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Electrostatic Effects on Electron-Transfer Kinetics in the Cytochrome *f*-Plastocyanin Complex

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ABSTRACT In a complex of two electron-transfer proteins, their redox potentials can be shifted due to changes in the dielectric surroundings and the electrostatic potentials at each center caused by the charged residues of the partner. These effects are dependent on the geometry of the complex. Three different docking configurations (DCs) for intracomplex electron transfer between cytochrome *f* and plastocyanin were studied, defined by 1) close contact of the positively charged region of cytochrome *f* and the negatively charged regions of plastocyanin (DC1) and by (2, 3) close contact of the surface regions adjacent to the Fe and Cu redox centers (DC2 and DC3). The equilibrium energetics for electron transfer in DC1–DC3 are the same within approximately ±0.1 *kT*. The lower reorganization energy for DC2 results in a slightly lower activation energy for this complex compared with DC1 and DC3. The long heme-copper distance (~24 Å) in the DC1 complex drastically decreases electronic coupling and makes this complex much less favorable for electron transfer than DC2 or DC3. DC1-like complexes can only serve as docking intermediates in the pathway toward formation of an electron-transfer-competent complex. Elimination of the four positive charges arising from the lysine residues in the positive patch of cytochrome *f*, as accomplished by mutagenesis, exerts a negligible effect (~3 mV) on the redox potential difference between cyt *f* and PC.

INTRODUCTION

Electron transfer in energy-transducing membranes occurs upon the formation of a complex between donor and acceptor proteins. Although structural data relevant to the electron transfer process exists for several such proteins in isolation, there are relatively few data available for the complexes. One such example, for which there are both in vitro and in vivo data on reaction rates (see references below), is the reaction between cytochrome *f* (cyt *f*) and plastocyanin (PC), the soluble PC serving as the link between the cytochrome b_6f complex and photosystem I in oxygenic photosynthesis (Cramer et al., 1996).

Studies of the cyt *f*-PC reaction in vitro have suggested a substantial role for electrostatic interactions between the reactants (Morand et al., 1989; He et al., 1991; Modi et al., 1992a,b; Qin and Kostić, 1992, 1993; Meyer et al., 1993; Gross, 1993; Wagner et al., 1996; Kannt et al., 1996). Electrostatic effects were also observed in reactions of PC with different cytochromes (e.g., Peery and Kostić, 1989; Bagby et al., 1990b; Nordling et al., 1990; Roberts et al., 1991; Peery et al., 1991; Meyer et al., 1993; Qin and Kostić, 1996) and with photosystem I (Haehnel et al., 1994; Farah et al., 1995; Sigfridsson et al., 1996; Drepper et al., 1996). The structural data show the existence of two patches of negatively charged residues on the PC surface, viz., Asp-42 and -44, Glu-43 and -79 and, for the second patch, Glu-59 and -60 and Asp-61 (Guss and Freeman, 1983; Guss et al., 1992; Bagby et al., 1994). A well defined positive region was revealed on the turnip cyt *f* surface (Martinez et al., 1994, 1996; Cramer et al., 1994) that includes Lys-58, -65, and -66 and Lys-185 and -187 of the large and small domains. This suggested that the docking of cyt *f* and PC may involve contact of the extended positive patch of cyt *f* and two negative regions of PC (Martinez et al., 1994; Cramer et al., 1994). This idea has experimental support in the finding that Asp-44 of PC can be chemically cross-linked to Lys-187 of cyt *f*, and the PC Glu-59 or -60 to an unidentified residue on cyt *f* (Morand et al., 1989).

However, despite these data pointing to the importance of electrostatic interactions for the in vitro reaction, and to its theoretical justification, this does not mean that the electrostatically complementary complex is really the complex in which the electron transfer takes place. Chemical cross-linking that fixes the cyt *f*-PC structure in a form that was assumed to be similar to this electrostatically complementary complex leads to a product in which electron transfer is strongly retarded (Qin and Kostić, 1993). There is some disagreement concerning the extent of this retardation (Takabe and Ishikawa, 1989), although the latter experiments are less direct and have lower kinetic resolution. The decrease of electron transfer rate in the cross-linked complex implies that some rearrangement of the initial electrostatic complex before electron transfer is necessary (Qin and Kostić, 1993).

There are some data that are not obviously accommodated by a simple model of electrostatic docking. 1) mutagenesis of PC Asp-42 does not affect the reaction rate of cyt *f* and PC *in vitro* (Modi et al., 1992b). On the side of PC opposite to Asp-42, mutation of Leu-12 to Asn, which is able to form a hydrogen bond with some of cyt *f* residues, increases the binding of the two proteins (Modi et al.,

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1992b). Leu-12 is situated far from the negatively charged regions, in the mainly hydrophobic region close to His-87, a surface-exposed ligand of Cu. 2) The decrease in reaction rate at very low ionic strength suggests an important role of the hydrophobic contact between PC and cyt *f* (Meyer et al., 1993; Wagner et al., 1996). A similar conclusion has been drawn from the data on the in vitro reaction between two molecules of cyt *c* and PC (Qin and Kostić, 1996). In addition, mutagenesis and elimination of the positive patch on cyt *f* resulted in a very small decrease of the reaction rate in vivo in cells of the green alga *Chlamydomonas reinhardtii* (Soriano et al., 1996). Because the rate of the oxidation measured in vivo is similar to the maximal rate measured in vitro in a pseudo-first-order reaction (2800 s^{-1}; Qin and Kostić, 1992; Meyer et al., 1993), the electrostatically mediated docking is not obligatory or rate limiting for the reaction in the chloroplast of the living cell.

Three representative docking configurations of cyt *f* and PC were considered by Pearson et al. (1996): 1) an electrostatically favorable complex similar to that proposed by Martinez et al. (1994) and Cramer et al. (1994), 2) a complex with the copper ligand His-87 placed close to the heme iron ligand, Tyr-1, preserving the contact Asp-44 (PC)/Lys-187 (cyt *f*), and 3) a complex in which His-87 and Tyr-1 are also close to each other but the link between Asp-44 and Lys-187 is absent and is replaced by hydrophobic residues in direct contact. The last two complexes invoke the possibility that there is hydrophobic contact between regions near the two metal ligands in the electron-transfer-competent complex. The possibility of formation of all three complexes, in which less favorable electrostatic interactions are compensated by more favorable hydrophobic ones, has been shown previously (Pearson et al., 1996), although a quantitative evaluation of the relative stability of these complexes was not obtained in that study.

In the present study, we will not consider the problem of collision between the reaction partners or of formation of the most stable complex. We will focus our attention on the next step after complex formation, intermolecular electron transfer in the cyt *f*-PC complex.

From general considerations, one should expect that, upon complex formation, the redox potentials of both reaction partners should be shifted compared with the potentials of the separated proteins. This shift has three causes: 1) formation of the complex changes the dielectric surroundings of the redox active sites (i.e., the cytochrome heme and plastocyanin Cu), thereby changing their charging energies; 2) in the reaction complex, each of the redox centers becomes subject to the field of the charged residues of the partner protein, a field that was absent in the isolated protein; 3) the field arising from the charged residues of each protein is altered because of the change of their dielectric screening. In this study, we present the first analysis of the role of these effects on electron transfer reactions in the cyt *f*-PC complex.

