Kinetics of Photooxidation of Soluble Cytochromes, HiPIP, and Azurin by the Photosynthetic Reaction Center of the Purple Phototrophic Bacterium *Rhodopseudomonas viridis*†

T. E. Meyer,* R. G. Bartsch, M. A. Cusanovich, and G. Tollin

Department of Biochemistry, University of Arizona, Tucson, Arizona 85721

Received January 8, 1993; Revised Manuscript Received February 23, 1993

ABSTRACT: The photosynthetic reaction center of *Rhodopseudomonas viridis* contains a bound tetraheme cytochrome *c* subunit which is the primary electron donor to the photooxidized special pair bacteriochlorophyll. We have tested a variety of soluble electron-transfer proteins for their ability to serve as secondary electron donors to the bacteriochlorophyll via the bound cytochrome by measuring the kinetics of reaction center heme reduction following photooxidation by a laser flash, as a function of soluble protein concentration and ionic strength. All of the soluble redox proteins utilized appear to interact with a negatively charged region on the reaction center and to transfer electrons to the 300-mV heme *c*-556 of the bound cytochrome. *Rps. viridis* cytochrome c_2 was the best electron donor among those proteins tested, with a second-order rate constant extrapolated to infinite ionic strength of 1.2×10^6 M^{-1} s^{-1}, which is two orders of magnitude larger than that of horse cytochrome *c*. *Rps. viridis* cytochrome c_2 apparently binds to the reaction center at low ionic strength, as evidenced by a nonlinear dependence of k_{obs} on protein concentration. The limiting first-order electron-transfer rate constant at 6 mM ionic strength is approximately 1300 s^{-1}. Horse cytochrome *c* and the reaction center also form a complex, with a limiting first-order rate constant for electron transfer which is 5 times smaller than for cytochrome c_2. Other cytochromes c_2 are intermediate in reactivity. More distantly related cytochromes, HiPIP, and azurin are relatively poor electron donors under the conditions of assay. We conclude from these experiments that (i) soluble cytochrome binding to the reaction center does not correlate with the magnitude of the ionic strength effect on the electron-transfer kinetics; thus there may be more than one site of interaction, or forces other than electrostatics (e.g., hydrophobic interactions) may also be involved; (ii) electron-transfer rate constants do not correlate with thermodynamic driving force, and therefore parameters such as distance and orientation may also contribute; and (iii) the function of the bound cytochrome is to rapidly reduce the photooxidized reaction center bacteriochlorophyll special pair in order to prevent back-reaction from photoreduced quinone species.

The three-dimensional structure of the *Rhodopseudomonas viridis* reaction center (Deisenhofer et al., 1985) is fundamentally different from that of *Rhodobacter sphaeroides* (Allen et al., 1987) in that it contains a tightly bound tetraheme cytochrome *c* subunit. The purpose of this subunit and how it might alter the specificity of the reaction center toward soluble electron donors are unknown. It has been shown that each of the four hemes of the bound cytochrome has a distinctive redox potential, absorption spectrum, and orientation relative to the bacteriochlorophyll-containing subunits (Dracheva et al., 1986, 1988; Vermeglio et al., 1989; Fritzsch et al., 1989; Nitschke & Rutherford, 1989; Shinkarev et al., 1990; Nitschke et al., 1992; Fritz et al., 1992). The redox potentials of the four hemes also have been rationalized on the basis of the three-dimensional structure (Gunner & Honig, 1991). The 370-mV, 559-nm-absorbing heme 3, which is nearest the special pair bacteriochlorophyll, is photooxidized with a rate constant of 3.7×10^6 s^{-1}; the 300-mV, 556-nm-absorbing heme 2, which is third most distant from the special pair, is photooxidized with a rate constant of 2.8×10^5 s^{-1}; the 20-mV, 553-nm-absorbing heme 4, which is located between the two high-potential hemes, has a photooxidation rate constant of 7×10^6 s^{-1} (Dracheva et al., 1986, 1988; Shopes et al., 1987). Measurement of electron transfer from the lowest potential heme 1 is technically not feasible without chemical modification because the electron acceptor quinones are partially reduced at the redox potentials required for the experiment.

Bacterial species which contain the bound reaction center cytochrome are more commonly found than are those which do not (Bartsch, 1991). On the other hand, all those species which do not have a bound reaction center cytochrome do have a soluble cytochrome c_2. This suggests that cytochrome c_2 may be as reactive as the bound cytochrome in some cases. It is unusual that both cytochromes should occur in the same species, for example, as in *Rps. viridis*. Soluble cytochrome c_2 was found to effectively couple the bc_1 complex to the reaction center in *Rps. viridis* (Shill & Wood, 1984; Knaff et al., 1991). The second-order rate constant for oxidation of this cytochrome c_2 by the reaction center cytochrome was determined to be approximately 10^6–10^7 M^{-1} s^{-1} at $I = 5$ mM, which is 2–3 orders of magnitude smaller than for comparable reactions in the *Rb. sphaeroides* system. *Rps. viridis* cytochrome c_2 has been observed to form a complex with the reaction center cytochrome and to transfer electrons with a reported limiting first-order rate constant of 270 s^{-1} and a dissociation constant of 30 μM at $I = 6$ mM (Knaff et al., 1991). Horse cytochrome *c* was found to be a much less efficient electron donor with a second-order rate constant of 1.7×10^5 M^{-1} s^{-1} at $I = 6$ mM (Knaff et al., 1991).

It could be argued that the bound cytochrome is superfluous and that the *Rps. viridis* cytochrome c_2 would be oxidized more rapidly if the bound cytochrome were not present.

† This work was supported in part by NIH Grants DK 15057 to G.T. and GM 21277 to M.A.C.

* To whom correspondence should be addressed.

0006-2960/93/0432-4719$04.00/0

However, not all phototrophic bacteria contain a cytochrome c_2, nor even an abundant, soluble, high-potential cytochrome *c* of any sort (Bartsch, 1991). In such cases, assuming that a soluble electron donor is necessary at all, iron–sulfur proteins or copper proteins might mediate electron transfer between the bc_1 complex and the reaction center. Each heme of the bound reaction center cytochrome might have a different specificity for electron donors related to its redox potential, to its proximity to the protein surface, and to the distribution of charged residues which could form an electrostatic complex with a soluble donor. However, if this were the case, then soluble reactants would have to be very efficient in order to compete with intramolecular electron transfer within the tetraheme cytochrome, which, as stated above, is quite rapid. Otherwise, the ambient redox potential would determine which heme would react with the soluble protein. In order to clarify the role of the bound cytochrome subunit, we have examined the specificity of the *Rps. viridis* reaction center for soluble electron donors.

