Relationship between Rate and Free Energy Difference for Electron Transfer from Cytochrome c_2 to the Reaction Center in *Rhodobacter sphaeroides*†

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ABSTRACT: The rate of electron transfer from cytochrome c_2 to the bacteriochlorophyll dimer of the reaction center from the photosynthetic bacterium *Rhodobacter sphaeroides* has been investigated using time-resolved optical spectroscopy. Measurements were performed on a series of mutant reaction centers in which the midpoint potentials of the bacteriochlorophyll dimer vary over a range of 350 mV. Dramatic changes in the characteristic time of electron transfer were observed, with the measured values ranging from 7730 to 80 ns compared to 960 ns for wild type. The binding constants (0.15 to 0.25 μM^{-1}) and the second-order rate constants for the slow component (5.5×10^8 to 9.4×10^8 M^{-1} s^{-1}) for the mutants are similar to the corresponding values for wild type (0.35 μM^{-1} and 11×10^8 M^{-1} s^{-1}), indicating that the binding of the cytochrome to the reaction center is not changed in the mutants. In the mutants with the fastest rates, an additional minor component was resolved that is probably due to formation of a reaction center−cytochrome complex in an unfavorable configuration with a binding constant an order of magnitude weaker than the major component. The altered midpoint potentials in the mutants result in values for the free energy difference for this electron transfer reaction ranging from −65 to −420 meV compared to −160 meV for wild type. The relationship between the rate and free energy difference was well fit by a Marcus equation using a reorganization energy of 500 meV. Based upon this fit, a distance of 9−14 Å was predicted for the edge to edge separation between the heme and the bacteriochlorophyll dimer. Since the reorganization energy is over 300 meV greater than the free energy difference for wild type, the rate of electron transfer from the cytochrome to the reaction center is not optimized. The mutants allow an experimental study of the consequences of altered free energy differences in interprotein electron transfer, giving insight into the factors that determine the rates of electron transfer in biological systems.

In photosynthesis, light energy is converted into chemical energy through a series of electron and proton transfer processes. The primary process of bacterial photosynthesis occurs in a pigment-protein complex called the reaction center [for reviews, see Feher et al. (1989), Parson (1991), Kirmaier and Holten (1993), and Woodbury and Allen (1994)]. Light excites the bacteriochlorophyll dimer (P),[1] and an electron is transferred from the dimer to an intermediate acceptor in ~3 ps and then to the primary and secondary quinone acceptors, Q_A and Q_B. Cyclic electron transfer is achieved through a series of subsequent electron and proton transfer processes involving the reaction center and cytochrome bc_1 complex. A critical component of the cyclic process is the water-soluble cytochrome c_2 that serves as an electron carrier from the cytochrome bc_1 complex to the reaction center.

Electron transfer from cytochrome c_2 to the reaction center has been extensively characterized (Prince et al., 1974; Rosen et al., 1983; Overfield et al., 1979; Moser & Dutton, 1988; Tiede et al., 1993; Venturoli et al., 1993). For electron transfer to occur, the water-soluble cytochrome must bind to the periplasmic surface of the membrane-bound reaction center, as has been examined in mutagenesis and chemical modification studies of residues contributing to the binding region (Long et al., 1989; Caffrey & Cusanovich, 1991; Wachtveitl et al., 1993; Wang et al., 1994). In the presence of exogenous cytochrome c_2 from *Rhodobacter sphaeroides*, the oxidized bacteriochlorophyll dimer P^+ of the reaction center from wild type *Rb. sphaeroides* is observed to decay rapidly with a first-order exponential component, attributed to electron transfer within a reaction center−cytochrome complex, that has a characteristic (1/e) decay time of ~1 μs. A slow second-order exponential component is also observed that is attributed to a diffusion-limited process involving an unbound cytochrome c_2 and the reaction center. While some have modeled the process as involving only these two components, others have proposed a kinetic scheme that includes an additional fast first-order component with a characteristic time of 100−500 μs that represents electron transfer from an unfavorable configuration of the reaction center−cytochrome complex. It has been suggested that the different number of components observed by different groups may arise from differences in preparations of the reaction centers (Tiede et al., 1993), although a recent study has shown no clear evidence for two distinct bound states (Venturoli et al., 1993).

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[1] Abbreviations: LDAO, lauryldimethylamine *N*-oxide; P, bacteriochlorophyll dimer; Q, quinone; Tris, tris(hydroxymethyl)aminomethane.

