Interaction between Cytochrome c_2 and Reaction Centers from Purple Bacteria†

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ABSTRACT: The kinetics of electron transfer of cytochrome c_2 from *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, and *Rhodospirillum centenum* to reaction centers from *Rb. sphaeroides* and *Rb. capsulatus* have been measured. Observed in the kinetics of decay of the oxidized donor are a rapid first-order rate and one or more slower rates that are due to diffusion-limited complex formation. For reaction centers from *Rb. sphaeroides*, the fast component had time constants of 1.0 and 0.5 μs for cytochrome c_2 from *Rb. sphaeroides* and *Rb. capsulatus*, respectively, but only a slow component was observed for cytochrome c_2 from *Rs. centenum*. For reaction centers from *Rb. capsulatus*, the kinetics from all three cytochromes had a fast component with time constants of 1.0, 0.7, and 1.9 μs for cytochrome c_2 from *Rb. sphaeroides*, *Rb. capsulatus*, and *Rs. centenum*, respectively, although the dissociation constant for cytochrome c_2 from *Rs. centenum* was approximately 20 times larger than that of the other cytochromes. The observation of the fast component for cytochrome c_2 from *Rs. centenum* in reaction centers from *Rb. capsulatus* but not *Rb. sphaeroides* demonstrates that the binding interactions for the two reaction centers differ, and the involvement of amino acid residues in the binding is discussed. The kinetics of electron transfer from cytochrome c_2 to reaction centers of *Rb. sphaeroides* from wild type and three mutant strains that have altered carboxyl-terminal regions of the M subunit of the reaction center have also been measured. For cytochrome c_2 from *Rb. sphaeroides*, the kinetics are very similar between the mutants and wild type. In contrast, for cytochrome c_2 from *Rb. capsulatus*, the dissociation constants vary from 2.4 to 18 μM in the mutants compared to 6.3 μM for wild type. A greater involvement for the M carboxyl region in the binding of the cytochrome c_2 from *Rb. capsulatus* is proposed.

The primary processes of photosynthesis in purple bacteria take place in an integral membrane protein–pigment complex called the reaction center (RC)[1] [for reviews, see Feher et al. (1989) and Parson et al. (1991)]. After excitation of the RC by light, the bacteriochlorophyll dimer P donates an electron through a series of electron acceptors to the secondary quinone. Cyclic electron transfer is achieved with the presence of a cytochrome serving as a secondary electron donor to P^+. In *Rhodobacter (Rb.) sphaeroides*, *Rb. capsulatus*, and *Rhodospirillum (Rs.) rubrum*, which have RCs containing three subunits, L, M, and H, cytochrome c_2 directly reduces the photooxidized donor P^+. Many bacterial species, such as *Rhodopseudomonas (Rps.) viridis* and *Rs. centenum*, have RCs with a bound tetraheme cytochrome, which serves as the secondary electron donor to P^+. In these cases, cyclic electron transfer occurs with the cytochrome c_2 donating an electron to the oxidized tetraheme cytochrome.

Electron transfer reactions involving cytochromes have been well characterized [for a review, see Moore and Pettigrew (1990)]. The electron transfer domain of *c*-type cytochromes has been modeled as involving the "frontside" region (Salemme et al., 1973) of the molecular surface area surrounding the solvent-accessible heme edge in cytochromes from mitochondrial and bacterial species (Margoliash & Bosshard, 1983; Hall et al., 1987a; Bosshard et al., 1987). The "frontside" region contains a significant number of lysine amino acid residues that are well conserved among cytochromes. The binding of cytochromes to RCs from *Rb. sphaeroides* has been observed to be strongly dependent upon ionic strength, suggesting the involvement of electrostatic interactions between the lysine residues of the cytochrome and negatively charged carboxylate groups on the periplasmic surface of the RC (Okamura et al., 1983). Kinetic data obtained with mixtures of lysine-modified derivatives of cytochrome c_2 from *Rs. rubrum* (Hall et al., 1987a) and *Rb. sphaeroides* (Hall et al., 1987b) are consistent with the "frontside" binding model.

Two different kinetic models have been proposed for the interaction of RCs from *Rb. sphaeroides* with cytochromes. For the two-state model, electron transfer has a fast phase, with a time constant of ~1 μs, due to bound cytochrome and a slow component due to the reaction of cytochrome in solution (Rosen et al., 1979). Other workers have proposed that the data are more consistent with a three-state model in which there are two fast components due to binding of cytochrome c_2 in both a distal and a proximal state (Dutton & Prince, 1978; Moser & Dutton, 1988; Overfield & Wraight, 1986). It has been suggested that the difference in these results is due to variations in nonintrinsic properties of the RC that are dependent upon the isolation process (Tiede et al., 1993). In a recent study, no evidence was found for the existence of two bound states of cytochrome c_2 to the RC from *Rb. sphaeroides* (Venturoli, 1993). In the case of electron transfer from the *Rs. rubrum* cytochrome c_2 to RCs from *Rs. rubrum* and *Rb.*

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[1] Abbreviations: RC, reaction center; P, primary electron donor; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; LDAO, lauryldimethylamine oxide.

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sphaeroides, only slow second-order kinetics were observed (Rickle & Cusanovich, 1979; Van der Wal et al., 1987; Long et al., 1989). These results indicate that the cytochrome binding may vary among different species.

