high ionic strength: artifacts such as incomplete mixing would decrease rather than increase the rate. A possible explanation of electrostatic nature is illustrated in Figure 4, which shows the contours of electrostatic potential for wild-type *P. laminosum* Cyt *f* at different ionic strengths: at low ionic strength positive charges on Pc, centered on residue Arg93 (colored cyan in Figure 1b), are attracted to the large negative cloud which buries the interaction site on Cyt *f* (see Figure 4, 0.04 M). As ionic strength increases, the effect of the negative charge on Cyt *f* and with it the attraction decreases (Figure 4, 0.2 and 1.0 M), while the areas of positive potential, which to a large degree are due to backbone charges, appear to be influenced little by ionic strength. The attractive forces left appear to direct the positive charge of Pc away from the interaction site around the heme (see Figure 1c,d), toward the remaining centers of negative charge depicted as red clouds in Figure 4 (1 M). However, as the ionic strength rises to 3 M, the negative charge on acidic side chains is screened further; hence the rate-lowering "trapping" of Pc away from the interaction site diminishes (Figure 4, 3.0 M). The fact that the effect is stronger for F3V-W4F, F3V, and W4L than for wild type and W4F seems to suggest that the electrostatic effect is modified by specific hydrophobic interactions. A possible interpretation is given in the Discussion.

Difference between Chloroplast and P. laminosum Cyt f. The mutations (with the exception of W4L) make the hydrophobic heme-shielding region of *P. laminosum* Cyt *f* successively more similar to that of chloroplasts. A comparison between the reaction of the *P. laminosum* Cyt *f* mutants with both *P. laminosum* and pea Pc at high ionic strength, and that of turnip Cyt *f* with the same Pcs, can therefore give insight into the role of the N-terminal heme-shielding region in the variations in nonelectrostatic contributions of Cyt *f*. Figure 5a shows the ionic strength dependence of *P. laminosum* wild-type, F3V-W4F mutant, and turnip Cyt *f* in reaction with *P. laminosum* Pc. Above 1 M ionic strength, the three reactions form essentially parallel lines. Replacing residues 3 and 4 of *P. laminosum* Cyt *f* by those used in the chloroplast of *C. reinhardtii* (mutant F3V-W4F) reduces the rate constant in the absence of long-range electrostatics nearly to that of the chloroplast Cyt *f*. The same conclusion can be drawn from Figure 5b, which depicts the analogous reactions with pea Pc. In a different formulation, ca. 75% of the enhancement in rate at high ionic strength that Cyt *f* from *P. laminosum* exhibits compared to the chloroplast protein is due to the presence of Phe in position 3 and Trp in position 4.

DISCUSSION

The aims of this study were (a) to determine which of the two reaction partners, *P. laminosum* Cyt *f* and *P. laminosum* Pc, contributes more to the fast rate at high ionic strength and (b) to identify individual amino acid residues of Cyt *f* that contribute to the kinetic difference between the chloroplast and *P. laminosum* reaction in the absence of long-range electrostatics. From the data presented under Results we can conclude that *P. laminosum* Cyt *f* contributes more to the fast rate of the homologous *P. laminosum* reaction in the absence of long-range electrostatics than *P. laminosum* Pc. However, nonelectrostatic interactions of turnip Cyt *f* were still more favorable with *P. laminosum* Pc than with pea Pc. Experimental work is under way to identify the structural basis of this difference between the two Pcs.

In this publication, we have focused on the role of Cyt *f* and have found the structural basis for most of the difference in nonelectrostatic contributions between *P. laminosum* and