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In this work we examine how the rate of electron transfer from the cytochrome c_2 to the reaction center is dependent upon the driving force, i.e., the free energy difference between the final and initial states. This relationship has been previously examined by use of cytochromes that are from different species and have different redox potentials; however, each cytochrome was found to have significantly different binding properties and the range of altered driving force was limited [for a review, see Tiede and Dutton (1993)]. Recently, a series of mutants from *Rb. sphaeroides* have been designed to study the involvement of the protein subunits in establishing the oxidation potential of P (Williams et al., 1992a,b; Murchison et al., 1993; Lin et al., 1994). Each bacteriochlorophyll of the dimer has an acetyl on ring I and a keto carbonyl on ring V that can serve as proton acceptors for hydrogen bonds. In wild type reaction centers, only one hydrogen bond is formed, between histidine L168 and one of the acetyl groups of P (Yeates et al., 1988; Chang et al., 1991; Chirino et al., 1994). It was found that introducing a histidine in hydrogen bonding position to either the keto or acetyl groups results in an increase in the P/P^+ midpoint potential by 60−125 mV (Williams et al., 1992a,b; Lin et al., 1994) as was also found for reaction centers from *Rhodobacter capsulatus* (Stocker et al., 1992) and loss of the existing bond lowered the P/P^+ midpoint potential by 95 mV (Murchison et al., 1993; Lin et al., 1994). Characterization of mutants with multiple combinations of the individual mutations shows that the effect of the hydrogen bonds on the P/P^+ midpoint potentials is additive, resulting in a range of midpoints from 410 to 765 mV for the mutants compared to 505 mV for the wild type. As a result of the altered P/P^+ midpoint potential in the mutants, the driving force for electron transfer from cytochrome c_2 to the mutant reaction centers will be correspondingly altered. The relationship between the altered driving force and the rate of electron transfer as measured by time-resolved optical spectroscopy is reported in this communication. These results are then compared to previous models and results.

MATERIALS AND METHODS

Strain Construction. The general construction of the mutants has been described previously (Williams et al., 1992a,b; Murchison et al., 1993; Lin et al., 1994). Differences from previously published procedures are described below. After mutagenesis, the mutations were verified by DNA sequencing, the appropriate fragment was cloned into a shuttle vector, and the plasmids were transferred by conjugation into the *Rb. sphaeroides puf*LM deletion strain ΔLM1.1 (Paddock et al., 1989). Wild-type reaction centers were isolated from the strain ΔLM1.1 containing the plasmid pRKEN (Paddock et al., 1989).

For the FH(M197) mutation, a 55 bp *Xmn*I−*Pvu*I fragment encoding residues M188−M206 was replaced with a fragment consisting of annealed complementary synthetic oligonucleotides. To create a vector in which the *Xmn*I and *Pvu*I sites were unique, the *Xmn*I sites at residues M4−M7 and M173−M176 were removed by creating silent mutations using oligonucleotide-directed mutagenesis in a 1 kb *Sal*I−*Bam*HI fragment containing *puf*M cloned in M13mp18 (Yanisch-Perron et al., 1985). The *Pst*I−*Eco*RI fragment encompassing this fragment was then cloned into a derivative of pBR322 in which the *Xmn*I and *Pvu*II sites had been fused (Taguchi et al., 1992). The remaining *Xmn*I−*Pvu*I fragment was replaced with a synthetic fragment containing the mutation Phe (TTC) to His (CAC) at M197.

A different set of shuttle vectors was used for the double and triple mutants than for the single mutants. The vectors have been modified by cloning the *Eco*RI−*Hin*dIII fragment from pRKENB (Williams et al., 1992a) into M13mp19 and using oligonucleotide-directed mutagenesis to add a *Sac*I site (CACCTC to GAGCTC) at residues 32−33 in *pufA*, a *Cla*I site (GAAGCATG to AATCGATG) immediately before *pufL*, and a *Hin*dIII site (AAGCTGCCG to AAGCTTCCT) at residues 268−270 in *pufL*. The *Eco*RI−*Bam*HI fragment containing these mutations was then cloned into a derivative of pRKENB in which the *Hin*dIII site from the polylinker region had been removed by digestion, filling in, and religating. The restriction sites *Asp*718I, *Hin*dIII, *Xho*I, and *Bam*HI can be uniquely cut in the resulting plasmid, pRKSCH. Derivatives of the vector used for cloning fragments containing mutated *puf*L or *puf*M were altered by addition of a kanamycin resistance marker to the *Asp*718I−*Hin*dIII fragment in *puf*L (pRKSCHKm) and addition of a spectinomycin marker to the *Xho*I−*Bam*HI fragment in *puf*M (pRKSCHSp) in a manner similar to that used for the previous shuttle vectors (Williams et al., 1992a; Paddock et al., 1989).