An understanding of the RC–cytochrome complex has been aided by the determination of the three-dimensional structures of cytochromes from *Rb. sphaeroides* (Axelrod et al., 1994), *Rb. capsulatus* (Bushnell et al., 1990), *Rs. rubrum* (Salemme et al., 1973), and horse (Dickerson et al., 1971), and RCs from *Rb. sphaeroides* (Allen et al., 1988, El-Kabbani et al., 1991) and *Rps. viridis* (Michel et al., 1986). Two molecular models have been proposed for the binding of cytochrome to the RC (Allen et al., 1987; Tiede & Chang, 1988). These models are largely based upon the assumption that the binding of the cytochrome to the RC is determined by electrostatic interactions. However, these models cannot explain the orders of magnitude differences in binding to the RC observed for the structurally homologous cytochromes from different species (Long et al., 1989; Tiede et al., 1993).

In order to better understand the differences observed for the cytochrome–RC interactions of different species, we have performed two sets of experiments. First, we have measured the reaction rates involving cytochrome c_2 from three different species, *Rb. sphaeroides*, *Rb. capsulatus*, and *Rs. centenum*, and RCs isolated from two different species, *Rb. sphaeroides* and *Rb. capsulatus*. Second, we have examined the role of the species-specificity of the M carboxyl terminus of the RC. The carboxyl terminus of the M subunit of the RC is located on the periplasmic surface of the RC near the putative cytochrome binding site. The extended M carboxyl region in *Rps. viridis* appears to have a significant interaction with the bound tetraheme cytochrome (Michel et al., 1986). Mutant RCs of *Rb. sphaeroides* were constructed with the M carboxyl terminus replaced with the corresponding region of *Rb. capsulatus* and *Rps. viridis*. Also constructed was a mutant with the last 10 residues deleted from the M carboxyl terminus of the *Rb. sphaeroides* RCs. The electron transfer between these mutant RCs and cytochrome c_2 from *Rb. capsulatus* and *Rb. sphaeroides* was measured. An initial characterization of these mutants has been published (Wang et al., 1992).

EXPERIMENTAL PROCEDURES

Mutant Design and Protein Isolation. Two mutants were made in which the DNA encoding the last 17 amino acid residues of the *Rb. sphaeroides* RC M subunit was replaced with the corresponding sequence of the genes from *Rb. capsulatus* (mutant SWCAP) and *Rps. viridis* (mutant SWVIR). Another mutant was constructed with the last 10 residues deleted from the carboxyl terminus (mutant SWTRC). The construction of the mutants has been previously described (Wang et al., 1992). The sequences of the carboxyl terminus of the M subunit of the wild type and mutants are as follows:

wild type	VDNWYVWGQNHGMAPLN
SWCAP	VDNWYVWAQVHGYAPV TP
SWVIR	VDNWYLWCVKHGAAPD YPAYLPATPDPASLPGAPK
SWTRC	VDNWYVW

RCs were isolated following protocols for *Rb. capsulatus* (Wang et al., unpublished results) and for the wild-type strain of *Rb. sphaeroides* (Paddock et al., 1989). Cytochrome c_2 from *Rb. sphaeroides* and *Rb. capsulatus* was isolated following the unmodified procedures of Bartsch (1978), and the isolation of cytochrome c_2 from *Rs. centenum* was based upon the protocol of Li and Allen (unpublished results). The optical absorptions of the RC and cytochrome were measured at 802 and 550 nm, respectively, with a CARY 5 spectrophotometer (Varian). The molar extinction coefficients used are $\xi^{802} = 288\ mM^{-1}cm^{-1}$ (Straley et al., 1973) and $\xi^{550} = 18.5\ mM^{-1}cm^{-1}$ (Margoliash & Frohwirt, 1959) for RCs and reduced cytochrome c_2, respectively.

Determination of Electron Transfer Rates. The samples were excited at 694 nm using 10 ns excitation flashes from a ruby laser while monitoring the absorption changes at 1283 nm using a transient digitizer in which the signals were amplified and measured. Further experimental details have been described elsewhere (Ortega & Mathis, 1992). The temperature was 21°C, and the optical path length was 10 mm. In order to simultaneously measure both nanosecond and millisecond absorption changes in the kinetics, the 2048 channels of the digitizer were divided into segments with different time intervals per channel (see, for example, Figure 1). RCs were prepared at concentrations of 0.5–2.0 μM in 15 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.025% lauryldimethylamine oxide (LDAO) for *Rb. sphaeroides* and in 10 mM phosphate buffer, pH 7.4, and 0.05% LDAO for *Rb. capsulatus*. Cytochrome c_2 was added to the RC samples at concentrations varying from 2 to 40 μM. Before each measurement, 1 mM sodium ascorbate and 20 μM ubiquinone-9 from a 5 mM stock in 5% LDAO were added to the samples.

The kinetics of P^+ re-reduction were analyzed by sums of exponential components, including millisecond components that were treated as a single constant, using a modified Marquardt algorithm (program by Dr. P. Sétif). The concentration of cytochrome was calculated for each experimental condition. The assumption was that all cytochrome was free, except for the fraction giving rise to the fast phase of electron transfer to P^+. Neglecting any nonfunctional binding of the cytochrome to the RC, one may write

$$[C] = [C_t] - [P_t]Y \quad (1)$$

where $[C_t]$ and $[C]$ are the total and free cytochrome concentrations, respectively, $[P_t]$ is the total RC concentration, and Y is the fraction of fast component.