MATERIALS AND METHODS

Photosynthetic reaction centers were prepared from *Rps. viridis* by a modification of the procedures of Thornber and Thornber (1980). Membranes from high-speed centrifugation of broken cells were suspended in 10 mM Tris-HCl buffer, pH 8, and 1 mM EDTA (TE buffer) to which 5% LDAO detergent had been added. The suspension was centrifuged and desalted into TE buffer plus 0.1% LDAO (TEL buffer). Crude reaction centers were adsorbed to DEAE-cellulose and chromatographed with a 0–50 mM NaCl gradient in TEL buffer and were assayed by the ratio of absorbance at 280–830 nm. The reaction centers eluted near the void volume of Sephadex G-200 in TEL buffer, and the final purity index was 2.1. The purified reaction centers were stored frozen in TE buffer with 0.025% LDAO.

Laser flash photolysis and data analysis were carried out as previously described (Tollin et al., 1986; Tollin & Hazzard, 1991; Watkins, 1986). Excitation was at 610 nm and absorbance changes followed at 556–558 nm. Kinetic data were analyzed using the program SI-FIT (Olis Co.). Errors in rate constants are estimated to be ≤10–20%. The experiments were performed aerobically in 5-mm cuvettes with 10 mM Tris-HCl buffer, pH 8, plus 1 mM ascorbate prepared fresh daily. Experiments using *Chlorobium c*-555 were performed anaerobically. Horse and *Candida krusei* cytochromes *c* were obtained from Sigma Chemical Co. Other redox proteins were prepared according to published procedures. Proteins were reduced with dithionite and exhaustively dialyzed against 10 mM Tris-HCl plus 0.5 M NaCl, pH 8, and then against 10 mM Tris-HCl, pH 8, to remove all multivalent ions.

RESULTS

In the absence of added secondary electron donors, a laser flash at 610 nm results in rapid photooxidation of the highest potential heme 3 (*c*-559) in the *Rps. viridis* reaction center cytochrome (Dracheva et al., 1986, 1988; Shopes et al., 1987; Ortega & Mathis, 1993). However, in the presence of ascorbate, which maintains both high-potential hemes 2 and 3 in the reduced state, the wavelength maximum for photobleaching shifts to 556 nm due to oxidation of heme 2, and the reaction is about an order of magnitude slower than for oxidation of heme 3. We have obtained similar results (data not shown) in the present study. Following a laser flash, back-reaction from reduced quinone to heme 3 occurs with a rate constant of about 0.1 s^{-1} (Figure 1, panel A). In the presence

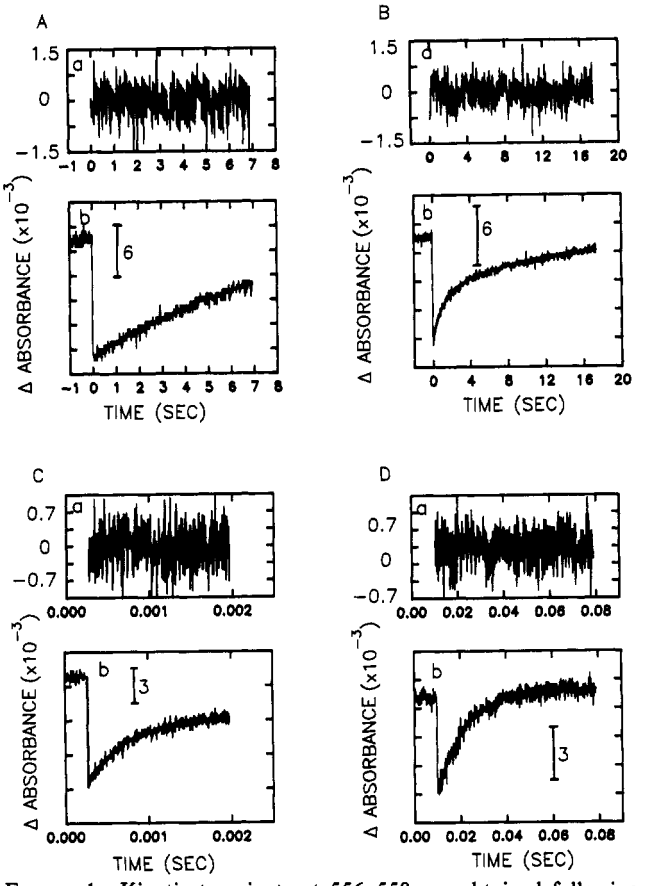

FIGURE 1: Kinetic transients at 556–558 nm obtained following photoexcitation of *Rps. viridis* reaction centers at 610 nm: (A) 15 μM reaction centers in 10 mM Tris-HCl buffer, pH 8; (B) conditions as in (A) plus 1 mM ascorbate; (C) conditions as in (B) plus 25 μM *Rps. viridis* cytochrome c_2; (D) conditions as in (B) plus 89 μM horse cytochrome *c*. The solid lines in panels b are theoretical fits to the data using a single exponential, except in (B), which was fit with two exponentials. Panels a show the residuals for the theoretical fits.

of 1 mM ascorbate, heme 2 is reduced in a biphasic reaction with rate constants of about 0.7 s^{-1} and 0.05 s^{-1} as shown in Figure 1 (panel B). We interpret these results as being due to reduction of heme 2 both by the reduced quinone species and by ascorbate (the concentration dependence for the latter reductant was not examined). The precise rate constant values are not important in the present context, except to demonstrate that these reactions are slow relative to reaction with soluble protein donors [however, see Gao et al. (1990) for a detailed analysis of recombination with reduced quinone].