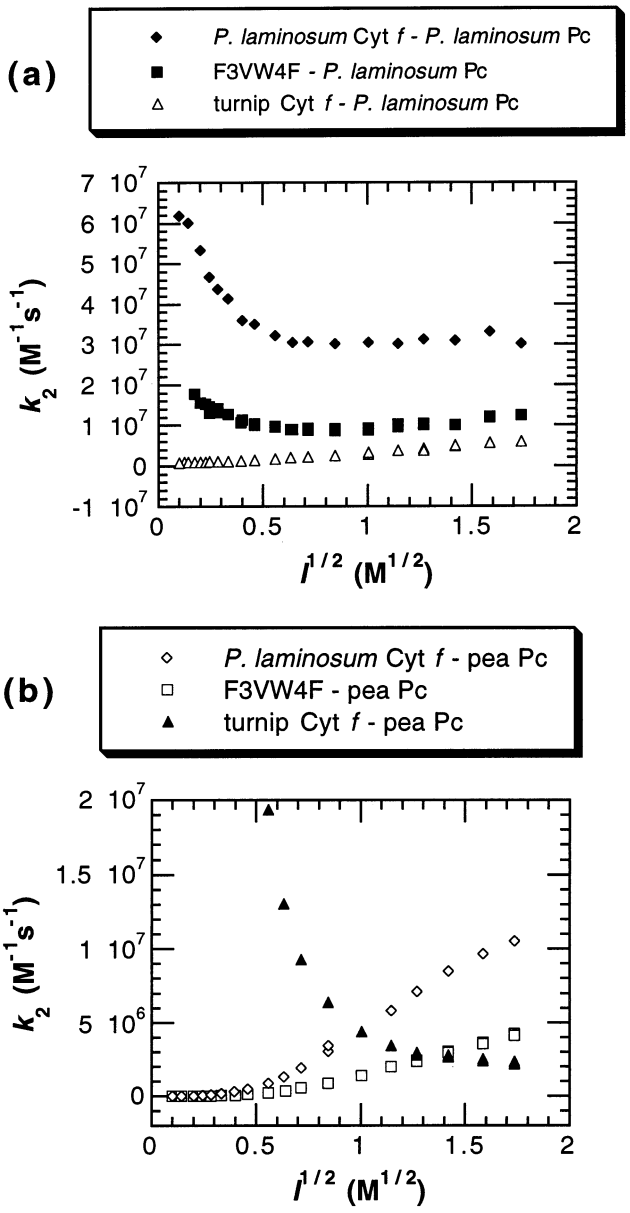

FIGURE 5: Ionic strength dependence of k_2: wild-type or F3V-W4F mutant *P. laminosum* Cyt *f* or turnip Cyt *f* reacting with (a) *P. laminosum* or (b) pea Pc. For experimental conditions see legend of Figure 2.

turnip Cyt *f*. Replacing the cyanobacterial residues Phe3 and Trp4 by those found in the chloroplast of *C. reinhardtii* reduced the difference in kinetic behavior at high salt concentrations between *P. laminosum* Cyt *f* and turnip Cyt *f* by ca. 75% (Figure 5). Although in turnip Cyt *f* position 3 is taken up by Ile rather than Val, the difference between these two nonaromatic hydrophobic residues is expected to be small.

A kinetic comparison between the homologous *P. laminosum* and the mixed *P. laminosum*−plant reactions has been made before. In 1996, Wagner et al. (*29*) published the ionic strength dependence of the reaction of the cytochrome *bf* complex isolated from *P. laminosum* with *P. laminosum* and spinach Pc. The assay was carried out at room temperature and pH 6.2. They found that, at high ionic strength, the two Pcs reacted with the cytochrome *bf* complex at a similar rate. The experiments carried out in this study with the soluble, fully redox active lumenal domain of *P. laminosum* Cyt *f* at 300 K and pH 6.0 with *P. laminosum* and pea Pc found that k_2 of the mixed reaction at 3 M NaCl was at least 60% slower than that of *P. laminosum* Pc with *P. laminosum* Cyt *f* (Figure 2a). The differences between these two results are not surprising, as Wagner et al. (*29*) used a steady-state measurement of a multistep reaction whereas the results presented here were obtained by a single turnover and single-step stopped-flow experiment. Furthermore, temperature, pH, and source of Pc differed. It remains to be clarified if the mode of nonelectrostatic interaction with Pc differs between soluble Cyt *f* and the cytochrome *bf* complex.