RESULTS

Interaction between Cytochrome and RCs from Rb. sphaeroides. After excitation of the RCs by a saturating light pulse, P^+ re-reduction by cytochrome c_2 was measured by monitoring the optical absorption changes at 1283 nm. In the presence of cytochrome c_2 from *Rb. sphaeroides* and *Rb. capsulatus* the decays were well fit by two exponential terms, a fast and slow component, and a constant (Figure 1). The amplitude of the constant term was less than 10% of the total amplitude and decreased as the cytochrome c_2 concentration increased. This term is thought to arise from the fraction of RCs in which P^+ is not re-reduced by cytochrome c_2 but decays by charge recombination on a time scale of ~1 s (Venturoli et al., 1993). A small fraction of slowly decaying P^+ has also been found by Tiede et al. (1993) and was attributed to RCs unable to react with cytochrome. This fraction is not taken into consideration in our analysis. With cytochrome c_2 from *Rs. centenum* serving as the electron donor, the decays did not show a detectable fast phase, and only second-order kinetics were observed.

The rate of the fast phase was independent of the cytochrome c_2 concentration in solution. The time constants for this component were determined to be 1 and 0.5 μs for the cytochrome c_2 from *Rb. sphaeroides* and *Rb. capsulatus*, respectively (Table 1). The amplitude of the fast phase relative

Table 1: Electron Transfer Kinetics between Cytochrome c_2 and Reaction Centers[a]

reaction center	cytochrome c_2	K_d (μM)	B_{max} (%)	τ_{fast} (μs)	K_{slow1} (M^{-1} s^{-1})[b]	K_{slow2} (M^{-1} s^{-1})[b]
Rb. sphaeroides	*Rb. sphaeroides*	3.8 (±0.4)	92 (±3)	1.0 (±0.1)	4.9×10^8	
Rb. sphaeroides	*Rb. capsulatus*	6.3 (±0.6)	100 (±3)	0.5 (±0.1)	6.7×10^8	
Rb. sphaeroides	*Rs. centenum*				7.1×10^7	
SWCAP	*Rb. sphaeroides*	4.8 (±0.3)	93 (±2)	1.1 (±0.1)	6.2×10^8	
SWTRC	*Rb. sphaeroides*	2.3 (±0.5)	74 (±4)	1.4 (±0.2)	6.3×10^8	
SWVIR	*Rb. sphaeroides*	5.0 (±0.3)	93 (±2)	1.1 (±0.1)	6.0×10^8	
SWCAP	*Rb. capsulatus*	5.8 (±0.6)	98 (±3)	0.6 (±0.1)	8.2×10^8	
SWTRC	*Rb. capsulatus*	2.4 (±0.4)	98 (±3)	0.7 (±0.1)	5.5×10^8	
SWVIR	*Rb. capsulatus*	18 (±6)	100 (±15)	0.6 (±0.1)	7.2×10^8	
Rb. capsulatus	*Rb. sphaeroides*	2.9 (±0.5)	48 (±2)	1.0 (±0.1)	2.3×10^9	1.6×10^8
Rb. capsulatus	*Rb. capsulatus*	1.1 (±0.4)	85 (±5)	0.7 (±0.1)	6.3×10^8	5.5×10^7
Rb. capsulatus	*Rs. centenum*	40 (±7)	86 (±9)	1.9 (±0.1)	7.5×10^7	1.9×10^7

[a] K_d, dissociation constant; B_{max}, maximum binding amount; τ_{fast}, time constant of the fast component; K_{slow}, second-order rate constant. The numbers in parentheses are the standard deviations. [b] Standard deviations are 5–10%.

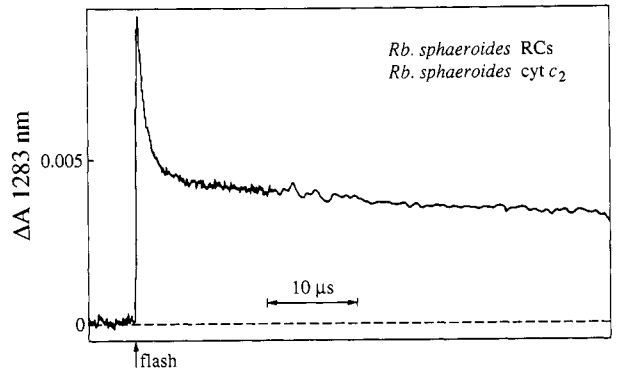

FIGURE 1: Representative kinetics of flash-induced P^+ decay for RCs from *Rb. sphaeroides* and cytochrome c_2 from *Rb. sphaeroides*. The absorbance changes were measured at 1283 nm with a maximum change of 9.2×10^{-3}. The trace was recorded with different sampling rates in order to measure both the millisecond and nanosecond absorption changes as is evident by the change in sampling rate approximately 15 μs after the flash. RCs at a concentration of 1.8 μM were in 15 mM Tris-HCl, pH 8.0, 0.025% LDAO, and 1 mM EDTA with 1 mM sodium ascorbate and 20 μM ubiquinone. The total cytochrome c_2 concentration 13.3 μM.