When soluble *Rps. viridis* cytochrome c_2 is added to the mixture of reaction center and ascorbate, the initial photobleaching at 556 nm is followed by a rapid protein concentration dependent exponential absorbance increase as shown in Figure 1 (panel C). The absorbance does not completely return to the baseline because cytochrome c_2 and heme 2 have similar redox potentials, although the signal does approach the baseline at the highest protein concentrations used. The kinetics at 550 nm (the wavelength maximum for cytochrome c_2) are the same as the second phase at 556 nm (data not shown). At low ionic strength (I = 6 mM), second-order plots are nonlinear and exhibit saturation (Figure 2). The simplest interpretation of this behavior is that the reaction center and cytochrome c_2 form an intermediate complex within which electron transfer occurs, as previously reported (Knaff et al., 1991). To fit the data, a two-step mechanism was assumed involving rapid reversible binding followed by reversible electron transfer. An equilibrium constant of 1.22, based on the difference in redox potentials of the reactants,

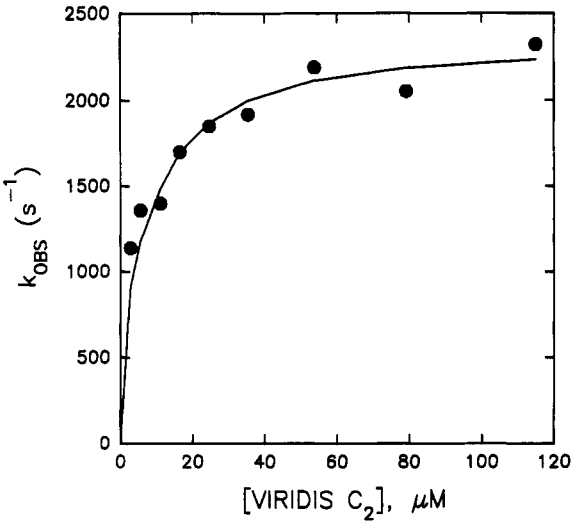

FIGURE 2: Concentration dependence of the reaction of *Rps. viridis* cytochrome c_2 with 15 μM reaction centers in 10 mM Tris-HCl buffer plus 1 mM ascorbate, pH 8. The solid line is a theoretical fit to the data as described in the text.

was also assumed. A nonlinear least squares analysis of the data shown in Figure 2 (Simondsen et al., 1982; Simondsen & Tollin, 1983) yields k_{12} (minimum value) = 1.3×10^8 M^{-1} s^{-1}, K_d = 8.6 μM, and k_{23} = 1300 s^{-1} (Table I). The k_{23} value is 5 times larger, and the dissociation constant 3.5 times smaller, than those previously reported (Knaff et al., 1991). The reason for this discrepancy is not clear. The k_{23} value is also about 2 orders of magnitude smaller than the rate constant for the initial reaction of heme 2 with the special pair (or more precisely between heme 2 and heme 3), thus precluding reaction between cytochrome c_2 and heme 3.

There is a large ionic strength effect on the observed rate constants for reaction of *Rps. viridis* cytochrome c_2 with *Rps. viridis* reaction center, as shown in Figure 3. This is qualitatively consistent with the earlier study (Knaff et al., 1991). The direction of the effect indicates that it is due to localized positive charge near the heme of cytochrome c_2 and localized negative charge near heme 2 of the reaction center cytochrome. A theoretical fit of the data by the method of Watkins [1986; cf. also Tollin et al. (1984)], using the parameters indicated in the legend to Figure 3, results in an electrostatic interaction energy, V_{ii}, = −5.2 kcal/mol (Table I). The charge product, $Z_1Z_2 = V_{ii}D_e\rho^2/\alpha r_{12}$, was calculated assuming D_e (the dielectric constant at the interface) = 10, ρ (the radius of the interaction site) = 7.25 Å, α = 128.47, and r_{12} (the distance of closest approach) = 3.5 Å. The values for these parameters were chosen to be consistent with our earlier work [cf. Tollin et al. (1984)]. Further, assuming that the interacting charges on cytochrome c_2 and reaction center are equal, then each protein would have 2.5 interacting charges. The limiting rate constant extrapolated to infinite ionic strength (k_∞ = 1.2×10^6 M^{-1} s^{-1}) is similar to values previously determined at moderately high ionic strength, 10^6 M^{-1} s^{-1} at I = 100 mM (Shill & Wood, 1984) and 2.5×10^5 M^{-1} s^{-1} at I = 1 M (Knaff et al., 1991).

When horse cytochrome *c* was substituted for *Rps. viridis* cytochrome c_2, its photooxidation was markedly slower as previously observed by Knaff et al. (1991). The signals were much larger than for *Rps. viridis* cytochrome c_2 and returned to the baseline at all protein concentrations as shown in Figure 1D. This is because horse cytochrome *c* has a much lower redox potential than do *Rps. viridis* cytochrome c_2 and reaction center heme 2. The data are also more reliable due to the larger signals at low protein concentrations. Second-order plots of k_{obs} vs cytochrome concentration are nonlinear at I = 6 mM as shown in Figure 4, again suggestive of complex formation. When the data are fit in the same manner as above, assuming an equilibrium constant of 4.76 based on redox potential differences, then k_{12} = 1.0×10^6 M^{-1} s^{-1}, K_d = 150 μM, and k_{23} = 330 s^{-1} (Table I). Both rate constants are markedly smaller than for *Rps. viridis* cytochrome c_2, despite the fact that horse cytochrome *c* has a much more favorable redox potential and should have been more reactive on the basis of considerations of thermodynamic driving force. Thus, other factors may be involved; i.e., horse cytochrome *c* may bind at a greater distance from heme 2 or it may bind in the wrong orientation for efficient electron transfer. The relatively weak binding constant is also remarkable (see below). Our value for the second-order rate constant is 6 times larger than that reported by Knaff et al. (1991). Again, the reason for the discrepancy is not apparent.

Horse cytochrome *c* also displays a large ionic strength effect, as shown in Figure 5, and interacts with a negative charge on the reaction center. The data were fit using the method of Watkins (1986), resulting in an electrostatic interaction energy of −5.8 kcal/mol and a rate constant at infinite ionic strength of 1.5×10^4 M^{-1} s^{-1} (Table I). The 2 orders of magnitude smaller rate constant at infinite ionic strength also indicates that the reaction with horse cytochrome *c* is less favorable than that with cytochrome c_2. The relatively large electrostatic interaction, comparable to that of *Rps. viridis* cytochrome c_2, is in marked contrast to the weak apparent binding constant, suggesting that other forces such as hydrophobic and van der Waals interactions contribute to binding, as has recently been reported for the yeast cytochrome *c* peroxidase/cytochrome *c* complexes by Pelletier and Kraut (1992). It is also possible that horse cytochrome *c* interacts at more than one site on the reaction center.