Protein Isolation. Reaction centers were isolated from semiaerobically grown cultures following published procedures (Paddock et al., 1989; Williams et al., 1992a) with the following modifications. After reaction centers were solubilized with 15 mM Tris-HCl, pH 8, 0.65% lauryldimethylamine *N*-oxide (LDAO), 0.1 M NaCl, and 1 mM EDTA, the protein was precipitated using 30% saturated ammonium sulfate and 1% LDAO. After centrifugation at 12000*g* for 10 min, the protein pellet was resuspended in 15 mM Tris-HCl, pH 8, 0.1 M NaCl, and 1 mM EDTA. These crude reaction centers were clarified by centrifugation at 26000*g* for 30 min, purified by DEAE-Sephacel (Supelco) ion exchange chromatography, concentrated, and dialyzed against 15 mM Tris-HCl, pH 8, 0.025% LDAO, and 1 mM EDTA. The cytochrome c_2 from *Rb. sphaeroides* was purified to a A_{280}/A_{410} ratio of 0.28 using published protocols and dialyzed against 10 mM Tris-HCl, pH 7.3 (Bartsch, 1978). The concentration of reaction centers and cytochrome c_2 (reduced by ascorbate) were determined spectrophotometrically using the molar extinction coefficients $\xi^{802} = 288$ mM^{-1} cm^{-1} (Straley et al., 1973) and $\xi^{550} = 30.8$ mM^{-1} cm^{-1} (Margoliash et al., 1959), respectively. Measurements were performed on reaction centers at a fixed concentration of ~1.8 μM in 15 mM Tris-HCl, pH 8, 0.025% LDAO, and 1 mM EDTA. The cytochrome c_2 concentration was varied from 1 to 17 μM. Ubiquinone-6 at a concentration of 40 μM was added as a redox mediator, and 2 mM sodium ascorbate was added to keep the cytochrome in the reduced state in the dark.

Spectrophotometric Measurements. The electron transfer kinetics from cytochrome c_2 to P^+ were determined by monitoring the absorption changes of the reaction center at 1283 nm where P^+ has a characteristic absorption band that is centered at ~1250 nm (Parson & Cogdell, 1975). The sample was excited by 8-ns laser pulses obtained by pumping a rhodamine 6G methanol solution with the frequency-doubled light from a Q-switch YAG laser. Further details

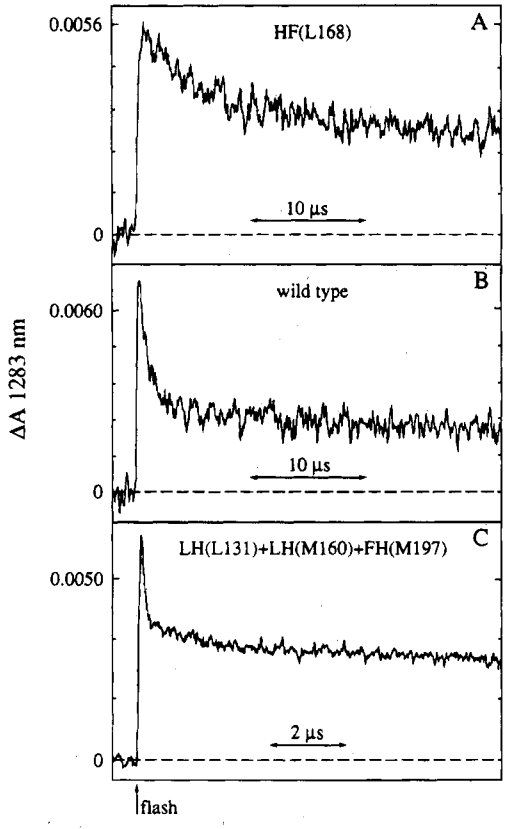

FIGURE 1: Representative reduction kinetics for the light induced P^+ for reaction centers from *Rb. sphaeroides* measured at 1283 nm at high cytochrome c_2/reaction center molar ratio. The absorbance changes were recorded with different sampling rates in order to measure on both the nanosecond and millisecond time scales. (A) Decay of P^+ in the mutant HF(L168), which has the lowest midpoint potential, with the concentration of reaction centers at 1.7 μM and cytochrome c_2 at 8 μM. (B) Decay of P^+ in the wild type with the concentration of reaction centers at 1.7 μM and cytochrome c_2 at 10 μM. (C) Decay of P^+ in the mutant LH(L131)+LH(M160)+FH(M197), which has the highest midpoint potential, with the concentration of reaction centers at 1.7 μM and cytochrome c_2 at 15 μM. For all measurements the reaction centers and cytochrome c_2 were in 15 mM Tris-HCl, pH 8.0, 0.025% LDAO, and 1 mM EDTA buffer with 40 μM ubiquinone and 2 mM sodium ascorbate.