to the combined fast and slow amplitudes (percent fast phase) was found to be dependent on the cytochrome c_2 concentration and could be described by a binding model of the cytochrome to the RC:

$$C + P \leftrightarrow CP \quad (2)$$

where C represents free cytochrome c_2, P represents the free RC, and CP represents the RC–cytochrome complex. For this model, the dissociation constant K_d is given by [C][P]/[CP]. The percent fast phase is related to the free cytochrome c_2 concentration according to the equation:

$$\%\ \text{fast phase} = \left(\frac{[C]}{K_d + [C]}\right) B_{max} \quad (3)$$

The parameter K_d was determined to be 3.8 and 6.3 μM for the cytochrome c_2 from *Rb. sphaeroides* and *Rb. capsulatus*, respectively (Figure 2, Table 1). The fits were corrected with the parameter B_{max} which is the maximum possible value of the percent fast phase. The deviation from 100% represents the small fraction of RCs where a fast phase does not occur at high cytochrome concentrations due to no binding of cytochrome c_2 or binding without fast electron transfer to P^+. The values of this parameter were ~100% for the cytochrome c_2 from *Rb. sphaeroides* and *Rb. capsulatus*. Our values for the time constant and a dissociation constant are in agreement with previous studies (Overfield et al., 1979; Rosen et al., 1983; Long et al., 1989; Tiede et al., 1993; Venturoli et al., 1993).

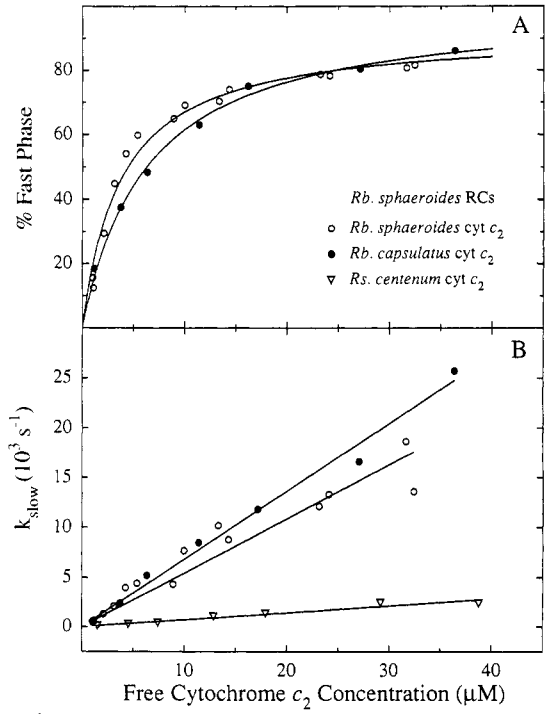

FIGURE 2: (A) Dependence of the relative amplitude of the fast component, percent fast phase, upon the cytochrome concentration for RCs from *Rb. sphaeroides* with cytochrome c_2 from *Rb. sphaeroides* and *Rb. capsulatus*. No fast phase was observed for the reaction with cytochrome c_2 from *Rs. centenum*. Also shown is a fit of the data using eq 3 and dissociation constants of 3.8 and 6.3 μM for the reaction with cytochrome c_2 from *Rb. sphaeroides* and *Rb. capsulatus*, respectively. (B) Dependence of the slow rate upon cytochrome concentration for electron transfer from cytochromes to RCs from *Rb. sphaeroides*. Also shown is a fit using eq 4.

The slow phase, that we assume to be due to a second-order collisional reaction between the RC and the free reduced cytochrome c_2, has been analyzed according to a pseudo-first-order approximation, as discussed by Venturoli et al. (1993). The second-order rate constant K_{slow} was calculated according to the equation:

$$k_{slow} = K_{slow}[C] \quad (4)$$

The rate constant K_{slow} was 4.9×10^8, 6.7×10^8, and 7.1×10^7 M^{-1} s^{-1} for the cytochrome c_2 from *Rb. sphaeroides*, *Rb. capsulatus*, and *Rs. centenum*, respectively (Table 1, Figure 2B). Our calculated values for the second-order rate constants of cytochrome c_2 from *Rb. sphaeroides* and *Rb. capsulatus* are in agreement with the values of 1×10^8 to 8×10^8 M^{-1} s^{-1} previously reported (Tiede et al., 1993; Long et al., 1989; Rosen et al., 1983; Overfield et al., 1979; Venturoli et al., 1993).

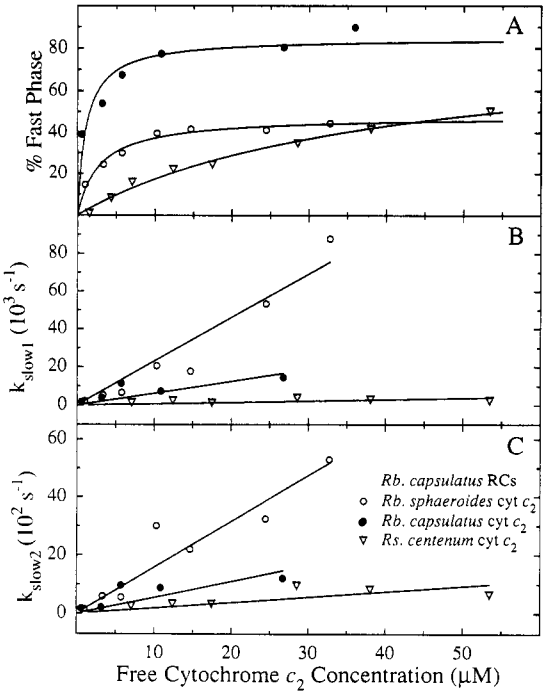

FIGURE 3: (A) Dependence of the relative amplitude of the fast component, percent fast phase, upon the cytochrome concentration for RCs from *Rb. capsulatus* with cytochrome c_2 from *Rb. sphaeroides*, *Rb. capsulatus*, and *Rs. centenum*. Also shown is a fit using eq 3 and dissociation constants of 2.9, 1.1, and 40 μM for the reaction with cytochrome c_2 from *Rb. sphaeroides*, *Rb. capsulatus*, and *Rs. centenum*, respectively. (B) Dependence of the rate of the intermediate (or first slow) component upon cytochrome concentration for electron transfer from cytochromes to RCs from *Rb. capsulatus*. Also show is a fit using eq 4. (C) Dependence of the rate of the slowest component upon cytochrome concentration for electron transfer from cytochromes to RCs from *Rb. capsulatus*. Also shown is a fit using eq 4.

