Electron Transfer from Cytochrome c_2 to the Primary Donor of *Rhodobacter sphaeroides* Reaction Centers. A Temperature Dependence Study†

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ABSTRACT: Kinetics of flash-induced electron transfer from the soluble cytochrome c_2 to the primary donor (P) of the reaction center purified from the purple bacterium *Rhodobacter sphaeroides* R-26 were investigated by time-resolved absorption spectroscopy. Re-reduction of P^+ induced by a laser pulse was measured at 1283 nm both in isolated reaction centers and in reconstituted proteoliposomes reproducing the lipid composition of the native membrane. The effects of temperature (230–300 K) and of the cytochrome c_2/reaction center stoichiometry were examined. At room temperature, over a wide range of cytochrome c_2 to reaction center molar ratios, the biphasic kinetics of cytochrome c_2 oxidation in the microsecond-to-millisecond time scale could be accurately described by a minimum reaction scheme which includes a second-order collisional process ($k = 1.4 \times 10^9$ M^{-1} s^{-1} and $k = 2.4 \times 10^9$ M^{-1} s^{-1} in isolated and reconstituted reaction centers, respectively) and a first-order intracomplex electron donation ($t_{1/2} = 590 \pm 110$ ns in isolated reaction centers; $t_{1/2} = 930 \pm 140$ ns in proteoliposomes). At cytochrome c_2 to reaction center molar ratios exceeding 5, the monomolecular process almost completely accounts for P^+ re-reduction. At lower stoichiometries, the relative contribution of the two parallel reaction pathways is modulated by a single binding equilibrium between cytochrome c_2 and reaction centers, yielding a binding constant of 3.5×10^5 M^{-1} in both systems. In the 230–300 K range, the kinetics of the mono- and bimolecular reactions are markedly affected by temperature, following Arrhenius behavior with activation energies of 4.9–5.8 and 6.5 kcal mol^{-1}, respectively. At low temperature, in isolated reaction centers (but not in the reconstituted system), a minor microsecond phase of P^+ re-reduction was detected, attributed to formation of the triplet-state ^3P. Upon lowering the temperature, the relative contribution of the fast monomolecular oxidation of cytochrome c_2 decreased rather abruptly below 260 K and essentially vanished at 230 K. Mechanisms leading to this dramatic impairment are discussed in relation to medium reorganization coupled to electron transfer. A reorganization energy of 25 kJ mol^{-1} has been estimated for electron donation within the cytochrome c_2–reaction center complex.

The reaction between soluble cytochrome (cyt)[1] c_2 and the photosynthetic reaction center (RC) from *Rhodobacter* (*Rb.*) *sphaeroides* appears to be an attractive system for studying the mechanism of electron transfer within interprotein complexes as well as the interaction between a peripheral and an integral membrane protein. The X-ray crystallographic analysis has indeed provided the three-dimensional structure of this reaction center to a resolution of 2.8 Å (Allen et al., 1987a,b, 1988; Yeates et al., 1988), and its photochemistry is perhaps better characterized than in any other photosynthetic reaction center [for reviews, see Feher and Okamura (1978), Okamura et al. (1982), Parson and Ke (1982), Parson (1987), and Feher et al. (1989)]. Within the RC, a special pair of bacteriochlorophylls ($BChl_2$) acts as the primary donor (P) and upon excitation delivers an electron through a bacteriopheophytin to a molecule of ubiquinone-10 (Q_A) in about 200 ps (Kirmaier & Holten, 1987). Stabilization of the primary charge-separated state ($P^+Q_A^-$) is accomplished on the cytoplasmic side of the RC by electron transfer from Q_A^- to the secondary quinone acceptor Q_B and on the periplasmic surface of the reaction center by re-reduction of P^+ by ferrocyt c_2.

The atomic structure of cytochrome c_2 has not been obtained for *Rb. sphaeroides*, but the structure of many similar cytochromes has been determined, including that from the very closely related bacterium *Rb. capsulatus* (Benning et al., 1991).

The literature concerning the oxidation of cyt c_2 by the RC is quite substantial and includes studies performed in whole cells and in native membrane vesicles (chromatophores) (Kihara & Chance, 1969; Ke et al., 1970; Dutton et al., 1975; Prince et al., 1978; Overfield et al., 1979; Bowyer et al., 1979; Meinhardt & Crofts, 1982), in purified RC preparations solubilized in detergent suspensions (Prince et al., 1974; Overfield et al., 1979; Moser & Dutton, 1988), and in phospholipid-reconstituted proteoliposomes (Moser & Dutton, 1988; Overfield & Wraight, 1980a,b). The oxidation of cyt c_2 by P^+ displays multiphasic kinetics. At low reactant concentrations, or at high ionic strength, the reaction proceeds by a second-order process. At low ionic strength and high reactant concentrations, a fast first-order phase of cytochrome oxidation ($t_{1/2} \simeq 1$ μs) has been observed (Overfield et al., 1979), attributed to electron transfer within a cyt c_2–RC complex. A kinetic scheme has been proposed which includes

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[1] Abbreviations: BChl, bacteriochlorophyll; cyt, cytochrome; LDAO, lauryldimethylamine oxide; Q_A and Q_B, primary and secondary quinone acceptors; RC, reaction center; *Rb.*, *Rhodobacter*; *Rps.*, *Rhodopseudomonas.*

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two states of the complex, ascribed to cytochrome bound in favorable (proximal) and unfavorable (distal) configurations for electron transfer (Overfield et al., 1979).

On the basis of the atomic structure of the RC and of cyt *c* or c_2, models have been elaborated for the binding of cyt c_2 to the L and M subunits of the RC (Allen et al., 1987b; Tiede et al., 1988). The complex has been proposed to be stabilized by electrostatic interactions between negatively charged carboxylate groups on the periplasmic surface of the RC and positively charged lysine residues surrounding the heme crevice of cyt c_2 (Hall et al., 1987; Long et al., 1989). The main difference between the models presented by Allen et al. (1987b) and Tiede et al. (1988) resides in the orientation of the heme with respect to the symmetry axis of the RC: while in the former the heme is kept coplanar with $BChl_2$ rings, the latter model keeps the heme axis at an angle of 53° with respect to the average $BChl_2$ axis. This orientation had been evaluated from linear dichroism measurements on cyt *c*–RC complexes in reconstituted phosphatidylcholine vesicles (Tiede, 1987).