Candida krusei cytochrome *c* is closely related to horse cytochrome *c* but has a smaller net charge and shows less steric hindrance in other protein–protein electron-transfer reactions (Meyer et al., 1984; Tollin et al., 1984). We find that it behaves similarly to horse cytochrome *c* in its interaction with reaction centers as shown in Table I, except that there is no apparent saturation effect at high protein concentrations (up to 286 μM) at low ionic strength (I = 6 mM). This indicates a smaller binding constant than obtained with horse cytochrome *c*. Furthermore, the *Candida* cytochrome gives a smaller observed electrostatic interaction energy (−4 kcal/mol), which is probably a consequence of a smaller charge of the protein. It is noteworthy that the rate constant at infinite ionic strength is about 3.4 times larger than for horse cytochrome *c*, consistent with our previous observations concerning relative steric effects.

Rps. viridis cytochrome c_2 has a much smaller net positive charge (+1) than does horse cytochrome c (+7). It was therefore of interest to test the behavior of a cytochrome c which has an equally large net negative charge. We chose *Rhodospirillum salexigens* cytochrome c_2, which has a net charge of −8 and a redox potential which is almost the same as *Rps. viridis* cytochrome c_2. As shown in Table I, *R. salexigens* cytochrome c_2 displays similar behavior with reaction centers as does *Rps. viridis* cytochrome c_2. Binding is a little weaker, but the limiting rate constant is twice as large. These results, as well as those noted above with *Candida* cytochrome *c*, demonstrate that net charge does not determine the sign or the magnitude of the electrostatic interaction or whether or not a complex will be formed in this system. Rather, localized charge near the preferred site of electron transfer is dominant, and nonelectrostatic forces also contribute to the

Table I: Rate Constants for Interaction of Soluble Redox Proteins with Photooxidized *Rps. viridis* Reaction Centers in 10 mM Tris-HCl and 1 mM Ascorbate Buffer, pH 8[a]

	E_{m7} (mV)	net charge	k_{12} (M^{-1} s^{-1})	K_d (μM)	k_{23} (s^{-1})	k_{32} (s^{-1})	k_∞ (M^{-1} s^{-1})	V_{ii} (kcal)
Rps viridis c_2	295	+1	1.3×10^8	8.6	1300	1100	1.2×10^6	−5.2
R. salexigens c_2	300	−8	4.6×10^7	38	2500	2500	5.2×10^5	−6.3
Paracoccus c_2	250	−7	2.2×10^6				2.8×10^5	−3.1
Rb. sphaeroides c_2	370	−2	2.9×10^7	45	1400	4200	4.1×10^7	−0.8
horse *c*	260	+7	1.0×10^6	150	330	68	1.5×10^4	−5.8
Candida c	265	+5	1.1×10^6				5.1×10^4	−4.0
Chlorobium c-555	145	+6	8.2×10^7				2.4×10^7	−1.6
Pseudomonas c-551	270	−2	1.0×10^4				5.2×10^4	+2.5
C. vinosum c_4	325	−5	9.0×10^3				1.6×10^4	
Rc. tenuis c-553	405	+4	1.1×10^4				3.8×10^3	
Rc. gelatinosus HiPIP	330	+4	6.9×10^3				6.3×10^3	
Rps. marina HiPIP	345	+4	1.5×10^4				2.2×10^3	
R. salinarum HiPIP	265	−5	1.1×10^3				1.1×10^3	
Pseudomonas azurin	327	−2	8.3×10^3				3.8×10^4	

[a] k_{12}, K_d, k_{23}, and k_{32} are shown for those proteins displaying saturation kinetics in second-order plots at $I = 6$ mM. The number for k_{12} is a minimal value. The rate constants for those proteins displaying strictly second-order kinetics at $I = 6$ mM are also shown in the column headed k_{12}. The rate constant at infinite ionic strength, k_∞, and the electrostatic interaction energy, V_{ii}, are shown where a range of ionic strengths were examined and parameters calculated according to the Watkins (1986) equation. Some proteins were examined at only two ionic strengths, $I = 6$ mM and $I = 506$ mM. The rate constants for these latter proteins at $I = 506$ mM are shown in the column headed k_∞ without an accompanying value for V_{ii}.

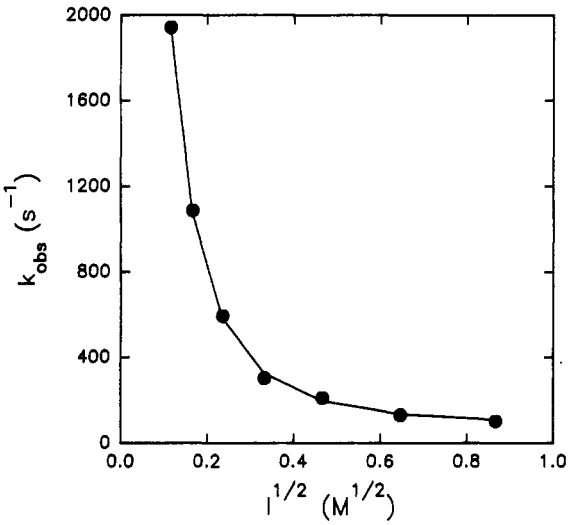

FIGURE 3: Effect of ionic strength on the observed first-order rate constants for reaction of 71 μM *Rps. viridis* cytochrome c_2 with 15 μM reaction centers. Ionic strength was varied with NaCl. The solid line is a theoretical fit to the data analyzed according to Watkins (1986), using an interaction radius of 7.25 Å.

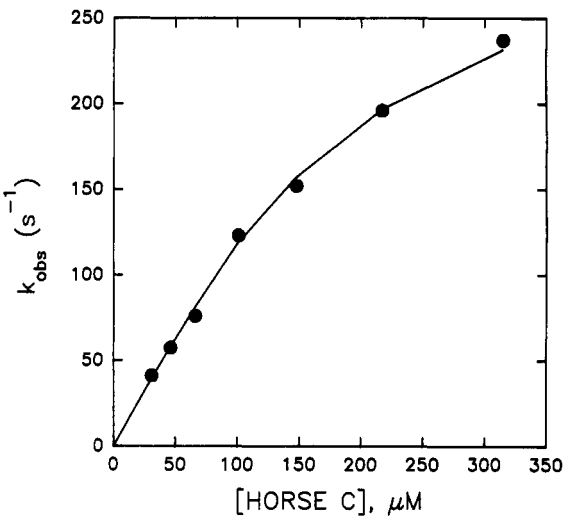

FIGURE 4: Concentration dependence of the reaction of horse cytochrome *c* with 15 μM reaction centers in 10 mM Tris-HCl buffer and 1 mM ascorbate, pH 8. The solid line is a theoretical fit to the data as described in the text.

interaction. We have obtained similar ionic strength behavior for reactions of cytochromes c_2 in other systems (Tollin et al., 1986; Tollin & Hazzard, 1991).