Interaction between Cytochrome c_2 and RCs from Rb. capsulatus. The rates of reduction of photooxidized *Rb. capsulatus* RCs with cytochrome c_2 from all three different species were best fit by three exponential components and a constant. The time constants of the fast component were independent of the concentration of free cytochrome and were determined to be 1.0 and 0.7 μs for the cytochrome c_2 from *Rb. sphaeroides* and *Rb. capsulatus*, respectively (Table 1). In contrast to the observed kinetics for RCs from *Rb. sphaeroides*, a fast component with a time constant of 2 μs was resolved between *Rs. centenum* cytochrome c_2 and RCs from *Rb. capsulatus*. The percent fast phase was modeled using eq 3 as a function of the concentration of free cytochrome (Figure 3A). The calculated dissociation constant of 40 μM was much higher for *Rs. centenum* cytochrome c_2 than the values of 2.9 and 1.1 μM for cytochrome c_2 from *Rb. sphaeroides* and *Rb. capsulatus*, respectively. The maximum binding amount B_{max} was calculated to be 86% for *Rs. centenum* cytochrome c_2, which is similar to the value of 85% for *Rb. capsulatus* cytochrome c_2. In contrast, the value of 48% for B_{max} for *Rb. sphaeroides* cytochrome c_2 was significantly lower than the values for the other cytochromes. Both of the slow rates increased with the cytochrome concentration (Figure 3B, C), and the values of the second-order rate constant for the two slow components differed by a factor of 10 (Table 1).

Interaction between Cytochrome c_2 and Mutant RCs. All measurements of the decay of P^+ in RCs from *Rb. sphaeroides* wild type and mutant strains were well fit by two exponential components, a fast and slow component and a constant term. The data for the mutant RCs were modeled using eq 2 and 3 as was done for wild-type RCs (Table 1). For *Rb. sphaeroides* cytochrome c_2, all the kinetic decays showed a fast component with a time constant of 1.1–1.4 μs that is close to the value of 1.0 μs for wild-type RCs. Similarly, the rate constants for the three mutants, ~ 6.0×10^8 M^{-1} s^{-1}, were essentially the same as the value of 4.9×10^8 $M^{-1}s^{-1}$ for wild type. The dissociation constant did vary but only from 2.3 μM for SWTRC to 5.0 μM for SWVIR compared to 3.8 μM for wild type. For *Rb. capsulatus* cytochrome c_2, the fast component had a time constant of 0.6–0.7 μs for the mutants that is essentially the same as the value of 0.5 μs measured for wild type. A large range of values, from 2.4 μM for SWTRC to 18 μM for SWVIR, was calculated for the dissociation constant compared to 6.3 μM for wild type (Figure 4). The rate constant for the slow component was (5.5–8.2) $\times 10^8$ M^{-1} s^{-1}, which is comparable to the value of 6.7×10^8 M^{-1} s^{-1} for wild-type RCs.

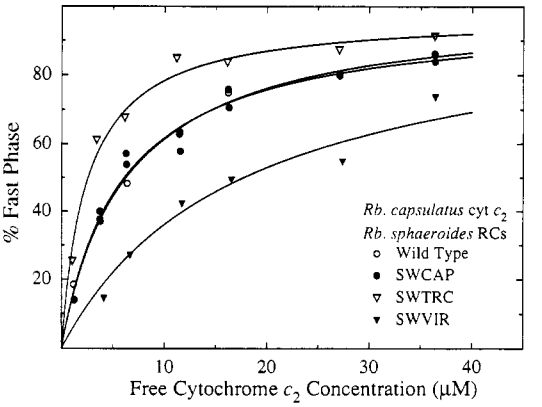

FIGURE 4: Dependence of the relative amplitude of the fast component, percent fast phase, upon the concentration of cytochrome c_2 from *Rb. capsulatus* for RCs from the wild-type and mutant strains of *Rb. sphaeroides*. Also shown is a fit of the data using eq 3 and dissociation constants of 6.3, 5.8, 2.4, and 18 μM for RCs from wild type, SWCAP, SWTRC, and SWVIR, respectively.

DISCUSSION

Interactions between Cytochrome c_2 and RCs from Rb. sphaeroides Wild Type. Our data showed only biexponential components, which is consistent with the two-state model and is similar to recent measurements by Venturoli et al. (1993). Our analysis of the fast phase did not permit the resolution of a second, minor (10%) fast phase component that was independent of cytochrome concentration as has been reported by Tiede et al. (1993). Measurement of different preparations did not change the number of components nor the maximum binding (as determined from the kinetic measurements) of nearly one cytochrome per RC. A variation in this parameter has been reported and attributed to a "non-intrinsic" variability of RC preparations (Tiede et al., 1993); no such variability is evident in our data. The presence of only two components in our results is not due to our use of RCs isolated from the wild-type strain as the measurements using RCs isolated from the R-26 strain gave identical results (Venturoli et al., 1993).