have been described elsewhere (Ortega & Mathis, 1992). In order to simultaneously monitor changes on both the nanosecond and microsecond time scale, the 2048 channels of the digitizer were divided into segments with different time intervals per channel. The absorption transients were fit by multiple-exponential decays by using a modified Marquardt algorithm (program by Dr. P. Sétif). Measurement of the decays for mutants with the fastest rates was partially limited by the 20-MHz high-pass electrical bandwidth of the detecting system.

RESULTS

The kinetics of electron transfer were measured at 22 °C by monitoring the optical absorption decay of P^+ at 1283 nm following its formation in response to a laser pulse (Figure 1). In the presence of cytochrome, the P^+ decay was well fit by a fast exponential component, a slow exponential component, and a constant that had an amplitude less than 10% of the total amplitude. An additional minor fast component could be resolved for mutants with high P/P^+ midpoint potentials as described below.

The characteristic (1/e) time of the major fast component, τ_1, was found to dramatically vary by two orders of magnitude, from 7730 to 80 ns for the different mutants (Table 1). The characteristic time for wild type was measured to be 960 ns (or equivalently a half-time of 660 ns) that is comparable to previous measured values of 700 to 2000 ns (Overfield et al., 1979; Rosen et al., 1983; Long et al., 1989; Tiede et al., 1993; Venturoli et al., 1993; Wang et al., 1994). The rate in all cases was independent of the cytochrome c_2 concentration indicating that this component arises from a first-order reaction. In general the τ_1 values were found to decrease as the midpoint potentials increased, except for the mutants LH(L131)+HF(L168) and HF(L168)+FH(M197) (Table 1).

The amplitude of the fast phase was found to increase as the total cytochrome c_2 concentration increased due to a greater fraction of the reaction centers having a bound cytochrome. The relationship between the amplitude and cytochrome concentration has been modeled (Venturoli et al., 1993) as follows:

$$\text{cyt } c_2 + \text{RC} \leftrightarrow \text{RC-cyt } c_2$$

$$K_b = \frac{[\text{RC-cyt } c_2]}{[\text{cyt } c_2]_f[\text{RC}]_f} \quad (1)$$

where K_b is the binding constant, and [RC-cyt c_2], [cyt c_2]$_f$, and [RC]$_f$ are the concentrations of the reaction center–cytochrome c_2 complex, the free cytochrome c_2, and the free reaction center, respectively. These concentrations can be related to the relative amplitude of the fast component, A (the fraction of P^+ which decays with a fast component), the total reaction center concentration, [RC]$_t$, and the total cytochrome concentration, [cyt c_2]$_t$, according to

$$[\text{RC-cyt } c_2] = A[\text{RC}]_t$$

$$[\text{cyt } c_2]_f = [\text{cyt } c_2]_t - A[\text{RC}]_t$$

$$[\text{RC}]_f = [\text{RC}]_t(1 - A) \quad (2)$$

For each mutant, the amplitude A was measured for a wide range of cytochrome c_2 concentrations varying from 2 to 40 μM [as in Venturoli et al. (1993) and Wang et al. (1994)], and the results were fit using this model. The binding constant for wild type was determined to be 0.35 μM^{-1} (Table 1), which was comparable to the previous results of 0.1−1.0 μM^{-1} (Overfield et al., 1979; Long et al., 1989; Tiede et al., 1993; Venturoli et al., 1993; Wang et al., 1994). Similar K_b values from ~0.15 to 0.25 μM^{-1} were measured for most mutants with the two mutants LH(L131)+HF(L168) and HF(L168)+FH(M197) having slightly lower binding constants of 0.08 and 0.11 μM^{-1} respectively. For those mutants that have two resolved fast components, K_b is derived from the total amplitude of the fast components so that it could be directly compared to the values calculated for the other mutants in which the minor component is presumably present but not resolved. Use of the amplitude of only the major fast component resulted in values that were ~60% of the binding constant calculated using the amplitudes of both components.