Paracoccus denitrificans cytochrome c_2 also has a large net negative charge (−7), but it has a redox potential similar to that of the mitochondrial cytochromes (250 mV). The interaction of this cytochrome c_2 with reaction centers was second order at all ionic strengths; i.e., no saturation was observed at the highest protein concentrations used (up to 281 μM; cf. Table I). Only a small ionic strength effect was observed, and the rate constant at infinite ionic strength was comparable to that of *R. salexigens* cytochrome c_2 (Table I). Thus, neither the net protein charge nor the redox potential appear to have much effect on the rate constants in the *Rps. viridis* reaction center cytochrome system.

Rb. sphaeroides cytochrome c_2 is interesting because it normally interacts directly with the special pair bacteriochlorophyll in the *Rb. sphaeroides* reaction center. Although the measured redox potential is unfavorable for reaction with heme 2, it also reacts quite well with the *Rps. viridis* reaction center as shown in Table I. Layover kinetics are observed, and the limiting rate constant is comparable to that of *Rps. viridis* cytochrome c_2, although the binding constant is weaker.

It should be noted that it was not possible to fit the data with an equation based on a two-step mechanism using the value of the redox potential shown in Table I; furthermore, the observed extent of reaction was considerably greater than would be expected from this potential. Instead, we assumed a redox potential for *Rb. sphaeroides* cytochrome c_2 estimated from the extent of reduction of reaction centers (331 mV). Although there is a weakly favorable electrostatic interaction, it appears that the second-order rate constant at low ionic strength is smaller than that extrapolated to infinite ionic strength. This anomaly probably results from the fitting procedure, which only gives minimum values for k_{12} and k_{21}. Evidently, k_{12} is somewhat larger than the value shown in Table I. These results again emphasize the lack of correlation between binding constant and electrostatic interaction noted above for horse cytochrome *c*.

Chlorobium thiosulfatophilum cytochrome *c*-555 is distantly related to the mitochondrial cytochromes *c* and to the cytochromes c_2, but it has a net positive charge (+6) and a relatively low redox potential (145 mV), both of which are expected to be favorable for interaction with reaction centers. The observed kinetics were second-order under all conditions (i.e., no complex could be detected up to 80 μM protein), and the electrostatic effect was small, yet the rate constant at

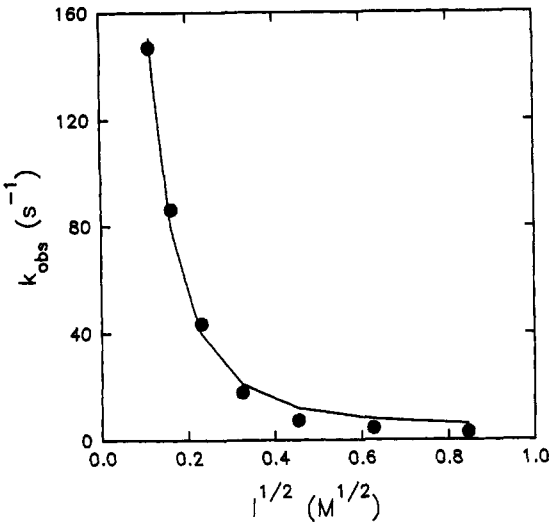

FIGURE 5: Effect of ionic strength on the observed first-order rate constants for reaction of 315 μM horse cytochrome *c* with 15 μM reaction centers in 10 mM Tris-HCl buffer and 1 mM ascorbate, pH 8. Ionic strength was varied with NaCl. The solid line is a theoretical fit to the data, as in Figure 3.

infinite ionic strength was an order of magnitude larger than for *Rps. viridis* cytochrome c_2 (Table I). A possible explanation is that in this case distance and orientation effects within a transiently formed complex may be as favorable for electron transfer as with the *Rps. viridis* cytochrome c_2, which allows the thermodynamic driving force to play a larger role in determining the rate constant.

Pseudomonas aeruginosa cytochrome *c*-551 is distantly related to the above cytochromes. It has a redox potential similar to that of horse cytochrome *c* (270 mV) but has a net negative charge (−2) and generally displays a negative charge at the site of electron transfer (Meyer et al., 1984; Tollin et al., 1984). We expected this protein to interact with the positively charged heme 3. However, at low ionic strength, *Pseudomonas* cytochrome *c*-551 is a poor electron donor for the *Rps. viridis* reaction center as shown in Table I. The effect of ionic strength is small, and the protein interacts repulsively with negative charge on the reaction center, probably in the vicinity of heme 2. Nevertheless, the rate constant at infinite ionic strength is similar to that of horse cytochrome *c*, indicating that once electrostatic effects are screened by salt ions, other factors controlling electron transfer (distance, orientation, etc.) are comparable.

Rhodocyclus tenuis is one of the species of photosynthetic bacteria in which cytochrome c_2 has not yet been found (Bartsch, 1991). *Rc. tenuis* does have a small, high redox potential (+405 mV), basic (+4) cytochrome *c*-553, which is homologous to *Pseudomonas* cytochrome *c*-551. It also has large amounts of HiPIP. It is likely that either cytochrome *c*-553 or HiPIP is the electron donor to the *Rc. tenuis* reaction center. However, *Rc. tenuis* cytochrome *c*-553 reacts very slowly with the *Rps viridis* reaction center. In fact, the rate constants are barely larger than for reaction with ascorbate or for recombination in the absence of soluble cytochrome (Table I). One would expect *Rc. tenuis* cytochrome *c*-553 to react much faster with *Rps. viridis* reaction center heme 3, which has a higher redox potential than heme 2, but the rate constant would have to be greater than 10^5 s^{-1} to compete with reduction of heme 3 by heme 2. The only possible way to observe such a reaction would be under very specific conditions in which the redox potential is poised midway between hemes 2 and 3 (i.e., near 340 mV, where heme 2 is already oxidized). We repeated the experiments in the absence of ascorbate, which would approximate the required conditions, but obtained the same results. *Rc. tenuis* cytochrome *c*-553 is a basic protein which shows a favorable plus–minus interaction with heme 2. Because there is a net positive charge surrounding heme 3 (see below), the unfavorable electrostatic interaction with *Rc. tenuis* cytochrome *c*-553 may be primarily responsible for lack of reaction at this site. In sum, the relative redox potentials of *Rc. tenuis* cytochrome *c*-553 and heme 2, the electrostatic repulsion at heme 3, and the rapid reaction between hemes 3 and 2 may all contribute to the slowness of the reaction of soluble cytochrome *c*-553 with the *Rps. viridis* reaction center.