For the *Rb. capsulatus* cytochrome c_2, kinetics with a fast and slow component were observed with similar parameters to those observed for cytochrome c_2 from *Rb. sphaeroides*. The time constant of the fast component, 0.5 μs, was faster than that for *Rb. sphaeroides* although the cytochrome from *Rb. capsulatus* did not bind as strongly to the RC as did the cytochrome from *Rb. sphaeroides* (i.e., K_d was 3.8 μM for *Rb. sphaeroides* and 6.3 μM for *Rb. capsulatus*). These values for K_d and a B_{max} of unity are in agreement with the results of Tiede et al. (1993). The lack of a fast component reported

by Caffrey et al. (1992) is presumably due to an insufficient time resolution of their instrument as has been previously suggested (Tiede et al., 1993). Only one fast component was observed unlike the two fast components observed by Tiede et al. (1993).

Interaction between Cytochrome c_2 and RCs from Rb. capsulatus. In contrast with the results for RCs from *Rb. sphaeroides*, the P^+ decay for RCs from *Rb. capsulatus* had three exponential components. One component had a fast time constant that was independent of the cytochrome concentration and two components decayed on slow time scales. The time constants of the fast component were 1.0 and 0.7 μs for the cytochromes from *Rb. sphaeroides* and *Rb. capsulatus*, respectively; these time constants are similar to those measured for RCs from *Rb. sphaeroides*. However, both cytochromes bound more tightly to the RC from *Rb. capsulatus*, as indicated by K_d values of 1.1 and 2.9 μM for cytochrome c_2 from *Rb. capsulatus* and *Rb. sphaeroides*, compared to 3.8 and 6.3 μM for RCs from *Rb. sphaeroides*. The most striking difference concerning the data for the two cytochromes was the $\sim$0.5 value of B_{max} for *Rb. sphaeroides* cytochrome binding to *Rb. capsulatus* RCs compared to the B_{max} values of $\sim$1.0 for the other combinations. One possible explanation could be the formation of a RC dimer in solution such that the binding of cytochrome on one RC may competitively inhibit the binding on the other as has been suggested for membrane systems (Lavergne & Joliot, 1991). Two reasons, however, argue against this explanation: (1) the small value of B_{max} is observed only in the heterologous system of RCs from *Rb. capsulatus* with cytochrome c_2 from *Rb. sphaeroides* and (2) the extent of the fast phase practically saturates at 10 μM cytochrome c_2 concentration (Figure 3). Under the same conditions, however, the rates of the two bimolecular reactions continue to increase linearly with cytochrome c_2 concentration (Figure 3B,C). Thus, the low value of B_{max} cannot be due to the formation of a RC complex. More probable is the explanation that the RCs from *Rb. capsulatus* have some heterogeneity that manifests itself in the binding of the cytochrome c_2 from *Rb. sphaeroides*. Heterogeneity of the RC is also consistent with the occurrence of two second-order rate constants. The nature of the heterogeneity is not known; it may arise from small differences in the positions or p*K*s of the carboxylate groups involved in the binding of the cytochrome. The specificity of this result for cytochrome c_2 from *Rb. sphaeroides* presumably is due to a difference in the binding of this cytochrome compared to the other species as discussed below.

The second-order rate constants obtained were 2.3×10^9 and 1.6×10^8 M^{-1} s^{-1} for the first component and 6.3×10^8 and 5.5×10^7 M^{-1} s^{-1} for the second for *Rb. sphaeroides* and *Rb. capsulatus*, respectively. The ratio of the amplitude of the two slow components varied although at low concentrations of cytochrome, which have the smallest fast amplitude and hence the most accurately determined amplitudes of the slow components, this ratio was in favor of the slowest component and was approximately 1:1.5 for *Rb. sphaeroides* and *Rb. capsulatus* cytochromes, and 1:5 for the *Rs. centenum* cytochrome. Presumably, these two components represent a heterogeneity of RCs with respect to the binding of cytochrome in the collisional process.

Interaction between Rs. centenum Cytochrome c_2 and RCs. In *Rs. centenum*, cytochrome c_2 donates an electron to the bound tetraheme cytochrome of the RC (Yildiz et al., 1992) as is true for *Rps. viridis*. For the *Rs. centenum* cytochrome c_2, only a slow monophasic component was observed for electron transfer to RCs from *Rb. sphaeroides*. For RCs from *Rb. sphaeroides*, the lack of a fast component has previously been reported for cytochrome c_2 from the purple bacteria *Rs. rubrum* and *Rps. viridis* although cytochromes from horse and tuna, that are distantly related to the purple bacteria, are capable of binding to the RC as evident by the presence of a fast component. For RCs from *Rb. capsulatus*, cytochrome c_2 from *Rs. centenum* was capable of forming a bound complex as evident by the presence of a fast component. The complex has a similar time constant as was measured for cytochromes from *Rb. sphaeroides* and *Rb. capsulatus*. However, the dissociation constant of 40 μM measured for the cytochrome from *Rs. centenum* is significantly larger than that for the other cytochromes. These results suggest that the complex formed between the *Rs. centenum* cytochrome and the *Rb. capsulatus* RC is very similar to that formed by the other cytochromes except that the complex is only weakly bound, as discussed later.