We have calculated the outer-sphere components of the equilibrium reaction energy (charging energies and ion interaction energies). We have included in our analysis mainly those components of the energy that change, relative to the free proteins, upon complex formation. These calculations have been carried out in the framework of the two-dielectric-constant formalism proposed recently for calculation of the electrostatics of proteins (Krishtalik et al., 1997). Furthermore, we have calculated the outer-sphere components of the reorganization energy.

Knowing the corresponding components of the equilibrium and reorganization energies, the activation energies for different complexes could be compared. It was found that the activation energies for electron transfer in all complexes are practically the same but somewhat higher (~1 kT) in DC1 and DC3 than in DC2. However, the main difference in behavior of these complexes arises from differences in electronic coupling. The much larger distance of electron transfer, including an appreciable spatial gap between the proteins along the direct pathway between the centers, makes the putative electrostatically preferred complex DC1 very unfavorable from the point of view of reaction rate. If it is formed initially, it should rearrange to one of the configurations that is competent for electron transfer.

It was also calculated that for a mutant of cyt *f* in which the four lysine residues that define most of the basic patch are neutralized, the redox potentials and the expected rate would be similar to that of the wild type, in agreement with the experimental data (Soriano et al., 1996).

MATERIALS AND METHODS

Docking

The complexes were constructed on a Silicon Graphics workstation with the aid of the program "O" (Jones and Kjeldgaard, 1995). The PC and cyt *f* molecules in a chosen orientation (described below in Results and Discussion) were brought to a close approach of selected residues. In the first step of this construction, wire models were used, and then all atoms of both molecules were represented as CPK spheres to ensure nonoverlapping of the structures.

The structural data for turnip cyt *f* were taken from Martinez et al. (1994, 1996), and the data for poplar PC were from Guss et al. (1992). In constructing the complexes, and in subsequent calculations, all external water molecules were discarded. For electrostatic calculations, it was essential to retain the five internal water molecules buried in the large domain of the cyt *f* globule (Martinez et al., 1996), which were treated as a part of the protein with the same dielectric properties as the whole globule.

Electrostatic calculations: equilibrium energetics

The calculations were performed with the aid of the DelPhi software package, version 3.0, QDIFFXS, 1990 (Gilson et al., 1987; Sharp and Honig, 1990; Nicholls and Honig, 1991). We have computed the self-energies of the ionic groups and the potentials that they generate, with an emphasis on the change of potentials upon complex formation.

Aqueous surroundings

Most calculations were performed with an ionic strength of 0.15 M. Calculations for the DC2 complex were also carried out at ionic strengths of 0.04 and 0.005 M. It was assumed that the reactant proteins are

surrounded by aqueous electrolyte, essentially as under in vitro conditions. One can ask whether the redox-active domain of cyt f in the intact b_6f complex is in sufficiently close contact with other membrane proteins that a substantial part of its surface becomes inaccessible to water, and hence the conditions of the dielectric screening are markedly different. However, it seems that if part of the cyt f surface interacts with another protein in the complex, the interaction would not involve a large enough fraction of the surface area of cyt f to significantly affect the dielectric screening. This is inferred from the close similarity of the redox potentials (~+0.37 V) of cyt f in 1) the intact membrane-bound b_6f complex and 2) soluble cyt f (Davenport and Hill, 1952; Rich and Bendall, 1980; Martinez et al., 1996; Metzger et al., 1997). This suggests that the cyt f dielectric surroundings are similar in both cases, not only around the heme but also near the surface ionic residues that generate substantial fields at the heme. That makes the model of cyt f and the cyt f-PC complex surrounded by the aqueous phase, i.e., for conditions in vitro, a reasonable approximation for the in vivo reaction.

Charges on amino acids and groups

We consider as fully ionized all amino groups (Lys Nζ, Arg guanidinium groups, and the amino terminus) and carboxylates (Asp, Glu, and the carboxy terminus). The ionization state of two heme propionates presents a special problem. In cyt f, in contrast to soluble c-type cytochromes, both these propionates are well exposed to the solvent (Martinez et al., 1994, 1996); the ionization of both propionates can be inferred from the pH independence of the heme redox potential over a wide range of pH, 4.5–8.5 (Martinez et al., 1996). His-142, the only histidine in the protein except for the heme ligand, is partly or fully ionized, depending on pH, but is situated far (35 Å) from the heme as well as from the Cu (39–50 Å) in all complexes, and hence its field is negligibly small.

A net charge of +1 was placed on the lysine ammonium nitrogen and equally distributed among the three nitrogens of the arginine guanidinium group. For carboxylates, charges of −0.5 were ascribed to both oxygens. In the construction of the dielectric boundary, H atoms were not taken into account explicitly, but, as usual in the DelPhi program, the effective radius of all heavy atoms was employed.

The interaction energy of the newly formed charge at the oxidized heme or copper site was calculated as the product of this charge (+1) and of the resultant potential at the Fe or Cu center. For free proteins, these interaction energies were also calculated by another method, distributing the entire charge among four ligands (four pyrrole N atoms ligating the heme and two N and two S atoms for the copper site) and multiplying them by the corresponding potentials. The resulting difference in interaction energy was ≤1.5% for Fe and 5% for Cu. Therefore, a more detailed calculation with the charge partially distributed over the ligands was not carried out.

As shown below (Tables 1 b 2 b), the effect of the ionized groups of the proteins is not very large. Therefore, the substantially weaker effect of the change of the dipole fields, which arises from partial charges of atoms of the polar bonds, was neglected. The dipole field decays much faster with distance and hence is strongly screened outside the protein globule.

DelPhi parameters

The calculations were performed using different grid sizes (65 and 151) and fractions of box filling (60 to 90%), with the grid spacing varied from 1.32 Å to 0.35 Å. In this interval of grid parameters, the calculated energies varied by 3–5% (in rare cases, when the calculated quantity was less than 1 kT, the difference reached 10%). The final calculations were performed with grid number 151 and a box filling of 75%. The errors in the quantities calculated for different complexes may arise from different orientations of the complexes in the box. As the grid parameters are determined by the largest dimension of the globule in the box, the same spacing (0.68 Å) was obtained, for the parameters chosen, for the elongated cyt f and for its complexes with PC. For the smaller and more spherical PC, the spacing was smaller (0.35 Å). Therefore, the calculations for free PC and for complexes were performed with a somewhat different degree of accuracy caused by different grid spacings. However, this factor did not influence the relative data for the different complexes because the parameters of the calculation were the same for each.

Optical and static dielectric constants

Two different contributions to the charging energy of each of the redox centers were taken into account (Krishtalik et al., 1997): 1) the energy of the new charge in the pre-existing electric field due to ions that were present in the globule before the appearance of the new charge; 2) the energy of the interaction of the new charge with the polarization of the surroundings, a polarization that did not exist before ion formation and is caused by the field of this newly formed ion. The charges of the protein that give rise to the pre-existing field are screened by the electronic polarization only. This is because in the equilibrium structure (used in the calculation of the pre-existing field), before formation of the ion under consideration, the positions of the atoms of the protein are already defined. Hence, one need not account for the shift of these atoms in response to the field of the pre-existing ions, i.e., not account for the atomic polarization. Therefore, the appropriate dielectric constant for the calculations is the optical constant. However, because the new charge causes not only electronic polarization but also shifts of atomic positions that were not accounted for in the equilibrium structure, the second energy component should be calculated using the static dielectric constant. In this case, both kinds of polarization are operative, and this is reflected by use of the static dielectric constant.