Chromatium vinosum cytochrome c_4 has two hemes in a 20-kDa peptide chain (Van Beeumen, 1991). It is distantly related to the above cytochromes, has a high redox potential (325 mV), and has a net charge of −5 (Van Beeumen, 1991; Cusanovich & Bartsch, 1969). The interaction with reaction centers is poor and is comparable to that of *Pseudomonas* cytochrome *c*-551 (Table I).

Some species such as *C. vinosum*, *Rhodocyclus gelatinosus*, and *Rhodospirillum salinarum* do not have an abundant, soluble, high redox potential cytochrome *c* but do have a large amount of the high redox potential iron–sulfur protein known as HiPIP (Bartsch, 1991). As noted above, some species (e.g., *Rc. tenuis* and *Rhodopseudomonas marina*) have both high-potential cytochromes and HiPIP. It was therefore of interest to ascertain whether the HiPIP's could serve effectively as electron donors to the *Rps. viridis* reaction center. Although the three species we tested vary in redox potential and charge, none were especially reactive toward reaction centers as shown in Table I.

The copper proteins known as auracyanins from *Chloroflexus aurantiacus* are homologous to azurin (McManus et al., 1992) and may serve the same role as cytochrome c_2 in mediating electron transfer to the reaction center in this organism. We therefore felt it was of interest to test *Ps. aeruginosa* azurin for its ability to interact with the *Rps. viridis* reaction center. As shown in Table I, it was found to react in a manner similar to that of *Pseudomonas* cytochrome *c*-551, which is better than the HiPIP's but still not very effective.

DISCUSSION

All of the redox proteins tested for photooxidation by *Rps. viridis* reaction centers in the presence of ascorbate appear to interact with a negatively charged surface and with the 300-mV heme 2. Furthermore, both *Rps. viridis* cytochrome c_2 and horse cytochrome *c* form similar, but not identical, complexes with the reaction center. The space-filling CPK model of the *Rps. viridis* reaction center cytochrome subunit and the electrostatic map shown in Figure 6 indicate that the top half of the molecule, which includes both hemes 1 and 2, is dominated by negative charge, whereas there is a predominance of positive charge in the bottom half of the molecule which contains hemes 3 and 4.

Considering only electrostatic interactions, at low ionic strength, all of the soluble cytochrome donors which have positive charges near the heme edge would be expected to form either transient or stabilized electron-transfer complexes with the top half of the reaction center cytochrome, i.e., in the vicinity of hemes 1 and 2. Knaff et al. (1991) proposed that the electron-transfer site was nearest heme 1 because of the larger net negative charge in this region, due to E67, E79, E85, and E93. As shown by our analysis of the present data on ionic strength effects, the charge on the reaction center experienced by the *Rps. viridis* and horse cytochromes is estimated to be about −2.5, which is consistent with binding