In elucidating the mechanisms of electron transfer catalyzed by the RC, the study of the temperature dependence of the reaction rates has proved to be highly informative, often yielding insights into the reaction pathways and serving as a valuable test of different electron-transfer models [see, e.g., Jortner (1980) or Gunner and Dutton (1989)]. Since the pioneering experiments of De Vault and Chance (1966) in *Chromatium*, light-induced oxidation of *c*-type cytochromes by the RC has been demonstrated to occur at cryogenic temperatures in many species of photosynthetic bacteria (De Vault et al., 1967; Kihara & Chance, 1969; Dutton et al., 1970; 1971; Kihara & McCray, 1973; Hales, 1976). The peculiar behavior observed in some species, showing thermal activation above 120 K and near temperature independence below (De Vault & Chance, 1966; De Vault et al., 1967; Dutton et al., 1971; Hales, 1976), has prompted considerable theoretical effort [for reviews, see Blankenship and Parson (1979b), De Vault (1984), and Marcus and Sutin (1985)]. More recently, low-temperature cytochrome *c* photochemistry has started to be reexamined both experimentally (Kaminskaya et al., 1990; Ortega & Mathis, 1992, 1993) and theoretically [see, e.g., Bixon and Jortner (1986, 1988), Knapp and Fisher (1987), and Cartling (1991)] in light of the functional and structural knowledge of bacterial RC gained over the last decade.

The effect of temperature on the kinetics of electron transfer from cyt c_2 to P^+ has not been studied in *Rb. sphaeroides*. In this respect, the only data available in the literature concern cyt *c* oxidation induced by continuous illumination in whole cells, which was reported to disappear upon lowering the temperature between 240 and 150 K (Vredenberg & Duysens, 1964). The absence of cytochrome *c* oxidation *in vivo* at 77 K was confirmed in a subsequent paper (Kihara & Chance, 1969).

In this work, we have studied the effect of temperature on the kinetics of the reaction induced by a laser pulse both in purified reaction centers solubilized in detergent suspensions and in reconstituted proteoliposomes restoring the native lipid environment of the protein. In the first part of the paper, we report a detailed analysis of the cytochrome c_2 concentration dependence of the kinetics measured at room temperature.

EXPERIMENTAL PROCEDURES

Reaction centers of *Rb. sphaeroides* R-26 were purified in lauryldimethylamine oxide (LDAO) as described by Feher and Okamura (1978). The RC concentration was determined spectrophotometrically using the value $\epsilon = 288\ mM^{-1}\ cm^{-1}$ at 802 nm (Straley et al., 1973).

Reconstitution of proteoliposomes was carried out by the following procedure [slightly modified from Overfield and Wraight (1980b)]. A phospholipid mixture of L-α-phosphatidylethanolamine (type V; Sigma, St Louis, MO), L-α-phosphatidylcholine (type IIIB), and L-α-phosphatidylglycerol at a ratio of 2:1:1, supplemented with ubiquinone-10, was dispersed in 10 mM glycylglycine, pH 7.8, 2 mM KCl, and 4% sodium cholate and sonicated for 30 s. For reconstitution, the RC complex was added to the suspension at approximately 20 μM. The final mixture (phospholipid:ubiquinone-10:RC ratio = 1000:50:1) was sonicated twice for 3 s and applied onto a column (1 × 15 cm) of Sephadex G-50 equilibrated in 50 mM glycylglycine, pH 7.8. Cytochrome c_2 was isolated and purified according to Bartsch (1971).

Flash absorption kinetics of electron transfer from cyt c_2 to the photooxidized primary donor (P^+) were measured essentially as described by Ortega and Mathis (1992). Re-reduction of P^+ following excitation by a ruby laser pulse (10 ns, 694 nm) was monitored at 1283 nm where P^+ has a broad absorption band centered at about 1250 nm (Parson & Cogdell, 1975). Since P has a negligible absorption in this spectral region, any actinic effect of the measuring light is avoided. The amplified photodiode output was fed into a PC-interfaced Tektronix transient recorder. The 2048 channels of the memory were partitioned into segments with different sampling rates in order to cover a millisecond time span and simultaneously capture the fast transients of P^+ re-reduction at an appropriate time resolution (40 ns).

For low-temperature measurements, glycerol was added to the reaction mixture to a final concentration of 60% (v/v), and the sample was inserted in a cryostat cooled with a thermostated water/ethylene glycol mixture or with helium gas. In all experiments on isolated reaction centers dispersed in detergent suspensions, the final LDAO concentration was kept lower than 0.03%. Unless otherwise specified, kinetic traces are the result of single flash measurements without averaging. Between successive excitations, samples were dark-adapted for a time interval (1–5 min) sufficient to allow a complete return to equilibrium. For measurements below 260 K, samples were dark-adapted at room temperature and cooled in darkness.

Deconvolution of P^+ re-reduction kinetics into multiple exponential decays and other nonlinear least-squares minimizations in data analysis were performed by computer routines based on a modified Marquardt algorithm (Bevington, 1969).

RESULTS

Cytochrome c_2 Concentration Dependence of P^+ Re-reduction. Electron-transfer kinetics from cyt c_2 to the primary donor P^+ were studied at room temperature by measuring P^+ re-reduction following a laser flash at a fixed RC concentration over a wide range of cyt c_2/RC stoichiometries. Measurements were performed both in detergent/RC dispersions and in reaction centers reincorporated into phospholipid vesicles. Proteoliposomes contained a mixture of phospholipids which simulated the native chromatophore membrane, i.e., about 50% phosphatidylethanolamine and 25% each of phosphatidylcholine and phosphatidylglycerol (Casadio et al., 1979). The average molar phospholipid:RC ratio was 1000, which compares to values of 800–1100 mol/mol of RC in chromatophores (Casadio et al., 1984, 1988). Ubiquinone-10 was present in the lipid phase at a molar ratio of 50 ubiquinone/