As discussed by Krishtalik et al. (1997), the optical constant ϵ_o should reflect not only electronic polarization but also a minor component of polarization due to the shift of protons (the "quantum boundary of dielectric constant," Kuznetsov et al., 1988). The purely electronic component of ϵ_o for liquid amides is ~2.0, and the quantum correction gives $\epsilon_o \approx 2.1$ (Krishtalik et al., 1997). Taking into account the higher density of the protein matrix compared with liquid amides, we estimate from the Clausius-Mossotti equation that $\epsilon_o = 2.4$, the value used in the present calculations.

The optical dielectric constant is used in the calculations of the electric potentials for the free proteins. However, when considering the cyt-PC complex, it is necessary to take into account that the initial positions of atoms in the free proteins will change somewhat upon docking under the action of the electric field of the partner's ions. The field of the ionized groups of each protein will also change as a result of changes in their screening from the aqueous surroundings. Therefore, the static dielectric constant ϵ_s was employed in calculations of the field of ions in the complex.

The usual value accepted for the static dielectric constant of proteins is ~4. However, this value corresponds to practically static conditions in

TABLE 1 Components of the shift of redox potential upon docking, calculated with ϵ_s = 3.5 (kT units)

	DC1		DC2		DC3	
	Fe	Cu	Fe	Cu	Fe	Cu
a. Change of self-energy	+0.9	+1.2	+1.1	+2.2	+0.8	+1.6
b. Change in interaction energy with all ionic groups	−0.4	+0.2	−1.9	−2.1	−0.2	−0.2
c. Total change of redox potential, $\Delta E = a + b$	+0.5	+1.4	−0.8	+0.1	+0.6	+1.4
d. Change of redox reaction free energy, $\Delta\Delta G = \Delta E_{Fe} - \Delta E_{Cu}$	−0.9		−0.9		−0.8	

To obtain ΔG of the docked complex, −0.5 kT should be added to values in line d.

TABLE 2 Components of the shift of redox potential upon docking, calculated with ϵ_s = 4 (kT units)

	DC1		DC2		DC3	
	Fe	Cu	Fe	Cu	Fe	Cu
a. Change of self-energy	+0.2	+0.3	+0.4	+1.2	+0.1	+0.6
b. Change in interaction energy with all ionic groups	−0.2	+0.3	−1.6	−1.9	−0.1	−0.1
c. Total change of redox potential, $\Delta E = a + b$	0	+0.6	−1.2	−0.7	0	+0.5
d. Change of redox reaction free energy, $\Delta\Delta G = \Delta E_{Fe} - \Delta E_{Cu}$	−0.6		−0.5		−0.5	

To obtain ΔG of the docked complex, −0.6 kT should be added to values in line d.

which the variation of the electric field proceeds so slowly that all kinds of protein polarization modes have enough time to respond to the changes of the field. Due to a very wide range of atom and group mobilities in proteins, there exists a wide range of dielectric relaxation times, making the effective static constant dependent on the characteristic time of the process. Recently, this dependence was estimated on the basis of the data on the electron transfer events that take place over a very large time range in the bacterial photosynthetic reaction center (Krishtalik, 1996). The characteristic time of the cyt f-PC intracomplex electron transfer is approximately 100–200 μs (Delosme, 1991; Kuras et al., 1995; Soriano et al., 1996). For this time interval, an effective ϵ_s = 3.5 was found (Krishtalik, 1996) and is used in some of the present calculations (Table 1).

The DelPhi software package computes only a part of the charging energy, namely, the reaction field energy due to polarization of the aqueous phase surrounding the protein. This is because only one dielectric boundary is accounted for in this program, namely, the protein/water boundary. However, there is an additional dielectric boundary, the ion/protein boundary. Polarization of the protein surrounding the ion (e.g., heme) causes an additional reaction field resulting in a second component of the total charging energy. In calculations where the dielectric constant of the protein remains the same for all quantities under consideration, this component remains practically constant and hence cancels out of all energy differences. However, in the present analysis one considers the possibility of different ϵ_s values for free proteins and the complex (e.g., for calculations of reorganization energy, two different dielectric constants, optical and static, should be used for the same complex, as discussed below). Therefore, one should include changes in this second component of the charging energies in media with different values of ϵ_s. It will be calculated as the charging energy of the ion in an infinite medium with the static dielectric constant of the protein, i.e., in the system involving only the ion/protein boundary. Representation of the total energy as a sum of reaction field energies for two systems with one boundary each (protein/water and ion/protein) presents an approximation that is strictly correct in the case of equal dielectric constants of the ion and its surrounding protein. As both of these values are similar (ϵ for ion can be estimated as a typical ϵ_o value of 2–2.5) and low in comparison to ϵ of external water, this approximation is acceptable. The charging energy in an infinite medium was approximated by a simple Born formula for spherical ions, i.e., $e^2/2a\epsilon_s$, where e is the ionic charge and a the ionic radius. The estimate of redox center radii done on the basis of standard crystallographic distances is, for heme with axial ligands, ~6 Å, and for Cu with ligands, ~4.5 Å. The effect of changing the Born energy introduces a relatively small correction to the redox potential difference between Fe and Cu. It is important that this correction be the same for all docking complexes and not influence their relative energy.

The estimate of ϵ_s = 3.5 has an approximate character. Therefore, calculations with ϵ_s = 4 were also performed (Table 2). Comparison of these two modes of calculation allows an evaluation of the accuracy of the calculations (see below, Results and Discussion).

Reorganization energy

Two different formalisms for the reorganization energy calculations have been used in the literature: the fixed-displacement-field and fixed-charge-density methods. Recently, arguments in favor of the fixed-charge-density formalism have been presented (Liu and Newton, 1994; Marcus, 1994; Medvedev and Kuznetsov, 1996). According to this method, to calculate the reorganization energy, one should calculate the sum of the charging energies of both reactants by the charge being transferred, including the (negative) Coulombic interaction energy of the charge at its initial and final positions (the interaction of the charge with the "hole" left after its transfer). This calculation should be performed separately with optical and static dielectric constants for all parts of the system. The reorganization energy is the difference of these optical and static total charging energies. The optical constant in these calculations is equivalent to the "quantum boundary constant" (Kuznetsov et al., 1988); i.e., for proteins, ϵ_o = 2.4, and for water, ϵ_o = 2.1.

In the present calculations, the total charging energies were calculated as a sum of three components: 1) the Born charging energies of both separated reactants in a protein of infinite extent (sum of these quantities for two ions without direct interaction between them), 2) the reaction field energies of both ions in a real globule calculated by the DelPhi program (i.e., the influence of polarization of the surrounding water by each ion on its own energy), and 3) the Coulombic interaction energies in a real globule calculated by the DelPhi program (i.e., direct Coulombic interaction between positive and negative (a hole) charges at the places of two ions plus interaction of one of ions with the reaction field of its partner). As pointed out above, the calculations of these three components were performed twice, using static and optical constants of both protein and water. The difference of these two sums is the medium reorganization energy.