The slow exponential component has a rate, k_{slow}, that was proportional to the concentration of free cytochrome c_2, [cyt c_2]$_f$. This dependence indicates that the component is due

Table 1: P/P^+ Midpoint Potentials and Electron Transfer Kinetics between Cytochrome c_2 and Reaction Centers

strain	P/P^+ E_m (mV)[a]	τ_1 (ns)[b]	K_b (μM^{-1})[c]	τ_2 (ns)[d]	K_{b2} (μM^{-1})[e]	K_{slow} (M^{-1} s^{-1})[f]
HF(L168)	410	7730	0.15			5.5×10^8
HF(L168)+LH(M160)	485	1545	0.20			7.7×10^8
LH(L131)+HF(L168)	485	4820	0.08			5.6×10^8
wild type	505	960	0.35			11×10^8
HF(L168)+FH(M197)	545	1515	0.11			8.4×10^8
LH(M160)	565	345	0.22			7.8×10^8
LH(L131)	585	470	0.25			6.9×10^8
FH(M197)	630	185	0.14	2020	0.008	7.6×10^8
LH(L131)+LH(M160)	635	210	0.15	2220	0.011	7.9×10^8
LH(M160)+FH(M197)	700	130	0.14	1860	0.019	7.3×10^8
LH(L131)+FH(M197)	710	165	0.16	1790	0.014	7.3×10^8
LH(L131)+LH(M160)+FH(M197)	765	80	0.20	1660	0.010	9.4×10^8

[a] Data are from Lin et al. (1994). Estimated error in E_m, the redox midpoint potential, is ±5 mV. [b] Estimated error in the characteristic time of the major fast component, τ_1, is ±10%. [c] The standard deviation of K_b, the binding equilibrium constant of all fast components, is 0.01−0.02 μM^{-1} [for wild type and LH(M160) the standard deviation is 0.05 μM^{-1}]. [d] Estimated error in the characteristic time of the minor fast component, τ_2, is ±10%. [e] The standard deviation of K_{b2}, the binding equilibrium constant of the minor fast component, is 0.001−0.003 μM^{-1}. [f] The standard deviation of K_{slow}, the second-order rate constant of the slow component, is (2.5−6.5) × 10^7 M^{-1} s^{-1}.

to a collision reaction between reaction center and free cytochrome c_2 that can be modeled by a pseudo-first-order approximation (Venturoli et al., 1993):

$$k_{slow} = K_{slow}[\text{cyt } c_2]_f \quad (3)$$

where K_{slow} is a true second-order rate constant. For wild type, the value of K_{slow} was found to be 11×10^8 M^{-1} s^{-1} in agreement with published results of (5.0−15) × 10^8 M^{-1} s^{-1} (Overfield et al., 1979; Moser & Dutton, 1988; Long et al., 1989; Venturoli et al., 1993; Wang et al., 1994). For the mutants the values of K_{slow}, (5.5−9.4) × 10^8 M^{-1} s^{-1}, are comparable to the wild type value (Table 1).

As mentioned above, a minor third exponential component was observed in the P^+ decay of the five mutants with the fastest τ_1 values. Since the rate for this third component did not vary significantly with the cytochrome c_2 concentration, it was identified as a first-order component. The characteristic time for this minor component, τ_2, varied from 2220 to 1660 ns for the five mutants (Table 1). At the lowest cytochrome concentrations, the amplitude of the minor component was only ~1% of the total amplitude, and increasing the cytochrome concentration increased the amplitude until it reached ~10% of the total amplitude. Using the binding model (eq 1) for this component yielded binding constants of ~0.01 μM^{-1}, which are approximately an order of magnitude less than the binding constants for the major fast component (Table 1).