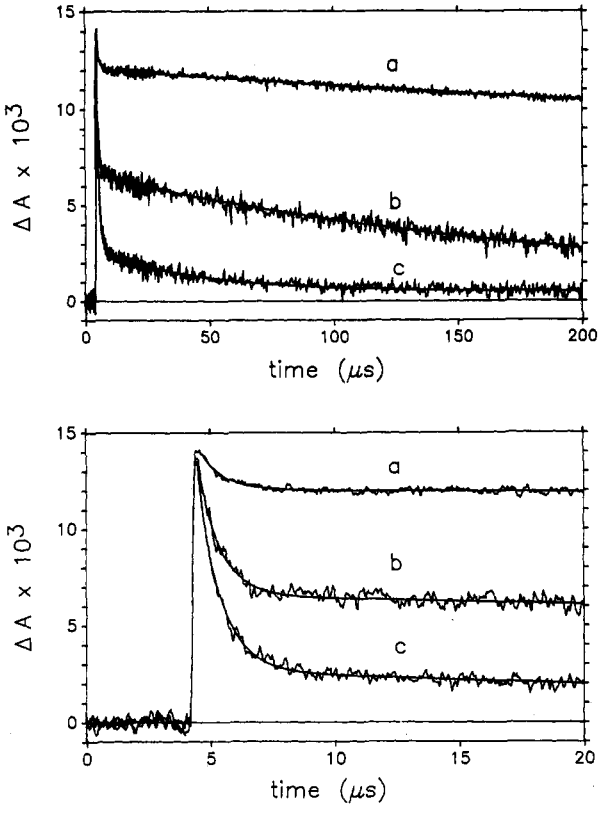

FIGURE 1: Kinetics of electron transfer between cytochrome c_2 and reaction centers isolated from *Rhodobacter sphaeroides* R-26 at 295 K. The flash-induced absorbance change detected at 1283 nm (P^+ absorption band) is shown for three cytochrome c_2 to reaction center stoichiometric ratios. Reaction centers were suspended at 3.3 μM in 50 mM glycylglycine (pH 7.8), 0.024% LDAO, 18 μM ubiquinone-6, and 0.8 mM ascorbate. Cytochrome c_2 was present at 1.2 μM (trace a), 5.7 μM (trace b), and 24.4 μM (trace c). In the lower panel, the fast transients of the signals are displayed on an expanded time scale. The decay of P^+ was fitted to a sum of two exponentials plus a constant (solid line).

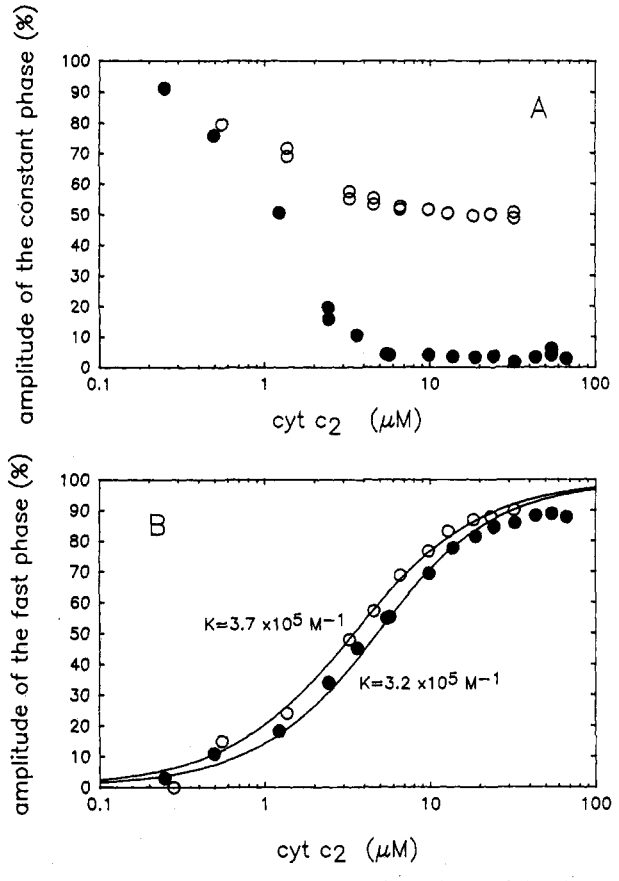

FIGURE 2: Cytochrome c_2 concentration dependence of the kinetics of P^+ re-reduction in detergent/reaction center dispersions (●) and in proteoliposomes (○). The recovery of P^+ following flash excitation was deconvoluted into a fast and a slow component plus a constant term (see traces in Figure 1). (A) Amplitude of the constant component. (B) Amplitude of the fast phase. The assay conditions for the measurements in isolated reaction centers are as in Figure 1. Proteoliposomes equivalent to 3.2 μM reaction centers were suspended in 50 mM glycylglycine (pH 7.8) and 1 mM ascorbate at 295 K. In the case of reaction centers reincorporated into phospholipid vesicles, the amplitude of the fast phase (B, open circles) has been normalized to the fraction (50%) of P^+ which can be re-reduced by the externally aded cytochrome c_2. The continuous lines in panel B are the best fits to the experimental data according to eq 1 in the text.

RC, corresponding to an approximate concentration of 65 mM in the lipids, again comparable to that of the native ubiquinone pool of the chromatophore membrane.

The results obtained in detergent/RC dispersions and in proteoliposomes will be presented in parallel, since a quite similar kinetic pattern could be observed in the two types of preparations as a function of the cyt c_2 concentration.

In the absence of cyt c_2, flash-oxidized P^+ decayed monoexponentially with $t_{1/2} \cong 1$ s. This is interpreted as recombination with Q_B^-, in agreement with Blankenship and Parson (1979a) and Baccarini Melandri et al. (1982). Figure 1 shows typical kinetic traces of flash-induced absorption changes in the P^+ region (1283 nm) obtained in a detergent/RC dispersion at three different cyt c_2 concentrations. Following the laser pulse, the absorption increases within the time resolution of the apparatus (P^+ formation) and subsequently decays (P^+ re-reduction) according to multiphasic kinetics. Analogous traces were obtained in proteoliposomes. For both types of preparation, over the whole range of cyt c_2 concentrations tested (from 0.1 to 50 μM), the decay of P^+ could be accurately deconvoluted into a constant term plus two exponential components, the half-times of which always differ by more than 1 order of magnitude. The results of this kinetic analysis are summarized in Figure 2A,B and in Figure 3.

The relative amplitude of the constant term decreases progressively at increasing cyt c_2:P^+ ratios and becomes cyt c_2 independent when the concentration of cyt c_2 overcomes that of P^+ (Figure 2A). This phase appears therefore to be related to the fraction of RC's in which P^+ is not re-reduced by cyt c_2. In this population of reaction centers, P^+ will decay by charge recombination of the $P^+Q_B^-$ state ($t_{1/2} \cong 1$ s). For the purpose of the present analysis, performed on the nanosecond-to-millisecond time scale, the correspondent kinetic phase can be considered constant in time. In detergent/RC dispersions, the relative contribution of the constant term reduces to 3–4% of the total at high cyt c_2 concentrations, showing that essentially the whole population of flash-oxidized P^+ can react with cyt c_2. In proteoliposome preparations, the relative amplitude of the constant term approaches a value of approximately 50% of the total at high cyt c_2 concentrations. This value is consistent with a random orientation of the reincorporated reaction centers with respect to the vesicle membrane, in agreement with previously published data (Overfield & Wraight, 1980a; Gabellini et al., 1989).