RESULTS AND DISCUSSION

Cyt f-PC complexes

The three docking complexes considered here are shown in Figs. 1-3. The first (DC1, Fig. 1) is similar to that initially proposed to be electrostatically complementary (Martinez et al., 1994; Cramer et al., 1994) with the region of positive charges near the interface of the large and small domains of cyt f contacting a patch of negative charges on PC (Pearson et al., 1996). The existence of this docking intermediate was shown by covalent binding of cyt f and PC in vitro (Morand et al., 1989). In the present version, DC1 differs slightly from the structure proposed by Pearson et al. (1996). The PC molecule was shifted closer to the heme to ensure better attraction of the negatively charged residues of PC to Arg-209 of cyt f and to weaken their repulsion by the cyt f Glu-186. The R209 and E186 residues are not conserved in all cyt f, but they are present in turnip cyt f, which has been used in virtually all in vitro kinetic electron transfer experiments. Complexes DC2 and DC3 (Figs. 2 and 3) have some similarity to those discussed by Pearson et al. (1996); in both cases, His-87 of PC is situated close to Tyr-1 of cyt f, thereby providing a short distance for electron transfer. However, in contrast to the study of Pearson et al. (1996),

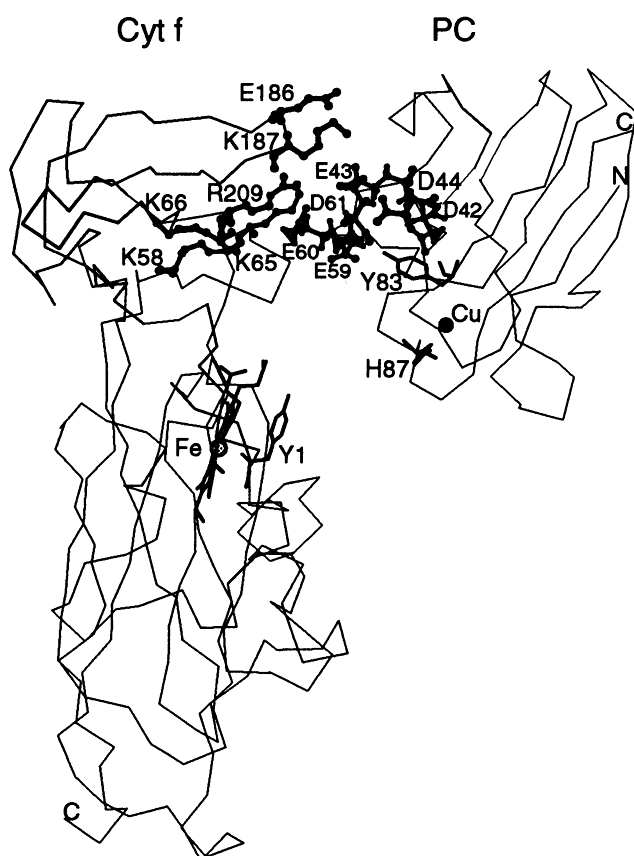

FIGURE 1 The structure of the DC1 complex between cyt *f* (*left*) and PC (*right*). The backbone is drawn in thin lines, the heme and the Tyr and His ligands in bold. Charged residues mentioned in the text are given in a stick-and-ball representation. Fe and Cu are shown as larger spheres. Distances (Å): Fe-Cu, 23.9; heme C2A-H87(Nϵ2), 17.8; K187(Nζ)-D44(Oδ1,Oδ2), 7.1; K187(Nζ)-D42(Oδ1,Oδ2), 10.9; R209(N)-D42(Oδ1,Oδ2), 12.8. Drawn with Molscript (Kraulis, 1991).

we have not tried to preserve a maximal electrostatic interaction of Asp-44 of PC with Lys-187 of cyt *f*. The larger distance between negative residues of PC and the positive ones of cyt *f* can particularly be seen for DC3 in Fig. 3 *B*, corresponding to a counterclockwise rotation of 40° around the long axis of cyt *f*, compared with the orientations shown in Fig. 3 *A*. The difference between DC2 and DC3 consists mainly of a clockwise rotation of ~30° of PC around the long axis of cyt *f* in DC3 relative to DC2; in DC2, the center of the positive patch on cyt *f* is farther from the Cu center than in DC3. In all docking complexes, Tyr-83 of PC is far from the heme, but in DC1 it is closer to the region of direct inter-protein contact than is His-87.

These three docking configurations have been chosen as representative of two classes of complexes: 1) DC1 as a primary collision complex the formation of which is guided by long-range electrostatic interactions between positively and negatively charged surface regions and 2) DC2 and DC3 as configurations favorable for the electron transfer itself.

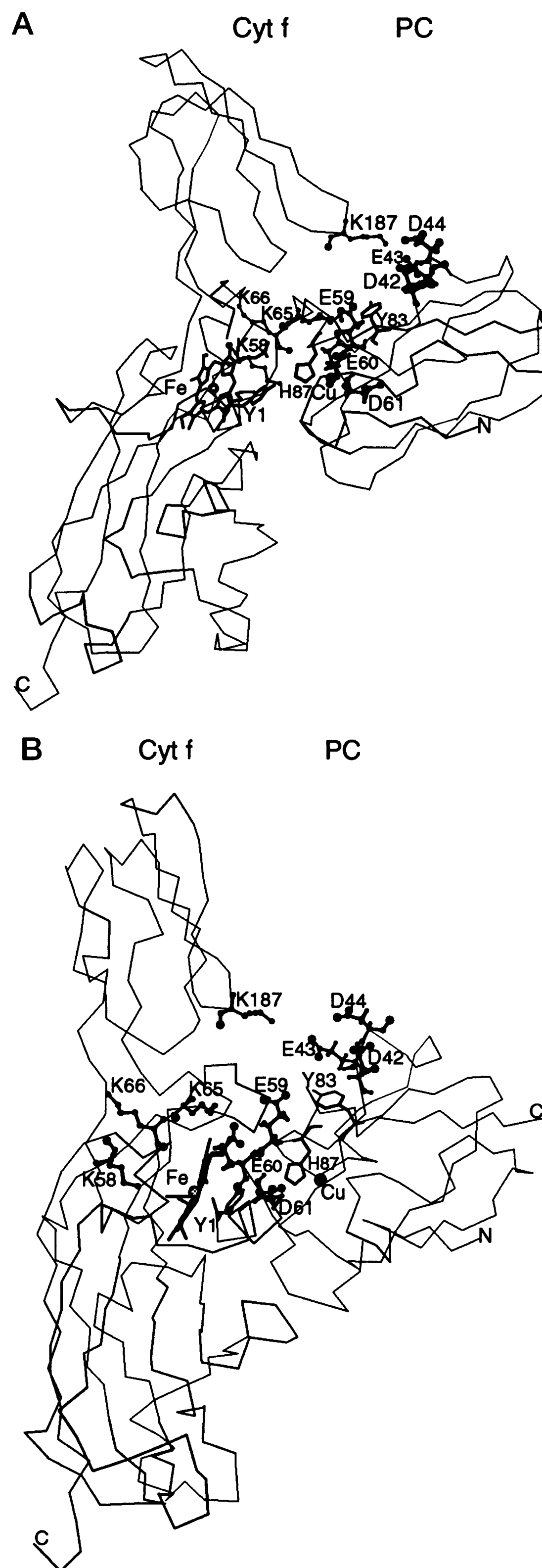

FIGURE 2 The structure of the DC2 complex. Designations are as in Fig. 1. (*A*) View showing the contact region of the two redox centers. (*B*) View from *A* rotated counterclockwise by 40° around the long axis of cyt *f* to show the absence of interprotein contact of charged residues. In this view, part of plastocyanin is shielded by cyt *f*. Distances (Å): Fe-Cu, 14.2; heme C3D-H87(Nϵ2), 8.3; H87(Nϵ2)-Y1(Cϵ2), 4.4; K187(Nζ)-D44(Oδ1,Oδ2), 7.8; K187(Nζ)-D42(Oδ1,Oδ2), 11.3.