DISCUSSION

The major difference observed in the P^+ decay of the mutants compared to wild type is that the characteristic times of the major fast component ranged from 7730 to 80 ns for the mutants compared to 960 ns for wild type. According to conventional electron transfer theory (Marcus & Sutin, 1985), the rate of electron transfer has an exponential dependence upon the free energy difference for the reaction. For electron transfer from the cytochrome to the reaction center the initial state is P^+ cyt $^{2+}$, the final state is P cyt^{3+}, and the free energy difference between these states is determined by the relative difference in oxidation/reduction midpoint potentials. For purified wild type reaction centers the P/P^+ midpoint potential is 505 mV (Lin et al., 1994), and for purified cytochrome c_2 from *Rb. sphaeroides* the cyt^{2+}/cyt^{3+} midpoint potential is 345 mV (Bowyer et al., 1981; Meinhardt & Crofts, 1982); thus the free energy difference is −160 meV. This value of the free energy difference is not corrected for other possible factors, such as alteration of the potentials due to the formation of the complex (although any small corrections to the potentials would presumably shift the free energy differences in a uniform way without changing the quality of the fit or the basic interpretation). In chromatophores, the midpoint potential of bound cytochrome is estimated to be lower by ~50 mV compared to isolated cytochrome (Dutton et al., 1975). This is supported by the observation of a 50-mV reduction in the cytochrome potential when isolated cytochrome is incorporated into phospholipid vesicles (Kimelberg & Lee, 1970). Similarly the value of the P/P^+ midpoint potential in chromatophores is ~450 mV (Dutton & Jackson, 1972), which is lower by ~50 mV than the value of ~500 mV for isolated reaction centers (Moss et al., 1991; Williams et al., 1992a; Jia et al., 1993; Nagarajan et al., 1993; Lin et al., 1994). Thus both the P and cytochrome midpoint potentials are apparently lower by 50 mV in chromatophores, and the relative free energy difference is unchanged. Possible changes in the midpoint potentials due to formation of the reaction center-cytochrome complex are estimated to be at most 10 mV on the basis of the measured binding constants (Rosen et al., 1980). The midpoint potentials may also change due to the presence of charged states, such as Q_B^-, but the effect of such charges on all of the potentials has not been determined.

Given the assumption that only the driving force is changed in the mutants due to the altered midpoint potentials, the calculated free energy differences between the final and initial states, $\Delta G°$, range from −65 to −420 meV. The relationship between the rate of electron transfer, k, and $\Delta G°$ can be modeled using conventional electron transfer theory (Marcus & Sutin, 1985) according to

$$k = (4\pi^2/h)V^2(4\pi\lambda k_B T)^{-1/2} \times \exp[(-(\Delta G° + \lambda)^2/4\lambda k_B T)] \quad (4)$$

where $\hbar$ is Planck's constant, V is the electronic coupling factor between the initial and final states, k_B is the Boltzmann constant, T is the temperature, and λ is the reorganization

energy. The data are well fit to eq 4 using a value of λ equal to 500 ± 20 meV (Figure 2). However, since there is no clear observation of an "inverted region" the value of λ should be regarded only as a lower limit. This fit predicts a maximal rate of 1.4×10^7 s^{-1} when the free energy difference equals the reorganization energy. The data were also well fit using a modified form of eq 4 that includes a term to describe coupling to a vibrational mode (Jortner, 1980). In that case a similar value of ~500 meV for λ was obtained with a vibrational frequency $\nu < 100$ cm^{-1}.

For application of these models of electron transfer, it is necessary that no other parameters of the system, such as the binding of the cytochrome to the reaction center, change as a result of the mutations. Previous studies of the relationship between the driving force and electron transfer rate used cytochromes with different redox potentials, but it was found that the binding differed by orders of magnitude [for a review, see Tiede and Dutton (1993)]. The mutations discussed in this work are next to the bacteriochlorophyll dimer, which is buried in the protein and not part of the periplasmic surface of the reaction center that forms the binding site for the cytochrome. Thus, the binding of the cytochrome to the reaction center should not change as a result of the mutations as was experimentally verified by the similar values of the binding constants K_b from ~0.15 to 0.25 μM^{-1} for the mutants compared to 0.35 μM^{-1} for wild type. Also consistent with the unchanged binding are the values of K_{slow}, (5.5 to 9.4) $\times 10^8$ M^{-1} s^{-1}, that are comparable to the wild type value of 11×10^8 M^{-1} s^{-1}. The exceptions are the lower values of 0.08 and 0.11 μM^{-1} for K_b observed in the LH(L131)+HF(L168) and HF(L168)+FH-(M197) mutants, respectively. These mutants have altered optical spectra indicating possible changes in the electronic or three-dimensional structures (Lin et al., 1994). It should be noted that these two mutants also show the largest deviations from the fit of eq 4 (Figure 2).

The fit of the data (Figure 2) yielded an estimated value for the reorganization energy of ~500 meV (or 48 kJ/mol). A theoretical estimate of the reorganization energy for electron transfer from a heme to the bacteriochlorophyll dimer in the reaction center−tetraheme cytochrome complex in *Rhodopseudomonas viridis* that was based upon the three-dimensional structure of that complex (Deisenhofer et al., 1984) and the measured rate constants (Holten et al., 1978) yielded a similar value of ~600 meV (Marcus & Sutin, 1985). A value of 800 meV for the reorganization energy in cytochrome b_5 was determined from cytochrome−ruthenium experiments (Jacobs et al., 1991) and metal substitution experiments involving cytochrome b_5 and cytochrome *c* (McLendon, 1988). Thus, our result is consistent with values of λ for other cytochrome systems, although it may be lower in the reaction center−cytochrome c_2 complex than in other types of cytochrome complexes.