Interaction between Cytochrome c_2 and Mutant RCs. The M carboxyl terminus is a species-specific region of the RC that lies near the putative cytochrome binding region in *Rb. sphaeroides* (Allen et al., 1987; Tiede & Chang, 1988) and interacts with the bound tetraheme in *Rps. viridis* (Deisenhofer et al., 1985). The last residue has a negative charge that would be favorable for formation of the cytochrome complex, and one residue of this region, M292, is a conserved Asp that has been proposed as forming a critical salt bridge with Lys-10 of cytochrome c_2 (Tiede & Chang, 1988). If the formation of the cytochrome complex is dependent upon specific interactions involving residues of this region, then a pronounced change would have been expected for the mutants that contain either the corresponding *Rb. capsulatus* region or the extended *Rps. viridis* region. If the presence of the negative charge of the last residue was critical, then mutants with the removal of residues at the carboxyl region or the presence of an extension region would have resulted in altered binding.

Comparison of the results for wild type and the SWCAP mutant shows no significant differences in the electron transfer kinetics with either cytochrome from *Rb. sphaeroides* or cytochrome from *Rb. capsulatus*. Thus, there does not appear to be sequence-specific interactions involving the M carboxyl-terminal residues. For both cytochromes, the dissociation constant was decreased for SWTRC and increased for SWVIR although the time constants and the rate constants of the second component were similar when compared to wild type. This suggests that alteration of the position of the carboxyl terminus does not change the electron transfer rates. For the cytochrome from *Rb. sphaeroides*, the relatively small difference in K_d for the mutants compared to wild type suggests that the M carboxyl-terminal region is not involved in the binding. In contrast, for the *Rb. capsulatus* cytochrome, the large differences in K_d calculated for the mutants compared to wild type suggest that the *Rb. capsulatus* cytochrome binds significantly closer to the M carboxyl terminus on the RC from *Rb. sphaeroides*. Since many residues are thought to contribute to the electrostatic stabilization of the complex, these results do not eliminate the possible involvement of the carboxyl-terminal charge in the binding, but show that the position of this charge is not critical for binding.

Model for the RC–Cytochrome Complex. The electron transfer process can be divided into three steps: (1) electrostatic interactions that attract the RC and the cytochrome c_2 toward each other; (2) the binding of cytochrome c_2 to the RC to form a RC–cytochrome c_2 complex; and (3) the transfer of an electron from the heme to the primary donor within the complex. The results presented in this paper will be discussed

Table 2: Comparison of Amino Acid Residues Proposed to Be Involved in the Cytochrome Binding Site of Reaction Centers from Different Species[a]

species	L72	L155	L257	L261	L268	M87	M88	M95	M100	M110	M173	M184	M292
Rb. sphaeroides	E	D	D	D	K	R	D	E	E	K	E	D	D
Rb. capsulatus	E	D	D	D	N	R	D	E	E	K	V	D	D
Rs. rubrum	S	D	G	D	K	R	Q	E	E	A	E	E	D

[a] The numbering of the amino acid residues refers to the sequence of the reaction center from *Rb. sphaeroides*. The comparison of reaction center sequences is based upon that of Komiya et al. (1988).

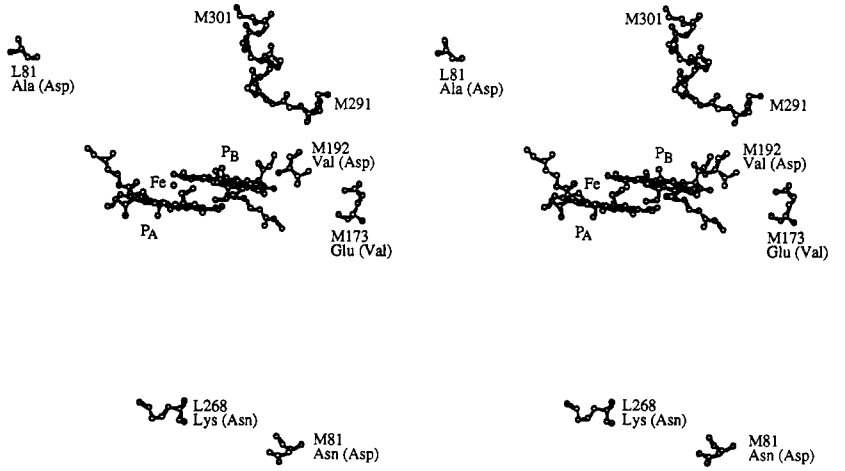

FIGURE 5: Stereoview of the primary electron donor, non-heme iron, and selected residues of the RC from *Rb. sphaeroides*. Shown are five amino acid residues, L81, L268, M81, M173, and M192, from the surface of the periplasmic side of the RC from *Rb. sphaeroides* that are different from those of the RC from *Rb. capsulatus*, and are charged residues either in *Rb. sphaeroides* or in *Rb. capsulatus*. Given in parentheses are the names of the corresponding residues in the RC from *Rb. capsulatus*. Residues near the M carboxyl terminus (M291–M301) are also shown. The stereoview is down the approximate C_2 symmetry axis of the RC. Data are from file 4RCR of the Brookhaven data base.

for these steps and then compared with previous models.