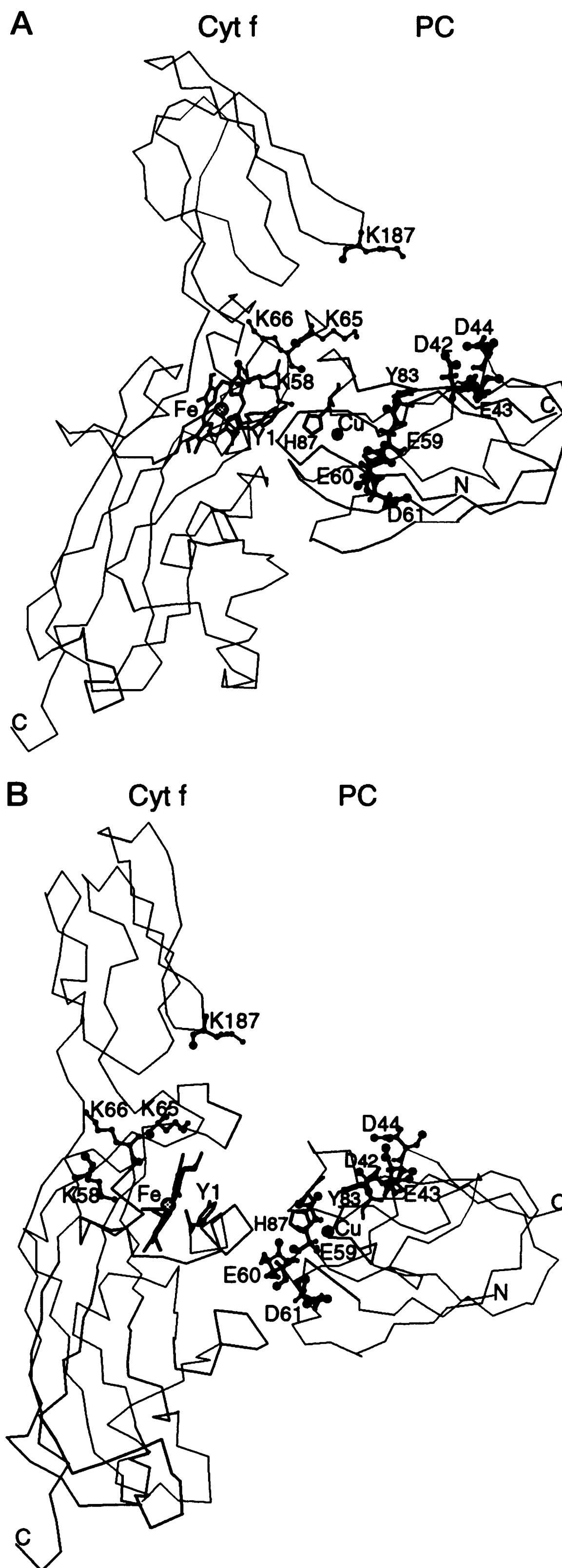

FIGURE 3 The structure of the DC3 complex. Designations are as in Fig. 1. (*A*) The view showing the contact region of two redox centers. (*B*) View from *a* rotated counterclockwise by 40° to show the absence of interprotein contact of charged residues. Distances (Å): Fe-Cu, 16.8; heme C1D-H87(Nϵ2), 12.7; H87(Nϵ2)-Y1(Cδ2), 8.6; K187(Nζ)-D44(Oδ1,Oδ2), 18.2; K187(Nζ)-D42(Oδ1,Oδ2), 21.1.

Equilibrium reaction energies

Energies of the free proteins

The values of the reaction field energy (not shown in Tables) of the cyt *f* heme is -6.6 *kT* and, for the Cu center in PC, -8.5 *kT*. The more negative value for PC arises from the closer distance of Cu to the protein surface, resulting in a stronger effect of polarization by the aqueous phase. The energy of interaction of all ions with the positive charge on the Fe center in oxidized cyt *f* is -1.4 *kT*, and the corresponding value for PC is -2.7 *kT*. The more negative value for PC probably reflects the larger excess of negatively charged carboxylate groups compared with basic residues (16 and 31 negative vs. 7 and 27 positive) in poplar PC and turnip cyt *f*, respectively.

Energies of the complexes

The components of the shift of redox potential upon docking of the complexes, relative to the equilibrium energy of reaction of two separate proteins, are shown in Tables 1 and 2. The data are computed with $\epsilon_s = 3.5$ (cf. Materials and Methods; incomplete dielectric relaxation during the reaction), and $\epsilon_s = 4$ in Tables 1 and 2, respectively.

The changes of the self-energies of ions (i.e., their charging energies in an infinite proteinaceous medium plus their reaction field energies) upon formation of complexes DC1, DC2, and DC3 are positive for both the Fe-heme and Cu centers (Tables 1 *a* and 2 *a*), as expected because of the smaller extent of solvent shielding upon formation of the complexes.

The shift of interaction energy of the Cu center set up by ionized groups has a small positive value in DC1, where the positive patch on cyt *f* has the closest contact with PC (Fig. 1). For the other complexes, the potential shift is negative (Tables 1 *b* and 2 *b*). For the Fe-heme center, the potential shift is negative for all complexes. The contribution of this component is smaller for DC3. The tendency toward negative potential shifts set up by the ionized groups can be ascribed to an excess of negative charges in each of the partner proteins.

The net effect of the two contributions (mainly opposite in sign) is a small shift (≤ 1 *kT*) of the heme redox potential, ΔE_{Fe}. The Cu redox potential shift, ΔE_{Cu}, is also small and mainly positive (Tables 1 *c* and 2 *c*).