The half-time of the fast phase (Figure 3, circles) was fairly independent of cyt c_2 concentration over more than 2 orders of magnitude and equal to 590 ± 110 ns in the detergent/RC suspension. A higher value (930 ± 140 ns), equally independent of cyt c_2 concentration, was obtained for the half-

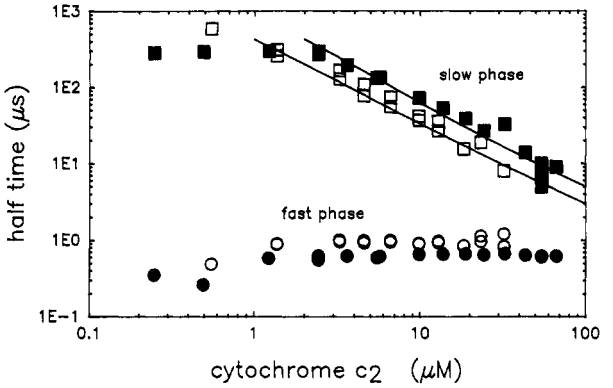

FIGURE 3: Cytochrome c_2 concentration dependence of the half-time of the fast (circles) and slow (squares) phases of P^+ re-reduction in isolated (closed symbols) and reconstituted (open symbols) reaction centers. The data were taken from the same experiments used for Figure 2. Continuous lines represent a best fit to the data according to eq 2 (see text for details).

time of the correspondent phase in proteoliposome preparations. On the contrary, in both types of preparation, the half-time of the slow phase showed a progressive decrease from 300–500 μs to 8 μs at cyt c_2 concentrations increasing from 0.2 to 80 μM (Figure 3, squares).

The portion of flash-induced P^+ undergoing true first-order re-reduction (fast phase) increased progressively at increasing cyt c_2/RC stoichiometries, as can be seen from Figure 2B. In the case of proteoliposome preparations, the amplitude of this fast phase has been normalized to the fraction (approximately 50%) of P^+ which are accessible to the externally added cyt c_2. In the detergent/RC dispersions, P^+ recovery occurred almost completely through the fast monomolecular reaction when the mole ratio of cyt c_2 to P^+ was above approximately 10. The same behavior was observed in the proteoliposome suspensions where more than 80% of the properly oriented flash-oxidized P^+ could be re-reduced in a true first-order (monomolecular) process at cyt c_2 concentrations exceeding 20 μM. The occurrence of a fast first-order phase of P^+ recovery, already observed in purified and reconstituted reaction centers (Overfield et al., 1979; Overfield & Wraight, 1980a,b), is consistent with electron donation to P^+ within a long-lived cyt c_2–RC complex. The relative amplitude of the fast phase was accordingly fitted to the fraction of bound RC evaluated according to the equation:

$$\left(\frac{CP}{P}\right)^2 K - \frac{CP}{P}\left[\frac{1}{P} + K\left(1 + \frac{C}{P}\right)\right] + K\left(\frac{C}{P}\right) = 0 \qquad (1)$$

where C and P are the total concentrations of cyt c_2 and RC, respectively, CP is the concentration of the cyt c_2–RC complex, and K is a binding equilibrium constant. When eq 1 was applied to the data obtained in proteoliposomes, P was set equal to the concentration of reaction centers exposing the docking site for cyt c_2 to the external phase. Binding constants equal to 3.2×10^5 and 3.7×10^5 M^{-1} were obtained in isolated RC and in proteoliposome suspensions, respectively (see continuous lines in Figure 2B). These values agree with previous estimates obtained at low ionic strength both in isolated RC (Overfield et al., 1979) and in reconstituted RC purified from *Rhodobacter sphaeroides* (Overfield & Wraight, 1980a,b; Moser & Dutton, 1988) and from the related species *Rhodobacter capsulatus* (Venturoli et al., 1990). In some of these studies, however, a more complex binding model, involving two bound states in the cyt c_2–RC complex, was considered (see Discussion).

The slow phase of P^+ re-reduction, which dominates the recovery at low cyt c_2 concentrations, appears to be compatible with a second-order collisional reaction between P^+ and unbound cyt c_2. In fact, the dependence of its half-time on cyt c_2 concentration (Figure 3, squares) could be accurately described in a pseudo-first-order approximation by the equation:

$$t_{1/2} = \frac{\ln 2}{kc_f} \qquad (2)$$

where k is a true second-order kinetic constant and c_f, the concentration of free cyt c_2, can be evaluated from eq 1 using the calculated values of the binding constant K. This analysis yielded $k = 1.4 \times 10^9$ M^{-1} s^{-1} for the detergent/RC dispersion. A higher value ($k = 2.4 \times 10^9$ M^{-1} s^{-1}) was obtained from the correspondent kinetics observed in proteoliposomes. In both cases, the fit has been restricted to cyt c_2 concentrations above that of reactive P^+. At lower cyt c_2:P^+ ratios, when the pseudo-first-order approximation does not hold, the half-time was roughly independent of cyt c_2 concentration. Although in these measurements the values of the half-time are likely to be affected by considerable experimental uncertainty (because the properties of the light source/detector system are well adapted to the 100 ns–1 ms time scale, but they degrade progressively above 1 ms), the behavior observed at low cyt c_2 concentrations is compatible with second-order kinetics, for which a break point in the concentration dependence of the half-time is expected at equal reactant concentrations (Prince et al., 1978).

In conclusion, the kinetics of P^+ re-reduction at varying cyt c_2 to P^+ ratios can be accurately described, under our experimental conditions, by a minimum reaction scheme, which includes a second-order collisional process and a first-order intracomplex electron donation. These two parallel reaction routes appear to be modulated by a single binding equilibrium between cyt c_2 and the reaction center.

Temperature Dependence of the Kinetics of Electron Transfer from Cytochrome c_2 to P^+. The effects of temperature on the kinetics of P^+ re-reduction following a laser pulse have been studied between 300 and 230 K in the presence of 60% glycerol. Measurements were performed at a cyt c_2 to P^+ molar ratio equal to 5. Under these conditions, at ambient temperature and in aqueous buffer, the decay of the photo-oxidized primary donor was dominated by the fast first-order kinetic phase ascribed to electron transfer within the cyt c_2–RC complex.