The changed free energy differences in the mutants compared to wild type should lead to changes in the temperature dependence of the rate. The temperature dependence of the reduction of P^+ has been studied previously for reaction centers from the R-26 strain and cytochrome c_2 from *Rb. sphaeroides* (Venturoli et al., 1993). The rate was observed to decrease following an Arrhenius dependence with an activation energy of 210 meV, and as the temperature was lowered from 295 K to 240 K, the amplitude of the fast component decreased until no fast

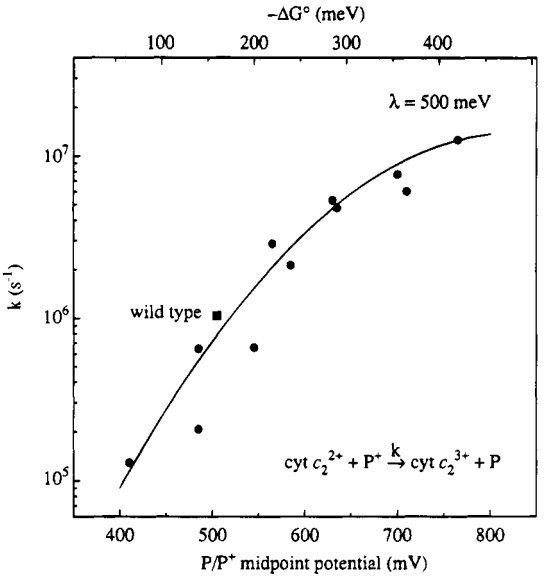

FIGURE 2: Relationship of the electron transfer rate from cytochrome c_2 to reaction centers of *Rb. sphaeroides* and the P/P^+ midpoint potential for wild type (■) and hydrogen bond mutants (●). The rate constants k are the reciprocal of the characteristic times τ_1 (Table 1). The free energy difference of the reaction, $\Delta G°$, is the difference between the cyt^{2+}/cyt^{3+} midpoint potential of 345 mV and the midpoint potentials of the bacteriochlorophyll dimer of the reaction centers. The curve shown is a fit using eq 4 with a reorganization energy $\lambda = 500$ meV and a maximum rate of 1.4×10^7 s^{-1} when $\Delta G° = \lambda$.

component was evident (Venturoli et al., 1993). A model, in which the reorganization energy of the protein has a significant temperature dependence, was proposed on the basis of estimates of various parameters, including the free energy difference (Venturoli et al., 1993). This model is being tested by measuring the temperature dependence of this rate for the mutants discussed in this work.

For most of the P^+ decays measured for the mutants, only two exponential terms were needed to describe the data as introduction of a third exponential component did not improve the quality of the fit. However, for mutants with the fastest τ_1 values (from 80 to 185 ns), an additional minor fast component was resolved that was an order of magnitude slower (from 1660 to 2220 ns) than the major fast component (Table 1). Although this third component could not be statistically resolved in the decays for which τ_1 was greater than 200 ns, the third minor component presumably is also present in that data. The amplitude of the third component was always small, and the binding constant determined from eq 1 was approximately 10-fold smaller than that of the major fast component (Table 1). Thus, the small amplitude of the component appears to be due to the significantly smaller binding constant for this state. A third minor component that had a similar time constant of ~2 μs was reported to marginally improve the fit for wild type reaction centers by Tiede et al. (1993). However, an improvement was not evident when a third component was included in the analyses of our wild type data nor was the variability of binding constants reported by Tiede et al. (1993) evident in our data.

Models have been developed in which two fast components are attributed to favorable ("proximal") and unfavorable ("distal") configurations for cytochrome bound to the reaction center and electron transfer only occurs from the "proximal" state (Moser & Dutton, 1988; Overfield & Wraight, 1986;

Tiede et al., 1993). In these models the rates for the fast components have time constants of ~1 μs and 100−500 μs, and the amplitude of each component would be ~50% of the total at high concentrations of the cytochrome c_2 relative to that of the reaction center. However the third component in our data had a characteristic time of ~2 μs and always represented a minor contribution to the total amplitude. Because of the time and amplitude of the third component, our data are not compatible with the specific features of the previous proximal−distal models. However, the third component probably represents a minor binding configuration of the reaction center−cytochrome complex.