It was argued (see Materials and Methods) that calculations using $\epsilon_s = 3.5$ (incomplete dielectric relaxation) may be preferable. Comparison of the data of Tables 1 and 2 shows that the results of the calculations carried out with two different ϵ_s values, 3.5 and 4, are similar, especially as they relate to the final quantities, the changes upon complex formation of the reaction free energy, $\Delta\Delta G$, for electron transfer. The changes of $\Delta\Delta G$ are small, negative, and approximately the same as calculated with $\epsilon_s = 3.5$ (approximately -0.9 $kT \approx -0.02$ eV) or 4 (approximately -0.5 $kT \approx -0.01$ eV). The negative value of $\Delta\Delta G$ means, from the thermodynamic point of view, that the binding

energy of reduced PC and oxidized cyt *f* is somewhat larger than the binding energy of reduced cyt *f* and oxidized PC. The experimental difference of redox potentials of free cyt *f* and PC, $\Delta E_{\text{Fe-Cu}}$, is approximately −0.01 to −0.02 V (Morand et al., 1989; Takabe and Ishikawa, 1989; Bagby et al., 1990a; Qin and Kostić, 1993). Adding to it the calculated $\Delta\Delta G$, we obtain the value of the reaction free energy between cyt *f* and PC in the complex of $\Delta G = -0.03$ to -0.04 eV and -0.02 to -0.03 eV for $\epsilon = 3.5$ and 4.0, respectively.

This estimate agrees reasonably well with the equilibrium constants obtained from the data on the rate constants for the forward and backward reactions. According to Qin and Kostić (1992) and Meyer et al. (1993), the energy difference is equal to 0 to −0.02 eV, respectively (note that the data of Meyer et al. (1993) refer to spinach cyt *f*; for soluble turnip cyt *f*, a smaller amplitude of cyt *f* reoxidation was observed, implying the possibility of a less negative ΔG, although it was not possible to obtain quantitative data for this system).

For the chemically cross-linked complex (CLC), there are slightly varying reports: 1) $\Delta E_{\text{Fe}} = -0.02$ V and $\Delta E_{\text{Cu}} = 0$ (Morand et al., 1989); 2) both potentials were shifted positively by 0.01 V (Qin and Kostić, 1993), or 3) by even smaller amounts (Takabe and Ishikawa, 1989). Thus, for the CLC, $\Delta\Delta G = -0.02$–0.00 eV, values close to those calculated for the non-cross-linked docking complexes.

It should be noted that for some other electron-transfer couples very small changes of redox potentials have been observed upon complex formation (Vanderkooi and Erecínska, 1974; Leonard and Yonetani, 1974; Burrows et al., 1991; McLendon et al., 1993). Small shifts of redox potentials (0.002–0.04 V) in the cyt *c*-cyt *c* peroxidase complex compared with those in the separate proteins were calculated by Zhou (1994). It should be mentioned that in the study of Zhou (1994), the difference between the appropriate dielectric constants for the free proteins and cyt *f*-PC in a complex was not taken into account.

All of the calculations discussed above were carried out with an ionic strength of 0.15 M. For DC2, and ionic strengths of 0.04 and 0.005 M, the change in reaction field energy for Fe and Cu centers is negligible (~0.01 *kT*). Calculations were carried out to document that at these lower ionic strengths the ion interaction energy of both Fe and Cu centers was shifted to more negative values. This is a result of a decrease in the Debye screening, which results in an increase in the contribution of the negative charges that are more abundant in both proteins. These effects were found to be small, or were the resulting changes in reaction and activation free energies and are not shown. An additional complication in the consideration of ionic strength effects is the finding of a decrease in the electron transfer rate at very low ionic strength (Meyer et al., 1993). This implies a significant role of hydrophobic interactions in the docking processes between cytochrome *t* and plastocyanin.

As discussed above, the total changes of redox potentials are relatively small, in particular, the contribution from the change of the redox center ionic self-energy. However, this should not be an intrinsic property of all inter-protein contacts but rather a feature of this particular system. As can be seen from Figs. 1–3, the region of contact is rather small, and hence it does not substantially shield the redox centers from electrostatic interaction with the surrounding aqueous solution. From this point of view, it is interesting to mention that a markedly larger shift of redox potential (approximately +50 to +60 mV) has been estimated for the complex of PC with photosystem I (Drepper et al., 1996), and a substantial difference of redox potentials (~+75 mV) was found for the soluble Rieske protein compared with its value when incorporated in an intact b_6f complex (Zhang et al., 1996). In both of these cases, the redox center is embedded in a large membrane structure, and therefore one should expect its more pronounced shielding by a low dielectric phase. The sign of the potential shifts corresponds to an increase in charging energy of a cation and an anion, respectively.

Effect of mutations in the cyt *f* positive patch

The calculations for mutated cyt *f* in which the four lysines in the interdomain basic patch were neutralized through site-directed mutagenesis were performed using the coordinates of turnip cyt *f* (Martinez et al., 1996). The charge on the Lys ζ-amino N of the side chains of residues 58, 65, and 187 (K58, -65, and -189 in the *Chlamydomonas* notation) was set equal to 0, which corresponds to the mutation of these lysines to residues with neutral side chains, as was done by Soriano et al. (1996). The mutation K66E was described by neutralization of Lys ζ-amino N and ascribing a charge of −1 to Cϵ of the Lys side chain (this C atom takes a position in the side chain equivalent to the center of gravity of the two Oϵ atoms of Glu). We have not tried to optimize the side chain configuration in the mutants, retaining for them the coordinates of the wild type. We believe that the main effect here is the change of charge, and hence we have not considered secondary effects due to small positional shifts of neutral atoms. The fifth mutation studied by Soriano et al. (1996) neutralizes Lys-188 in *C. reinhardtii* cyt *f*. Calculations show that this residue, far from the heme and well screened by water, contributes a very small value, ~0.02 *kT*, to the electrostatic energy of the heme. Hence, its effect on the difference of redox potentials should be even smaller.

In calculations of the effect on the reaction free energy of mutations in the region of the cyt *f* poly-Lys positive patch, a static dielectric constant, ϵ_s, should be employed. This is in contrast to the calculations of the electric field in the isolated proteins discussed above, where only the optical polarization was taken into account and ϵ_o was used. The reason for the use of ϵ_s for calculation of the effect of the mutations changing the charge on the lysines is that, upon neutralization of an ionized group, all of the polarization produced by this group disappears. Then, some shift of atoms from their equilibrium positions, characteristic for the

wild type, takes place. Therefore, the effect of atomic polarization should be added to that of electronic polarization; i.e., the total static dielectric constant should be involved in these calculations.

Elimination of the positive charge on the four Lys residues of the basic patch of cyt *f* (Soriano et al., 1996) would result in a ΔE_{Fe} of free cyt *f* of only -0.45 kT/e (-11 mV) because these ionized side chains are far away from the heme and very well screened by water. Similar values of ΔE_{Fe} have been calculated for all docking complexes, and somewhat smaller ΔE values are characteristic of the more distant Cu center (Table 3). The total change of the reaction energy is very small ($\Delta\Delta G = -0.1$ to -0.4 kT, i.e., on the order of 3–10 meV) and practically the same for all dockings. This change would produce a change in activation energy of -0.1 to -0.2 kT (see below, Eq. 3), causing an acceleration in the rate of electron transfer by 10–20%. For DC2, the most likely electron-transfer-competent complex, the increase in electron transfer rate would be less than 10%, i.e., at the limit of experimental detection.