The data obtained in isolated RC dispersions were analyzed as a sum of exponential components. In the 300–280 K temperature range, biexponential fits to kinetic data were generally acceptable, in agreement with the measurements in aqueous buffer at room temperature. Below 280 K, a considerable improvement in the accuracy of the fit could be obtained by including a third exponential phase, the relative amplitude of which, however, never exceeded 20% of the total. For the sake of clarity, the three kinetic phases considered will be designated here fast, intermediate, and slow.

The rate constant of the fast component (Figure 4A) was 1.7×10^6 s^{-1} at 295 K and decreased with decreasing temperature to a value of 1.6×10^5 s^{-1} at 238 K. Below this temperature, it was not possible to analyze the kinetics of the fast phase since its contribution to P^+ re-reduction was reduced to a few percent of the total and the other two components almost completely accounted for the kinetics (see below).

Between 295 and 280 K, the fast phase makes up 42% of P^+ re-reduction. At the same concentration of cyt c_2, the

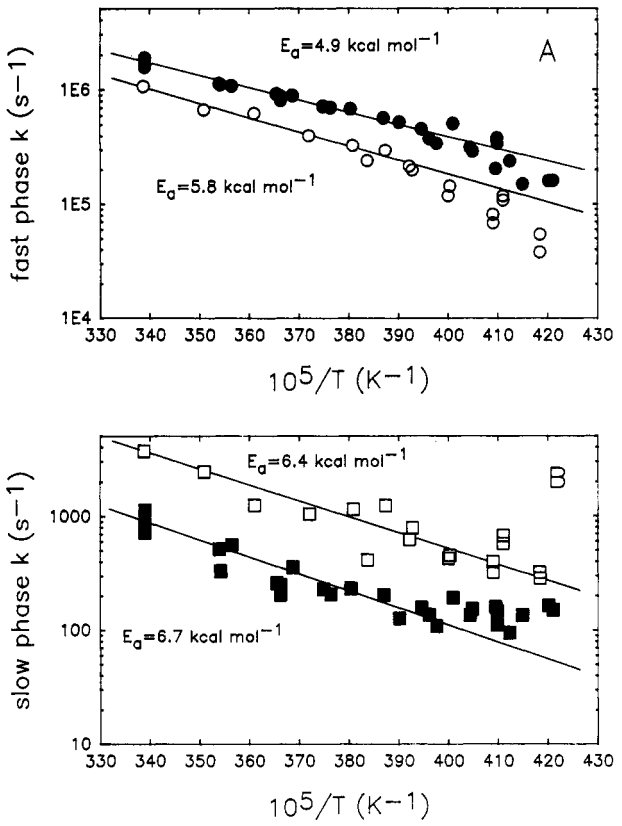

FIGURE 4: Temperature dependence of the rate constant of the fast (A) and slow (B) components of P^+ re-reduction in isolated (closed symbols) and reconstituted (open symbols) reaction centers. Purified reaction centers were dispersed at 4.2 μM in 50 mM glycylglycine (pH 7.8), 0.027% LDAO, 20 μM ubiquinone-6, and 2 mM ascorbate. The concentration of cytochrome c_2 was 21 μM. Proteoliposomes equivalent to 3.2 μM reaction centers were suspended in the same buffer in the presence of 1 mM ascorbate and 18 μM cytochrome c_2. Glycerol was added to the reaction mixture to a final concentration of 60% (v/v).

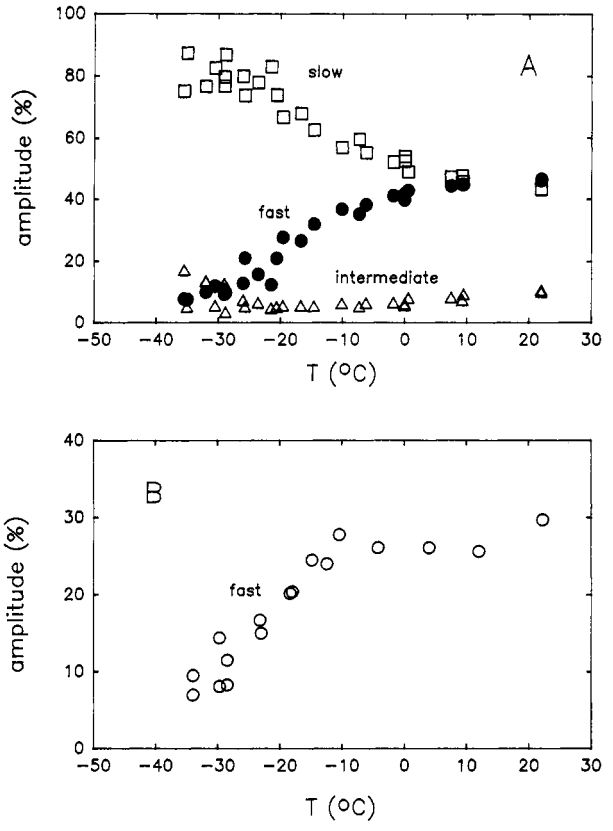

FIGURE 5: Temperature dependence of the amplitudes of the different kinetic components of electron transfer between cytochrome c_2 and P^+ in isolated (A) and reconstituted (B) reaction centers. Kinetic analysis was performed on the same traces used for Figure 4. In isolated reaction centers, P^+ re-reduction was deconvoluted into a fast (●), an intermediate (△), and a slow (□) exponential component. In proteoliposomes, the kinetics of P^+ recovery could be fitted to the sum of two exponentials plus a constant term kept fixed to 50% of the total.

contribution of the first-order fast phase ($t_{1/2} < 1$ μs) observed in aqueous buffer at room temperature was considerably higher (75% of the total), suggesting that the presence of 60% glycerol markedly affects the binding equilibrium between cyt c_2 and the RC. The increased viscosity affects the rate of the slow phase, which at ambient temperature was slowed down by more than 1 order of magnitude in the presence of glycerol. This dramatic effect is consistent with the collisional nature of the second-order reaction which is supposed to be responsible for the slow kinetic component of P^+ re-reduction.

Figure 5A shows how the relative amplitudes of the three kinetic components of P^+ recovery depend on temperature. The amplitude of the fast component, which was essentially constant between 300 and 280 K, decreased progressively at lower temperatures down to 5% of the total at 240 K. The contribution of the slow component increased in parallel from 42% at 300 K to 80% at 240 K.