According to electron transfer theories, the maximal possible rate is achieved when $\Delta G^\circ = \lambda$. Since the exponential term is unity, the maximal rate is determined strictly by the preexponential coefficient of eq 4. For biological systems this coefficient, and hence the maximal rate, is determined largely by a characteristic distance between the donor and acceptor (Marcus & Sutin, 1985; Moser et al., 1992; Beratan et al., 1992; Evenson & Karplus, 1993). From the fit of our data (Figure 2), a maximal rate of 1.4×10^7 s^{-1} is predicted. According to the model of Moser et al. (1992) this maximal rate of electron transfer is determined by the edge to edge distance between the donor and acceptor. Using this empirical model, we can predict the edge to edge distance between the heme and the bacteriochlorophyll dimer to be ~14 Å. The model of Moser et al. (1992) may be unsatisfactory for some electron transfer reactions, including those involving cytochrome *c*, as it may overestimate the edge to edge distance (Beratan et al., 1992). Comparing our maximal rate of 1.4×10^7 s^{-1} to the relationship between maximal rate and distance of Beratan et al. (1992), we would estimate a smaller edge to edge distance of ~9 Å. Thus, the predicted distances range from 9 to 14 Å. This range is in general agreement with distances of 10−14 Å derived from modeling studies of the reaction center−cytochrome complex that are based upon the individual three-dimensional structures of the reaction center from *Rb. sphaeroides* and cytochrome (Tiede & Chang, 1988; Allen et al., 1987). A similar distance of ~12 Å is found between the dimer and closest heme of the bound tetraheme cytochrome in *Rps. viridis* (Deisenhofer et al., 1984). It has been argued that for biological electron transfer the rate may be better correlated with the electron tunneling pathway rather than the edge to edge distance (Beratan et al., 1992; Evenson & Karplus, 1993). Using the model of Beratan et al. (1992) and the maximal rate of 1.4×10^7 s^{-1}, the tunneling pathway of the electron from the heme to the bacteriochlorophyll dimer is predicted to be ~20 Å for the reaction center−cytochrome complex in *Rb. sphaeroides*.

Our results demonstrate that the electron transfer rate for wild type is 10−20 times slower than the optimal rate due to the driving force being significantly smaller than the reorganization energy. Since reorganization energies of at least 500 meV appear to be an intrinsic property of cytochrome systems, a faster rate would require a larger free energy difference. As demonstrated by the hydrogen bond mutations, a greater free energy difference and thus a faster rate could have been achieved with different pigment−protein interactions that partially determine the energy levels of the cofactors. However, altering the midpoint potentials of either the cytochrome c_2 or the dimer to achieve a greater free energy difference would have repercussions for the rest of the cyclic electron transfer process involving the reaction center and the cytochrome bc_1 complex. For example, increasing the potential of the dimer results in a decrease in the initial rate of electron transfer in the reaction center (Williams et al., 1992a,b), and the midpoint potential of the cytochrome c_2 (E_m = 345 mV) must be higher than the midpoint potential of the cytochrome c_1 (E_{m7} = 260 mV; Meinhardt & Crofts, 1982). Since the rate of electron transfer from the cytochrome c_2 to the reaction center is much faster than the competing charge recombination rate (100 ms from the primary quinone and 1 s from the secondary quinone), an increase in the electron transfer rate for the cytochrome would not improve the quantum efficiency of photosynthesis. Thus, although many electron transfer processes are optimized in biological systems, electron transfer from the cytochrome c_2 to the reaction center has not evolved to achieve the maximal electron transfer rate.

This investigation of the electron transfer rate for cytochrome to the reaction center has provided an opportunity to characterize the relationship between the rate and driving force for interprotein electron transfer with a fixed donor−acceptor distance. The results show that, in mutants that do not change the binding geometry, a variation in the rate over two orders of magnitude can be achieved by a 350-meV range of free energy differences. The rate versus driving force relationship has been studied within a protein complex in photosynthetic systems primarily between the primary quinone and bacteriochlorophyll dimer in the reaction center (Popovic et al., 1986; Gunner et al., 1986; Feher et al., 1988; Franzen & Boxer, 1993; Lin et al., 1994). This charge recombination rate also has a large reorganization energy; however, it is not a forward electron transfer and so is not necessarily optimized by the protein. Studies of interprotein electron transfer in nonphotosynthetic systems are limited, as they involve difficult manipulation of the protein such as metal substitution and covalent attachment of redox active groups [for a review, see McLendon (1988)]. This study provides an estimate for electron transfer parameters such as the reorganization energy that should be applicable to other biological systems, especially those involving cytochromes, which are commonly found as electron carriers.

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