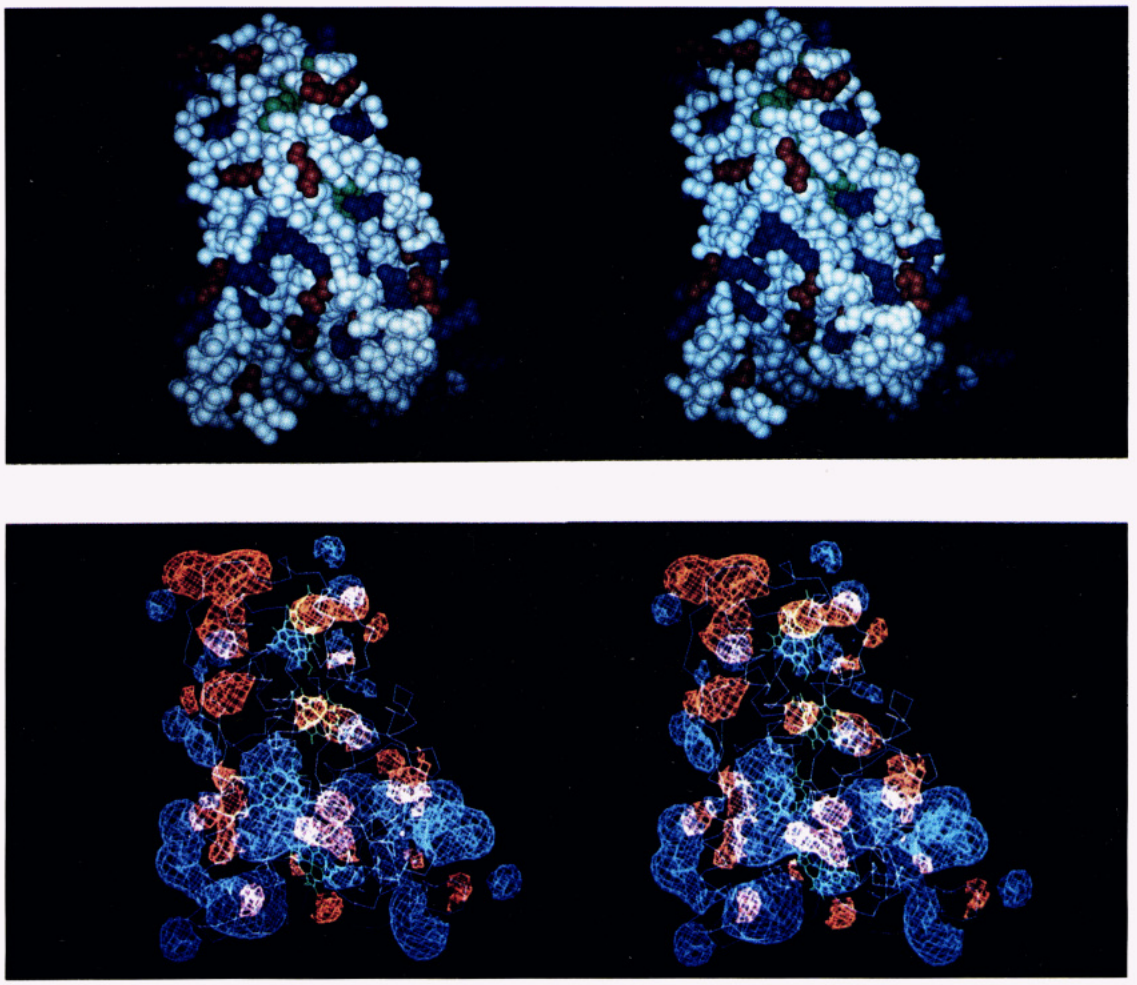

FIGURE 6: Stereoviews of (A, top) a space-filling model of the *Rps. viridis* reaction center cytochrome (acidic residues colored red, basic residues colored blue, and heme colored green) and (B, bottom) an electrostatic map of the *Rps. viridis* reaction center cytochrome calculated using the program DELPHI (Biosym, Inc.). Contours were generated at $\pm 2kT$. Heme propionates were charged as suggested by Gunner and Honig (1991). Internal charges were set to zero. Negative electrostatic potential is shown in red and positive potential in blue. The order of the hemes from top to bottom is 1, 2, 4, and 3.

near heme 1. Although heme 1 provides an electrostatically attractive binding site, the unfavorably low redox potential of –60 mV does not allow it to mediate electron transfer with any of the soluble donors. Thus, electron transfer is likely to be directly to heme 2, regardless of whether the binding site is near heme 1 or closer to heme 2. A possible binding site nearer to heme 2 overlaps that at heme 1 in that it is bounded by E85, D92, E93, K101, and R293. D92 and K101 appear to be ion-paired to one another, and one of the heme propionates is ion-paired with R293. The other heme propionate is likely to be uncharged (Gunner & Honig, 1991). The net charge at this site is therefore about –2 and thus would also be consistent with the present ionic strength results.

Other potential binding sites in the vicinity of hemes 1 and 2 also exist, and further experiments will be required to distinguish between these various possibilities. Furthermore, the observation that there is no correlation between electrostatic interaction energy, V_{ii}, and the dissociation constant, K_d, for the complexes between cytochromes and the reaction center (Table I) suggests that additional factors such as hydrophobic and van der Waals forces also contribute to binding. As noted above, this result also could be explained by interaction at more than one site. Thus, basing an assignment of electron-transfer site solely on the magnitude of electrostatic charge is clearly improper [cf. Pelletier and Kraut (1992)]. Because the rate of outer-sphere electron transfer drops off exponentially with distance, a 1.5–2-fold greater distance would result in a 4.5–7.5-fold smaller electron-transfer rate constant. This could in part be responsible for the anomalously slow reaction of horse cytochrome *c*, if it were to bind closer to heme 1, and *Rps. viridis* cytochrome c_2 were to bind nearer heme 2.

We have previously established a correlation between rate constants and differences in redox potentials of reactants using either free flavin semiquinone (Meyer et al., 1983, 1984) or flavodoxin semiquinone (Tollin et al., 1984) as reductants. However, in the present experiments we do not find any correlation between redox potentials of reactants and rate constants extrapolated to infinite ionic strength. This is most likely due to the contribution of other factors such as distance and orientation of reactants, as previously observed with the *Rb. sphaeroides* reaction center system (Tiede & Chang, 1988). Because horse cytochrome *c* has a lower potential than does *Rps. viridis* cytochrome c_2, it should have been more reactive, rather than 2 orders of magnitude slower. However, we previously observed that the reaction of horse cytochrome *c* was 1.5-fold slower than it should have been with FMN semiquinone, and was 30-fold slower with flavodoxin semiquinone (Meyer et al., 1984; Tollin et al., 1984), than were homologous *c*-type cytochromes of similar redox potentials. We attributed this anomalous behavior of horse cytochrome *c* to steric hindrance provided by side chains of Q16, T28, and I81, which project outward from the site of electron transfer near the heme. It is likely that these side chains also provide steric hindrance in the reaction center system, although the 100-fold lower rate constant may also

reflect additional factors. *Candida* cytochrome *c* shows less steric hindrance in the above systems and was also 3.4 times more reactive with the reaction centers than was horse cytochrome *c*. However, even taking this into account, the mitochondrial cytochromes are less reactive than expected. Distance could be an important factor if the mitochondrial cytochromes bind further away from heme 2 and nearer to heme 1, as discussed above. Another factor could be orientation. In the yeast cytochrome *c* peroxidase system, horse and yeast cytochromes *c* bind close to the same site but in slightly different orientations (Pelletier & Kraut, 1992). Proteins are not perfect spheres, and localized topography at the preferred site of electron transfer may force the reactants into different specific orientations. Therefore, the relatively small rate constant for the mitochondrial cytochromes *c* may be due to a combination of steric, distance, and unfavorable orientational effects.

Both horse cytochrome *c* and *Rps. viridis* cytochrome c_2 form complexes with the reaction center as shown by the change in rate-limiting step at high protein concentrations and low ionic strength. The limiting first-order rate constant for *Rps. viridis* cytochrome c_2, $k_{23} = 1300\ s^{-1}$, is 5-fold larger than the $270\ s^{-1}$ reported by Knaff et al. (1991). The first-order rate constant for horse cytochrome *c*, $k_{23} = 330\ s^{-1}$ is 4-fold smaller than for *Rps. viridis* cytochrome c_2, which as discussed above may also be due to a combination of steric, distance, and orientation effects.