The intermediate phase exhibited a half-time in the 10–50-μs range. The temperature dependence of the half-time could not be measured to a sufficient accuracy, due to the small relative amplitude of the phase. We suggest that this minor kinetic component is due to the decay of the triplet-state ^3P, which could be formed in the RC with reduced Q_A by charge recombination between P^+ and reduced bacteriopheophytin. In *Rhodobacter sphaeroides* reaction centers, the ^3P state has been shown to have a rather large absorption around 1300 nm (Shuvalov & Parson, 1981). The decay of the ^3P state is characterized by a half-time of 10 μs at 293 K which increases to approximately 30 μs at 240 K (Parson et al., 1975; Shuvalov & Parson, 1981). Within experimental uncertainty, these values are consistent with the kinetics of our intermediate phase. In line with the suggested interpretation, the relative contribution of the minor intermediate phase to P^+ re-reduction was rather variable and increased at low temperatures (from a few percent at 280 K to 15–20% at 230 K). In Figure 6, the kinetics of P^+ recovery observed at 244 K in a dark-adapted sample cooled in the dark (upper trace) are compared with the averaged effect of four flashes given after the first with a spacing of 30 s (lower trace). In this last case, the light-induced absorbance change was much smaller, and 45% decayed exponentially with a half-time of about 15 μs. This finding further supports the proposal that the minor intermediate component detected in dark-adapted samples is due to the decay of the triplet state of P.

The temperature dependence of the kinetics of P^+ re-reduction has been studied under the same conditions in proteoliposome suspensions. Over the 300K–230K temperature range, P^+ recovery could be quite accurately fitted to the sum of two exponential phases plus a constant term. In the fitting procedure, the fraction of P^+ not decaying on the considered time scale was kept fixed and set equal to 50% of the total, on the basis of the random orientation of reincorporated RC (see Figure 2A). At variance with the behavior observed in isolated reaction centers, no kinetic phase related to the formation and decay of the triplet-state ^3P was detected in proteoliposome preparations, even at the lowest temperatures. Consistent with this finding, no major changes were

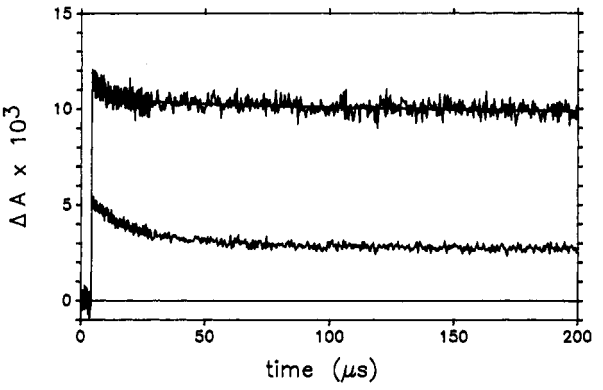

FIGURE 6: Kinetics of P^+ re-reduction following a laser pulse in isolated reaction centers at 244 K. Conditions as in Figure 4. The upper trace is the result of a single flash excitation in a dark-adapted sample cooled to the indicated temperature. Deconvolution into three exponential components (continuous line) yields a fast phase with a half-time of 4.3 μs making up 10% of P^+ recovery. The lower signal shows the averaged effect of four flashes following the first one and fired 30 s apart.

observed in the kinetics of P^+ re-reduction following a second flash, given 30 s after the first, as compared to that recorded in a dark-adapted sample cooled in the dark.

The effects of temperature on the rates of the fast and slow components were similar to those measured in the isolated reaction center dispersions. The rate constant of the fast phase, 1.1×10^6 s^{-1} at 295 K, decreased to approximately 4.6×10^4 s^{-1} at 240 K (Figure 4A). Its relative amplitude was temperature-independent and equal to 30% of the total P^+ (i.e., to 60% of the properly oriented population) down to approximately 260 K (Figure 5B). As already observed in isolated RC, also in proteoliposomes glycerol addition affected the binding equilibrium between cyt c_2 and the RC, resulting in a 20% decrease of the amplitude of the fast phase at room temperature in the presence of 60% glycerol. Under these conditions, the half-time of the slow phase increased by a factor of about 7 when compared to the half-time of the correspondent phase measured in aqueous buffer.

At temperatures below 260 K, the amplitude of the fast phase decreased rather steeply (Figure 5B), making up only 8% of the signal at 230 K. This abrupt decrease in the weight of the fast P^+ re-reduction phase appears therefore to take place approximately over the same temperature range in isolated and in reconstituted reaction centers.

Arrhenius plots of the rate constant for the rapid P^+ re-reduction are shown in Figure 4A. Activation energies equal to 4.9 and 5.8 kcal mol^{-1} can be obtained in the detergent/RC preparations and in the reconstituted vesicles, respectively. These values accurately fit the data between 295 and 250 K. At lower temperatures, a slight deviation correspondent to a high activation energy seems to take place, particularly in the reconstituted vesicles. If significant, this would occur in parallel with the steep decrease observed at low temperatures in the amplitude of the fast P^+ re-reduction.

Both in detergent/RC and in proteoliposome suspensions, the half-time of the slow kinetic component increased by about 1 order of magnitude between 300 and 230 K. Arrhenius plots of the pseudo-first-order rate constant (Figure 4B) yield activation energies of 6.7 and 6.4 kcal mol^{-1} in isolated and reconstituted reaction centers, respectively. In proteoliposomes, the preexponential factor was larger by a factor of about 2. It is evident from the data of Figure 4B that determination of the rate constant of the slow phase was rather inaccurate. These data should be regarded with some caution and more likely as upper limits for the value of the rate constant, particularly at low temperature. Under these conditions, in fact, the slow phase decayed with an apparent half-time of some milliseconds and was not sampled on a time interval long enough to allow a precise deconvolution. It has to be mentioned, however, that this uncertainty has negligible effects on the determination of the half-time and of the relative amplitude of the fast phase.