A decrease in the rate of cyt *f* oxidation by ~25% was observed in vivo for a fivefold Lys minus mutant (Soriano et al., 1996). This is a much smaller effect than is expected on the basis of in vitro data if the experimentally observed ion strength dependencies were due to electrostatic interaction of PC with the cyt *f* positive patch. (An effect of mutagenesis of the cyt *f* lysines on the cyt *f* oxidation rate that is intermediate between the in vitro and in vivo results has been reported in permeabilized (nebulized) cells of *C. reinhardtii* (Fernandez-Velasco et al., 1997).) The smaller effect has been interpreted as the absence of a rate limitation by collision complex formation because of the short diffusion distances in the small volume of the thylakoid lumen (Soriano et al., 1996). As can be seen, one can expect practically the same electron-transfer rate in the reaction complex for the wild type and mutant, with only a small increase in the rate for the mutants (Table 3). The slight decrease in the experimentally observed rate could be ascribed to a residual effect on the collision probability.

The calculations given above have shown a very small effect of mutational changes in the lysine-rich region that neutralize the positive charge. The shift of potential is small already in the free cyt *f*, and the effect on the potential difference in docking complexes is even smaller. This should be valid for any reasonable complex with other small electron-transferring proteins. From this point of view it is understandable that practically the same effects of the mutational changes were observed in vivo in Cu-deficient *C. reinhardtii* cells for the cyt *f* reaction with cytochrome c_6 (Soriano et al., 1996).

Reorganization and activation energies

In general, the reaction rate constant can be presented as a product of some pre-exponential (frequency) factor κ and the Boltzmann-type exponential involving the activation energy, ΔG,

$$k_{et} = \kappa \exp\{-\Delta G^{\ddagger}/kT\} \quad (1)$$

In the charge transfer theory, activation energy presents a function of two parameters, the reorganization energy λ and the free energy of the elementary act of reaction ΔG, which for simple electron transfer processes equals the difference of redox potentials. Then,

$$\Delta G^{\ddagger} = \frac{(\lambda + \Delta G)^2}{4\lambda} \quad (2)$$

The calculations of reorganization energies for electron transfer in the three docking complexes, DC1–DC3, described in Materials and Methods, are presented in Table 4. The component in Table 4 *a* is the difference of the sum of charging energies of two ions in infinite optical and static proteinaceous media; it is equal to $e^2(1/\epsilon_o - 1/\epsilon_s)(1/2a_1 + 1/2a_2)$, where a_1 and a_2 are ionic radii. This component is the same for all dockings and hence does not affect their relative energies. However, this value is important for evaluation of the order of magnitude of the total reorganization energy (see below).

In calculations of reaction field energy (Table 4 *b*) and Coulombic interaction (Table 4 *c*) in optical media, the relaxation of the ionic atmosphere should not been taken into account. Indeed, the processes in optical media are by definition very fast, in a subfemtosecond time range, whereas the ionic atmosphere relaxation time is on the order of nanoseconds. In the DelPhi program, this relaxation can be excluded by setting the ionic strength equal to zero; in this case, the program ascribes a Debye radius of 0.01 cm, fully excluding the field screening by the ionic atmosphere. In static media, the ionic relaxation should be fully included.

The reaction field energy in the media with the optical dielectric constants, both for protein and water (Table 4 *b*), is positive because this constant of the aqueous surroundings, $\epsilon_o = 2.1$, is smaller than that ($\epsilon_o = 2.4$) of the protein. In a static medium, the relationship between dielectric constants is reversed (80 and 3.5, respectively), and hence this entry is negative (Table 4 *d*).

The Coulombic interactions of the charge being transferred at its initial and final positions (i.e., an effective positive charge at one of the redox centers and a negative one at the other center; Table 4, *c* and *e*) are smaller for

TABLE 3 Shift of redox potential in the four-Lys$^-$ mutant relative to the wild type, calculated with ϵ_s = 3.5 (kT units)

	DC1		DC2		DC3	
	Fe	Cu	Fe	Cu	Fe	Cu
a. Change in interaction energy with all ions	−0.50	−0.40	−0.45	−0.30	−0.45	−0.10
b. Change of redox reaction free energy, $\Delta\Delta G = \Delta E_{Fe} - \Delta E_{Cu}$	−0.1		−0.2		−0.4	

TABLE 4 Reorganization and activation energies of complexes DC1, DC2, and DC3 (*kT* units)

	DC1	DC2	DC3
a. Difference of the charging energies of Cu and heme ions*	14.3	14.3	14.3
b. Sum of reaction field energies of Cu and heme ions (ϵ_s = 2.4 and 2.1)[#]	+2.7	+2.2	+2.35
c. Coulombic interaction energy in same medium as line b[§]	−10.0	−16.1	−13.6
d. Sum of reaction field energies of Cu and heme ions (ϵ_s = 3.5 and 80)[¶]	−14.5	−15.6	−16.6
e. Coulombic interaction in the same medium as line d	−0.1	−0.9	−0.7
f. Medium reorganization energy, $f = a + b + c - d - e$	21.6	16.9	20.3
g. Medium-dependent part of the activation energy	4.6	3.5	4.3

*Difference of the sums of charging energies of Cu and heme ions calculated for infinite optical (ϵ_o = 2.4) and static (ϵ_s = 3.5) protein media, calculated using $e^2(1/2a_1 + 1/2a_2)(1/\epsilon_o - 1/\epsilon_s)$.
[#]Sum of reaction field energies of Cu and heme ions in optical protein (ϵ_o = 2.4) and aqueous (ϵ_o = 2.1) media.
[§]Coulombic interaction between charge and "hole" in the same medium as line b.
[¶]Sum of reaction field energies of Cu and heme ions in static protein (ϵ_s = 3.5) and aqueous (ϵ_s = 80) media.

DC1, because in this complex, the distance between Fe and Cu is much larger than for DC2 and DC3, respectively (see discussion below). The large difference in the Coulombic energies in the medium with optical dielectric constants (Table 4 *c*) is the main determinant of the difference in reorganization energies (Table 4 *f*).

The calculated values of the medium reorganization energies λ lie in the range of 17–22 *kT* (Table 4 *f*). The total values of reorganization energy (~1.0 eV ≈ 40 *kT*) should be much larger. A contribution from the inner sphere reorganization (e.g., changes of metal-ligand distances) should be added to them. In either case, $\lambda \gg |\Delta G|$ (Tables 1 and 4), and the general formula for activation energy, Eq. 2, can be presented in a simpler form:

$$\Delta G^{\ddagger} = \frac{\lambda}{4} + \frac{\Delta G}{2} \tag{3}$$

The contributions to the activation energy calculated by Eq. 3 from the data of Tables 1 and 4 are shown (Table 4 *g*). It can be seen that the estimated activation energies for electron transfer inside all complexes are similar. For DC2, it is smaller by ~1 *kT*, resulting in an approximately threefold increase in the reaction velocity, but this effect lies at the limit of accuracy of the calculation.

As was discussed above, the cross-linked complex (CLC) is similar in some aspects but not identical to DC1. One can expect for the CLC a large Fe-Cu distance, similar to that in DC1, implying that the reorganization energies in these two complexes should be similar. Assuming that the reorganization energy for CLC is equal to that for DC1, and using the experimental difference of redox potentials in the cross-linked complex, i.e., $\Delta G \approx -0.03$ eV (Morand et al., 1989; Takabe and Ishikawa, 1989) or $\Delta G \approx -0.01$ eV (Qin and Kostić, 1993), one obtains an activation energy for CLC only ~1 *kT* larger than that for DC2. Therefore, the inefficient electron transfer in CLC is not explained by a large $\Delta G^{\ddagger}$.