Electrostatic Interactions. Various carboxylate groups of the RC have been identified as possibly being involved in salt bridges or charged pairs with lysines of cytochrome (Table 2) (Allen et al., 1987; Tiede & Chang, 1988). Six of these residues, L155, L261, M87, M95, M100, and M292, are conserved among *Rb. sphaeroides*, *Rb. capsulatus*, and *Rs. rubrum*. Comparing *Rb. sphaeroides* to *Rb. capsulatus*, only two residues, L268 Lys and M173 Glu, are not conserved. In addition, three residues on the periplasmic surface of the RC, L81, M81, and M173, are charged in *Rb. capsulatus* but not in *Rb. sphaeroides* (Figure 5). Previous models in *Rb. sphaeroides* indicated that L268 Lys interacts with a Glu residue in the cytochrome (Tiede & Chang, 1988) and M173 Glu interacts with a Lys residue in the cytochrome (Allen et al., 1987). In *Rb. capsulatus*, L268 Lys is replaced by Asn, and a nearby residue, M81 Asn, is replaced by Asp. The loss of the positive charge at L268 together with a nearby negative charge at M81 may result in the cytochrome binding site in *Rb. capsulatus* RCs being closer to the M terminus compared to that of *Rb. sphaeroides*. Also, M173 Glu is replaced by Val, and a nearby residue, M192 Val, is replaced by Asp in *Rb. capsulatus*. The loss of the negative charge at M173 together with a nearby negative charge at M192 may again result in the cytochrome binding site in *Rb. capsulatus* RCs being closer to the M terminus compared to that of *Rb. sphaeroides*.

Positively charged Lys residues on the surface of cytochrome c_2, especially those surrounding the heme crevice, are thought to play an important role in the electrostatic interaction between the RC and cytochrome c_2 (Allen et al., 1987; Hall et al., 1987; Tiede & Chang, 1988; Caffrey et al., 1992). Several lysine residues are conserved (10, 35, 95, 97, and 103, according to the *Rb. sphaeroides* sequence), and all of them could form charged pairs with corresponding residues in the RC (Allen et al., 1987; Hall et al., 1987; Tiede & Chang, 1988; Caffrey et al., 1992). No significant differences were observed in electrostatic potential maps calculated among the cytochromes from *Rb. sphaeroides*, *Rb. capsulatus*, and *Rs. rubrum*. (Tiede et al., 1993). Thus, although the ionic strength dependence of the kinetics (Overfield & Wraight, 1980) shows the importance of electrostatics, other interactions are also critical for the binding of the two proteins.

Binding of Cytochrome c_2 to the RC. Although the RC–cytochrome complex is primarily stabilized by electrostatic interactions, other interactions also contribute. These interactions do not involve the residues forming the M carboxyl terminus but probably several residues distributed on the periplasmic surface of the RC. The importance of such forces is demonstrated by the measured kinetics involving the cytochrome from *Rs. centenum*. For RCs from *Rb. sphaeroides*, no fast component was observed for this cytochrome. However, for RCs from *Rb. capsulatus*, a fast component was clearly evident. This component has a time constant very similar to that measured for cytochromes from *Rb. capsulatus* and *Rb. sphaeroides*. However, the dissociation constant was measured to be 40 μM, which is over 10-fold larger than the values of 1.1 and 2.9 μM measured for *Rb. capsulatus* and *Rb. sphaeroides*, respectively. We propose that the lack of a fast phase for electron transfer between cytochrome c_2 from *Rs. centenum* and RCs from *Rb. sphaeroides* is due to a large dissociation constant for this complex. Furthermore, this model suggests that the lack of an observed fast phase for cytochrome c_2 from *Rs. rubrum* and RCs from *Rb. sphaeroides* could also be due to a large binding constant for that complex. A bound complex may, however, be formed between cytochrome c_2 from *Rs. rubrum* and RCs from *Rb. capsulatus*, as the dissociation constants involving RCs from *Rb. capsulatus* appear to be overall smaller. The involvement of interactions other than electrostatic was indicated in the crystal structures of the cytochrome *c* peroxidase–horse cytochrome *c* complex and the cytochrome *c* peroxidase–yeast cytochrome

c complex (Pelletier & Kraut, 1992). Hydrophobic and van der Waals interactions were the predominant forces holding the yeast cytochrome complex together while a stronger charge–charge interaction was present in the horse cytochrome *c* complex.

The affinity of the cytochrome to bind to the RC, as measured by the value of K_d that is determined from the amplitude of the fast phase, is not the major factor that controls the rate of the bimolecular reaction. For RCs from *Rb. capsulatus*, cytochrome c_2 from *Rb. sphaeroides* has a larger K_d and a larger $K_{slow(1,2)}$ compared to cytochrome c_2 from *Rb. capsulatus*. A similar relationship is found for RCs from *Rb. sphaeroides*, although the difference in K_{slow} is smaller. This correspondence also holds for the mutant SWVIR with cytochrome c_2 from *Rb. capsulatus*, that has the largest K_d of the mutants and a large K_{slow}. Thus, the complexes with the lower affinities have larger second-order rate constants.

Comparison with Other Models. Our data show that the binding of cytochrome c_2 from *Rb. sphaeroides* to the RC from *Rb. sphaeroides* is not dependent upon the sequence of the M carboxyl-terminal region. The limited involvement of the M carboxyl-terminal region is more consistent with the cytochrome–RC model of Allen et al. (1987) rather than the model of Tiede and Chang (1988). The larger changes in the binding constants observed for the cytochrome c_2 from *Rb. capsulatus* for the mutants suggest that the model of Tiede and Chang (1988) may be more appropriate for the binding of this cytochrome. Recent measurements have shown that residue Tyr M162, that is located close to the C_2 symmetry axis on the periplasmic side of the RC, is critical for the proper binding of cytochrome c_2 from *Rb. sphaeroides* to the RC from *Rb. sphaeroides* (Wachtveitl et al., 1993a,b). The involvement of this residue is consistent with the model of Allen et al. (1987) and the binding of the cytochrome with no close interaction with the M carboxyl-terminal region.

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