DISCUSSION

Interaction of Cytochrome c_2 with the Reaction Center. The oxidation of c-type cytochromes by reaction centers has been extensively studied in purified reaction center preparations dissolved in detergent suspensions (Overfield et al., 1979; Overfield & Wraight, 1986; Hall et al., 1987; Moser & Dutton, 1988; Long et al., 1989) and in phospholipid-reconstituted proteoliposomes (Overfield & Wraight, 1980a,b; Moser & Dutton, 1988; Venturoli et al., 1990) by exploiting flash spectrophotometry at different time resolutions. Two basic mechanisms of reaction have been firmly identified: (a) a bimolecular mechanism, controlled by the diffusion of cytochrome in solution or at the membrane interface, prevailing at low cyt c_2 concentrations; and (b) a first-order electron-transfer process, occurring within a cyt c_2–RC complex, dominating at high cyt c_2 concentrations. Our analysis of the cyt c_2 concentration dependence of the reaction kinetics is fully consistent with this notion. In isolated RC dispersions, a fast first-order component and a second-order kinetic component have been shown to account quite accurately for P^+ re-reduction over a wide range of cyt c_2 to RC molar ratios. The half-time of the first-order process was close to 600 ns, a value similar to that obtained by Overfield et al. (1979) and in excellent agreement with a more recent determination (Long et al., 1989). The bimolecular rate constant ($k = 1.4 \times 10^9$ M^{-1} s^{-1}) compares favorably with the results of kinetic measurements in which the oxidation of cyt c_2 was directly monitored in the α (Overfield et al., 1979) and γ bands (Overfield & Wraight, 1980a,b; Moser & Dutton, 1988; Venturoli et al., 1990) at low cyt c_2 to RC stoichiometries. The concentration dependence of the amplitude of the fast phase, which fits a single binding curve, yields a binding constant K equal to 3.2×10^5 M^{-1}. When the ionic strength dependence of K is considered (Overfield & Wraight, 1980a,b; Moser & Dutton, 1988; Venturoli et al., 1990), this is consistent with the binding constant of 1.0×10^6 M^{-1} determined by equilibrium dialysis at approximately the same pH but in 10 mM Tris-HCl (Rosen et al., 1980).

To account for the biphasic kinetics of cyt c_2 oxidation, Overfield et al. (1979) proposed a model which considers two kinds of interchanging RC–cyt c_2 complex. These two states have been attributed to cytochrome bound in favorable ("proximal") and unfavorable ("distal") configurations for electron transfer. This scheme has been used in subsequent studies to model the interaction between c-type cytochromes and the RC (Overfield & Wraight, 1986; Tiede, 1987; Moser & Dutton, 1988). The crucial experimental support to the proposed reaction scheme comes from the kinetic analysis of the reaction at equimolar concentrations of cyt c_2 and RC. Under these conditions, it was found that the half-time of the slow phase of cyt c_2 oxidation obeyed second-order kinetics but was limited to a pseudo-first-order process, characterized by a $t_{1/2} = 200$–400 μs, at about 6 μM concentrations of each reactant. At these concentrations, the kinetics were half fast ($t_{1/2} \cong 1$ μs) and half slow, and not more than 50% fast phase was observed by titrating reaction centers with excess cyt c_2.

In contrast with this observation, we have found that more than 80% of P^+ could be re-reduced in a first-order process ($t_{1/2} \cong 600$ ns) at a cyt c_2 to RC ratio higher than 5. The remaining slow phase, which exhibited true second-order kinetics over the whole range of cyt c_2 concentrations tested, could be accelerated far beyond the apparent pseudo-first-order limit observed by Overfield et al. (1979) at equimolar reactant concentrations and reached a half-time close to 10 μs at 60 μM cyt c_2. Attempts to include a third kinetic phase in the analysis of P^+ re-reduction did not result in meaningful parameters nor did they improve the fit. In summary, the cyt c_2 concentration dependence of P^+ re-reduction did not yield any evidence of a "distal" to "proximal" transition limiting the rate of electron transfer. The interpretation of the behavior observed under our experimental conditions in the frame of the three-state model would imply a cyt c_2 concentration dependence of the transition rate and equilibrium constants between the "distal" and "proximal" states in favor of the "proximal" configuration, a possibility which does not seem likely to us.

Rosen et al. (1979, 1980) also used a description of the cytochrome c_2–reaction center interaction with a single (proximal, fast donating) binding site. It is not clear, however, that their experiments have effectively discriminated against the presence of a distal site. Quite recently, a detailed report appeared on the interaction between soluble cytochromes and the *Rb. sphaeroides* reaction center (Tiede et al., 1993). These authors propose that the occurrence of a distal bound state is rather variable and depends on the physical state of the reaction center. In our own experiments, which involved several reaction center preparations, we found no kinetic evidence for a distal site.

The results obtained in proteoliposome suspensions confirm the simple kinetic pattern observed in isolated RC's when the fraction of reincorporated RC's accessible to the external cyt c_2 is taken into account. In the reconstituted system, the fast first-order phase had a longer half-time (approximately 900 ns) when compared to the corresponding component in the detergent dispersions. Interestingly, an even larger half-time ($\cong 4$ μs) was found for fast electron donation in chromatophores from *Rb. sphaeroides* (Overfield et al., 1979) and from the related species *Rb. capsulatus* (G. Venturoli and P. Mathis, unpublished results). It is worth pointing out that the kinetics of re-reduction are different in cells and in the isolated system used in this work. In cells, the fast phase is still slower than in chromatophores ($t_{1/2} \cong 6$ μs), and it corresponds to only (approximately) 50% of the total decay (Farchaus et al., 1993). These discrepancies will have to be studied in detail.

On the contrary, reinsertion of the RC into phospholipid vesicles leads to an increase of the second-order rate constant by a factor of about 2. This effect, already documented in phosphatidylserine proteoliposomes (Overfield & Wraight, 1980b), is most likely related to the net negative charge of the phospholipid vesicles due to the presence of phosphatidylglycerol. Overfield and Wraight (1980b) have suggested that the collisional oxidation of cyt c_2 by reaction centers incorporated into negatively charged vesicles is coupled to diffusion on the membrane surface. These authors have shown that the restriction to two-dimensional diffusion can give rise to either an acceleration or a retardation of the reaction, depending on the ionic strength of the medium. Our measurements were performed under conditions (50 mM glycylglycine) which maximize the reaction rate in phosphatidylserine vesicles (Overfield & Wraight, 1980b).

A comparison of the kinetics of P^+ re-reduction as measured in aqueous buffer and in 60% glycerol at room temperature indicates significant sensitivity of the collisional oxidation and of the binding of cyt c_2 to glycerol addition. The effects observed both in the isolated and in the reconstituted system are quantitatively consistent with the dependence of the second-order rate and of the equilibrium constant on glycerol concentration as determined by Moser and Dutton (1988).