Distance dependence of the electronic factor

Despite an approximate equivalence of all the complexes from the point of view of activation energies, the DC1 complex seems to be much less favorable than the others as it relates to the pre-exponential factor in the rate equation. The larger distance of electron transfer in the DC1 complex makes the electronic wave function coupling in this complex much poorer than in DC2/DC3, and this drastically decreases the reaction rate. This effect has been estimated using an empirical rate-distance relationship, $k_{et} \propto \exp(-\beta R)$ (Moser et al., 1992), where $\beta = 1.4$ Å^{-1} and R is the donor-acceptor distance. Several different estimates of this distance have been employed. Over a distance equal to that between the Fe and Cu centers for DC1 (Fe-Cu distance = 23.9 Å), log k_{et} decreases by 5.9 and 4.4 compared with DC2 (Fe-Cu distance = 14.2 Å) and DC3 (Fe-Cu distance = 16.8 Å), respectively. If the shortest distance is that between one of the C atoms of the porphyrin ring (these are different atoms for different dockings) and the Nϵ2 atom of His-87 (ligand to Cu in PC), the corresponding distances for DC1, DC2, and DC3 are 17.8, 8.3, and 12.7 Å and the decrements in log k_{et} relative to DC1 are 5.8 and 3.1, respectively. When the pathway through Tyr-1 to His-87 is preferable (distances, 15.5, 4.4, and 8.6 Å), then the difference in log k_{et} between DC1 and DC2 or DC3 is 6.8 and 4.2. In these considerations, coupling inside the porphyrin or phenyl ring was assumed to be the same and did not influence the relative values of the k_{et}. Thus, the kinetics of electron transfer in the DC1 complex should be retarded by 6–7 orders of magnitude, compared with DC2, which has the most favorable distance dependence. It should be stressed that these estimates are conservative. Indeed, the direction of the shortest distance between Fe and Cu or between porphyrin (or Tyr-1) and His-87 in the complex DC1 crosses a water-filled gap between the two proteins (Fig. 1). As water is a medium with poorer electronic coupling than protein, the real electronic pathway in DC1 should circumvent this gap. The situation in DC2 and DC3 is quite different because in these complexes the shortest pathways lie entirely inside the proteins (Figs. 2 and 3).

The calculated reaction rate difference between DC2 and DC3 consists of two contributions: 2) the smaller one arises from the difference of activation energies (Δlog $k_{et} \approx 0.5$; see Table 4 *g*). The differences in electronic factor, calculated above, result in Δlog $k_{et} \approx 1.5$–2.7. Thus, the effi-

ciency of electron transfer from cyt *f* to PC is two to three orders of magnitude greater in DC2 compared with DC3. However, this difference is not as large as the difference relative to DC1. Therefore, it is reasonable to suppose that several complexes with structures intermediate between DC2 and DC3 can have comparable rate constants, and hence a considerable part of the surfaces of both proteins, close to their redox centers, may dock and form reaction-competent complexes. Surface regions of this type could also serve as contact sites for electron transfer from the Rieske (Fe-2S) protein, the donor to cyt *f*.

The contributions of outer sphere components to the reorganization (λ) and activation ($\Delta G^{\ddagger}$) energies (Table 4), considered in detail above, are not sufficient to predict the measured k_{et}. An edge-edge distance for DC2 of 8.3 Å implies an approximate activationless $k_{et} = 10^9$–10^{10} s^{-1} for cyt $f \rightarrow$ PC electron transfer according to the criteria of Moser et al. (1992). Using the value of $\Delta G^{\ddagger}$ for DC2 in Table 4 derived for $\lambda \approx 17$ kT (~0.42 eV), $k_{et} \approx 10^7$–10^8 s^{-1}. The measured value of $\sim 3 \times 10^3$ s^{-1} implies that an effective value of λ for this reaction is $\geq$1.0 eV. The difference is attributed to 1) too small a value of λ due to omission of inner sphere reorganization energy and/or 2) overestimation of the electronic (distance) factor in the region of the inter-protein electron transfer. The empirical rules of Moser et al. (1992) and Gray and Winkler (1996) are derived from intra-protein electron transfer. The situation for two weakly interacting soluble proteins may be much less favorable.

In a theoretical analysis of the cyt *f*-PC reaction (Ullmann et al., 1997), different docking configurations were obtained by Monte Carlo and molecular dynamics simulations of the approach of the proteins. An analysis of electronic factors for these docking complexes has shown that maximal overlap of the electronic wave functions is expected in a complex analogous to DC2 or DC3.

Ubbink and colleagues (D. S. Bendall, personal communication) have determined a structure for the complex formed in solution between spinach plastocyanin and turnip cyt *f* on the basis of NMR observations and other experimental results. This complex in solution is similar to DC2 that was proposed in this paper. In this structure, electrostatic contact between the basic ridge of cyt *f* and the acidic patches of plastocyanin is maintained, and the copper ligand His-87 makes van der Waals contact with the heme ligand Tyr-1 and also Phe-4.

One can imagine the electron transfer process in vitro as an approach to a configuration similar to DC1, guided by electrostatic attraction, with a subsequent diffusion of the partners until they accept one of the range of configurations such as DC2 or DC3 that are favorable for the electron transfer. The existence of several reactive configurations has been proposed for other electron-transfer partners (Bendall, 1996; Northrup, 1996). As discussed above, in vivo, the steering effect of the electrostatic interaction is less important, perhaps because of the smaller space available for diffusion and higher protein concentrations (Soriano et al., 1996). Hence, given these special conditions in vivo, the intermediate formation of a DC1-like complex seems not to be obligatory.

CONCLUSIONS

1. The shift of redox potentials upon docking as compared with free proteins is caused by the electric field of all ionized groups (amino acid residues and heme propionates) of the reaction partner and by the change in the dielectric surroundings upon docking. These effects are relatively small and, in some cases, partly compensatory.

2. For all docking complexes, the change of the reaction free energy upon complex formation is small (approximately -15 to -25 mV) and similar for all complexes.

3. Mutations in the positive patch on the cyt *f* surface are predicted to exert a very small effect on the redox potentials and reaction rate. This is due to the large distance of this patch from the redox centers of both proteins and to strong dielectric screening of these patches by the aqueous surroundings.

4. DC1 and DC3 have approximately the same reorganization energy but ~4 kT larger than that for DC2. However, electron transfer is much more favorable in DC2-DC3 than in DC1 because of the longer Fe-Cu distance in DC1 and also more favorable for this reason in DC2 compared with DC3. A large electron-transfer distance results in a large decrease in the electronic coupling factor and a low rate of intra-complex electron transfer for DC1. It is likely that the DC1 configuration is required only in the course of the mutual approach of the two proteins in vitro or in the chloroplast under conditions when the lumenal volume is expanded. Subsequently, the partners should reorient to another configuration, providing a much higher reaction rate. It is possible that there can exist not only a single reactive configuration but also a set of different inter-protein complexes having similar kinetic parameters.

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Coordinates of the docking complexes I–III are available upon request.

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