The N-terminal peptide of Cyt *f* has attracted the interest of a number of researchers over the past decade, triggered mainly by the unusual features of Cyt *f*'s three-dimensional structure (*1*). Cyt *f* is the only *c*-type cytochrome known so far in which the sixth heme ligand is the N-terminal amine. The crystal structure also revealed that the four N-terminal amino acids are involved in shielding the heme from solvent, thereby contributing to the protein's high midpoint potential (*15*). They are part of a flat hydrophobic surface surrounding the heme which has been shown to bind the similarly flat, hydrophobic, so-called "northern" end of its redox partner Pc, and structural data on the complex of Cyt *f* and Pc from higher plants (*30*) have suggested the electron might leave the heme via Tyr1, which is in van der Waals contact with the copper ligand His87 on Pc. Mutagenesis of N-terminal residues has been carried out in vivo (*31*, *32*) and in vitro (*15*, *33*), mainly focusing on Tyr1 and *C. reinhardtii*. Of the two residues that are of particular interest for this study, Val3 has been mutated to Pro in *C. reinhardtii*. The mutation had little influence on in vivo experiments (*31*). Phe4 of turnip Cyt *f* has been mutated to Tyr; the mutation decreased the midpoint potential and increased both k_2 and the binding constant with pea Pc in vitro (*33*). In Ponamarev et al. (*15*), mutagenesis was carried out in vitro for both *C. reinhardtii* and *P. laminosum* Cyt *f*: Val3 was mutated to Phe, Phe4 to Leu and Trp, and a double mutant V3F-F4W was created (all in *C. reinhardtii* Cyt *f*). Inversely, for *P. laminosum* Cyt *f*, Trp4 was mutated to Leu and Phe, and the double mutant F3V-W4F was generated. The analysis focused on spectral and redox shifts; the mutation V3F was found not to influence either the position of spectral peaks or the midpoint potential. In the study presented here, we were interested in the kinetic effects of these mutations and have found that replacing Phe3 by Val lowered both redox potential and rate of reaction. All mutations lowered k_2; the double mutant F3V-W4F had the largest kinetic effect.

As recent experiments (to be published elsewhere) have shown the reaction between *P. laminosum* Pc and *P. laminosum* Cyt *f* to be mainly diffusion controlled, the kinetic effects on k_2 will predominantly be effects on k_{on} (see Materials and Methods). Furthermore, k_{on} equals k_a, the rate of formation of the encounter complex. The encounter complex is the end point of the diffusional process and can be described as an ensemble of structures in which two final contacts have been formed (*34*). The encounter process hence comprises both long- and short-range interactions. The experimental results presented here can be explained if the size and hydrophobicity of the heme-shielding area influence the latter part of the encounter process through hydrophobic channeling. Larger aromatic residues such as Phe or Trp can provide an initial point of hydrophobic contact more readily

than smaller aliphatic residues such as Val or Ile. They can also facilitate two-dimensional diffusion of the hydrophobic surfaces relative to each other and thereby enhance the rate of finding a second final contact. Thus Phe3 and Trp4 could enhance the rate of reaction relative to Val3 and Phe4.

Hydrophobic channeling can also offer an explanation for the fact that the minimum of k_2 around 0.7 M ionic strength for each of the curves in Figure 3 is more pronounced for mutants F3V-W4F, F3V, and W4L than for wild type and W4F. The presence of the additional aromatic residue in wild type and W4F might be able to counteract electrostatic trapping away from the interaction site by increasing the probability of forming an initial hydrophobic contact.

The NMR structure of the *P. laminosum* Cyt *f*−*P. laminosum* Pc complex (*20*) shows van der Waals contact between Phe3 and the copper ligand His92, and Crowley et al. (*20*) propose that the electron leaves the heme via Phe3 rather than Tyr1. Mutating Phe3 should therefore have a marked influence on the rate of electron transfer. However, this could only be detected in k_2 measurements if the reaction were not diffusion controlled. Viscosity dependence measurements of the reaction between the F3V mutant Cyt *f* and wild-type *P. laminosum* Pc (data not shown) revealed a similarly high degree of diffusion control as observed for the wild-type reaction. Hence our results can neither confirm nor contradict the proposition of Crowley et al. (*20*).

This work is a further step toward a detailed understanding of the striking differences in the reaction between Cyt *f* and Pc from plants and the cyanobacterium *P. laminosum*. The structural basis for 75% of the difference in nonelectrostatic contributions between *P. laminosum* and plant Cyt *f* has been identified, and an understanding of the encounter process of the *P. laminosum* proteins has been extended. Diffusion-controlled encounter between two proteins has usually been thought of as mainly influenced by long-range electrostatics, and we have previously shown that in the interaction between Cyt *f* and Pc from *P. laminosum* electrostatics specifically influence the encounter complex. The work described in the current paper provides evidence for a role of specific hydrophobic interactions in the formation of the encounter complex.

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