One of the questions we wished to address in this study is the purpose of the bound cytochrome subunit in the *Rps. viridis* reaction center. The possibilities are (1) that it may facilitate reaction with a variety of substrates, which alone would not be very reactive with reaction centers lacking the cytochrome subunit; (2) that each heme of the reaction center cytochrome subunit might provide an optimal site of electron transfer with different substrates; (3) that it may couple directly with the bc_1 complex without need for a soluble mediator; and (4) that it is an evolutionary dead end or its vestigial. There could be some truth to each of these possibilities, but none is likely to be entirely correct. In the absence of an effective electron donor to the special pair bacteriochlorophyll, recombination with the reduced quinone species produced as a result of the photoreaction would compete with productive electron flow. Therefore, it is necessary to rapidly reduce the special pair to prevent this. This is accomplished directly by the cytochrome c_2 in *Rb. sphaeroides* or by the bound cytochrome subunit in *Rps. viridis*. Once the special pair is reduced, then secondary electron transfers to regenerate reduced cytochrome need not be rapid.

Even though *Rps. viridis* and other cytochromes c_2 are the most reactive of the potential donors tested with the *Rps. viridis* reaction center cytochrome subunit, those which react relatively slowly could still be functional with their own reaction centers in vivo because the critical requirement, reduction of the special pair, has been met. Both horse cytochrome *c* and *Rb. sphaeroides* cytochrome c_2 are quite reactive with the *Rb. sphaeroides* reaction center (Overfield & Wraight, 1980; Moser & Dutton, 1988), which does not have the bound cytochrome subunit. HiPIP, azurin, and cytochromes other than c_2 are poor electron donors to the *Rps. viridis* reaction center and therefore probably would not reduce *Rb. sphaeroides* reaction centers rapidly enough to prevent recombination with reduced quinone. We are currently testing this assumption. Thus, it is possible that the bound cytochrome subunit may be necessary in those species which do not have a highly reactive cytochrome c_2 and must rely on a less efficient mediator.

Do each of the four hemes of the bound reaction center cytochrome subunit provide an optimum binding and reaction site for soluble donors? The answer is probably no. Soluble cytochromes generally have redox potentials above 100 mV and would interact only with either of the two highest potential hemes. Furthermore, the two high-potential hemes 2 and 3 are too reactive with one another to allow interaction between a soluble donor and heme 3. The ambient redox potential in the cell is likely to be much lower than the potential of heme 2, so that conditions for reaction between soluble donors and heme 3 would be highly unusual or nonexistent in vivo. The highest potential component of the bc_1 complex is comparable with the high-potential hemes 2 and 3 and would interact with a soluble mediator which is between cytochrome c_1 and heme 2 in potential. Finally, the ultimate electron acceptor quinone would be reduced at the very low ambient potentials required to reduce heme 1, and no photochemistry could occur under these conditions. It appears then that heme 2 is the only likely site of interaction with soluble donors in vivo. However, the large surface area of the reaction center cytochrome could still provide alternative binding sites for soluble donors to heme 2.

The possibility that reaction centers may not require a soluble mediator at all is disproven in the case of *Rb. sphaeroides* by genetic studies in which cytochrome c_2 was deleted, resulting in cells which would not grow photosynthetically (Donohue et al., 1988). Spontaneous mutations reversed the photosynthetic-minus phenotype through expression of a cytochrome c_2 isozyme (Fitch et al., 1988; Rott & Donohue, 1990; Rott et al., 1992). In *Rhodobacter capsulatus*, it appeared that cytochrome c_2 was superfluous (Daldal et al., 1986; Prince et al., 1986), although it has since been found that restriction fragments of *Rb. capsulatus* DNA are able to complement both *Rb. capsulatus* and *Rb. sphaeroides* photosynthetic-minus mutants (Jenney et al., 1993), suggesting a membrane-bound cytochrome c_2 homolog may be functional in the *Rb. capsulatus* c_2 minus mutant. It has been reported that *C. vinosum* has a cytochrome c_2 (Van Grondelle et al., 1977; Tomiyama et al., 1983; Gray et al., 1983) which mediates between the bc_1 complex and the reaction center cytochrome. However, it has not been proven to be a cytochrome c_2, it is only a minor component, such as the wild-type levels of isocytochrome c_2 in *Rb. sphaeroides*, and it is not even necessary, because we have shown that HiPIP is both abundant and interacts with reaction centers, albeit at a relatively slow rate. Therefore, the bound reaction center cytochrome is necessary to *permit* reaction of poor donors with the reaction center special pair, but it probably does not facilitate reaction.

If at least two of the reaction center cytochrome hemes are nonfunctional, i.e., the low-potential hemes, then why are they there? There is evidence from the crystal structure that the reaction center cytochrome resulted from two successive gene doublings (Deisenhofer et al., 1985). It is likely that gene doubling was neither beneficial nor detrimental, but neutral, in which case there would be no functional significance to the presence of the extra hemes. Thus, we may expect to find some species which have deleted one or more of the four hemes. It would appear that cytochrome c_2 is a relatively recent acquisition by purple bacteria and that the whole reaction center cytochrome subunit could be deleted once cytochrome c_2 was found to be a more efficient electron mediator between the bc_1 complex and the reaction center.

Chloroflexus contains a reaction center similar to those of purple bacteria (Bruce et al., 1982; Shiozawa et al., 1989) and has a tetraheme cytochrome *c* subunit (Freeman &

Blankenship, 1990; Dracheva et al., 1991). There are no soluble cytochromes in *Chloroflexus*, but there are two copper proteins related to azurin, called auracyanins, which may couple the bc_1 complex to the reaction center (McManus et al., 1992). We have shown that *Pseudomonas* azurin is a poor donor to the *Rps. viridis* reaction center, but it is likely that the presence of the tetraheme cytochrome in *Chloroflexus* allows auracyanins to mediate electron transfer.

CONCLUSIONS

A variety of soluble redox proteins were tested for their ability to interact with *Rps. viridis* reaction centers. The cytochromes c_2 are clearly the best electron donors of those proteins studied. They generally form relatively tight complexes with the reaction centers. The mitochondrial cytochromes *c* are not effective substitutes for the cytochromes c_2 and may bind somewhat differently. All of the soluble proteins interact with a negatively charged site or sites on the reaction center cytochrome, and they all seem to interact with the 300-mV heme 2. Binding appears to be mediated by hydrophobic, van der Waals, and electrostatic interactions. The reaction center cytochrome does not facilitate reaction with soluble proteins but permits relatively inefficient donors to mediate between the bc_1 complex and reaction centers. There is no correlation between driving force and rate constants for electron transfer, which suggests that factors such as distance and orientation of electron donors and acceptors may also contribute to the mechanism, as is to be expected if there are subtle differences in the sites of interaction with soluble donors.

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