Temperature Dependence of P^+ Re-reduction. In whole cells of *Rb. sphaeroides*, cyt *c* oxidation induced by continuous illumination was reported to stop at temperatures between −50 and −170 °C (Vredenberg & Duysens, 1964). In the present work, we have provided an account of the effect of temperature on P^+ re-reduction kinetics by purified cyt c_2 between 300 K and 230 K, a temperature at which fast intracomplex electron donation was found to vanish.

Deconvolution of P^+ re-reduction kinetics into a fast (microsecond) and a slow exponential component proved to be correct over the examined temperature range. The half-times of the two phases differed by more than 2 orders of magnitude, since in the temperature dependence measurements the increased viscosity of the medium due to 60% glycerol slowed down the collisional reaction without affecting the rate of the first-order process as compared to the kinetics observed in aqueous buffer. In the detergent/reaction center dispersions, a third intermediate exponential component had to be introduced to account accurately for the kinetics at low temperature. This phase, which marginally contributes to P^+ recovery, is proposed to reflect formation of the triplet-state ^3P in a small fraction of reaction centers. No intermediate kinetic component of P^+ re-reduction was detected in the proteoliposome suspensions even at the lowest temperatures, suggesting that reinsertion of the reaction center into vesicles can prevent ^3P formation, presumably by a more effective interaction of the large ubiquinone pool dissolved in the lipid phase with the quinone acceptor complex of the RC.

The results of the kinetic analysis clearly indicate a large effect of temperature on the rate of the two kinetic components of P^+ reduction by cyt c_2. The activation energies of the rapid first-order reaction were 4.9 and 5.8 kcal mol^{-1} in isolated and reconstituted reaction centers, respectively. These values compare with an activation energy of 3.3 kcal mol^{-1} for the high-temperature region of cyt *c* oxidation in *Chromatium* (De Vault & Chance, 1966). Lower activation energies, lying between 2.1 and 1.6 kcal mol^{-1}, have been recently measured over the same temperature range for electron transfer from the highest potential heme to P^+ in *Rps. viridis* RC (Ortega & Mathis, 1992, 1993). It is tempting to relate the trend which emerges from these values to the structural properties of the different cyt *c*–RC complexes operating in these species. The activation energy obtained for the oxidation of the docked soluble cyt c_2 in *Rb. sphaeroides* appears in fact to be 3 times higher than that of the correspondent reaction in *Rps. viridis*, where cyt *c* is an integral part of the RC. An intermediate activation energy is found in *Chromatium vinosum*, where the tetraheme cytochrome is much less firmly bound to the RC than in *Rps. viridis* (Romijn & Amesz, 1977; A. Vermeglio, personal communication). In the related species *Chromatium tepidun*, the cytochrome subunit is also not very tightly bound, as shown by Nozawa et al. (1987).

Arrhenius plots of the rate of slow P^+ reduction yield an activation energy close to 6.5 kcal mol^{-1} both in isolated and in reconstituted *Rb. sphaeroides* RC's. A similar value was obtained in proteoliposomes when the second-order oxidation of cyt c_2 was directly monitored at low reactant concentrations

between 0 and 30 °C in the absence of glycerol (G. Venturoli, unpublished observation). Overfield and Wraight (1980a) have determined a similar (20% higher) activation energy for the slow phase of cyt c_2 oxidation by reaction centers either in solution or incorporated into vesicles of various phosphatidylcholines.

The more prominent feature of the temperature dependence of P^+ rereduction was that the amplitude of the fast kinetic component abruptly decreased below 260 K, essentially vanishing at 230 K. The decrease is common to RC in solution and in the lipid matrix and displays the genuine character of a phase transition. Indeed, in both systems it occurs over the same narrow temperature range without a significant change in the activation energy of the residual fast reaction. A structural change might in principle be advocated to explain the sudden disruption of the electron-transfer pathway between cyt c_2 and the pigment. In order to induce a decrease in the rate of electron donation by more than 3 orders of magnitude, freezing would have to switch the geometry of the cyt c_2–RC complex to a conformation highly unfavorable for electron transfer or even lead to dissociation of the complex. This last possibility does not seem likely when considering that a similar behavior has been recently observed at low temperatures in the reaction between tetraheme cyt *c* and the primary donor of *Rps. viridis*, where the cytochrome is firmly bound to the RC (Ortega & Mathis, 1992, 1993). In RC isolated from this last organism, the extent of fast P^+ reduction has been shown to decrease sharply at temperatures which vary according to the redox state of the hemes. Quite interestingly, when the three lowest potential hemes are oxidized, the inability of the heme closest to the pigment (*c*-559) to reduce P^+ occurs over the same temperature range which stops electron transfer between cyt c_2 and the special pair in *Rb. sphaeroides* RC.

Mechanisms alternative to structural changes of the complex and leading to a dramatic impairment in electron transfer at low temperature have been discussed extensively by Ortega and Mathis (1993). A reasonable possibility, which could equally account for the observation reported in the present paper, is that freezing prevents medium reorganization which partly compensates for the charge displacement coupled to electron transfer. As already proposed by Kaminskaya et al. (1990) for bound cyt *c* in *Rps. viridis*, rearrangement of the redox local environment mainly associated with cytochrome c_2 and involving the protein medium as well as bound water molecules could be hindered at low temperature, inducing a substantial increase of the effective redox potential of cyt c_2. Structural differences between reduced and oxidized cytochrome *c* have been studied in great detail. Recent studies [e.g., see Berghuis and Brayer (1992) and Schlereth and Mantele (1993)] conclude substantial differences. Most prominently, an internal water molecule moves by 1.7 Å in iso-1-cytochrome *c*, leading to several changes in H-bonding (Berghuis & Brayer, 1992). In that case, it was also concluded that the water movement is accompanied, upon oxidation of the heme, by several concerted adjustments of the surrounding protein. As found in other systems by neutron diffraction (Savage, 1986), a network of water molecules could be present, not perfectly ordered; its structure might experience phase transitions at low temperature.

An estimate of the reorganization energy associated with the fast electron-transfer process within the cyt c_2–RC complex can be attempted on the basis of the activation parameters of the reaction. Eyring analysis of the temperature dependence measured in isolated reaction center dispersions (Figure 4A) yields $\Delta H^* = 19.9$ kJ mol^{-1} and $\Delta S^* = -58.0$ J K^{-1} mol^{-1}. The entropy of activation (ΔS^*) includes a contribution from the transmission coefficient k, which in turns depends on the distance r separating the electron donor and acceptor (Marcus & Sutin, 1985):

$$\Delta S^* = \Delta S^* + R \ln k(r) \quad (3)$$

In the simplest view, the transmission coefficient can be expressed as

$$k(r) = \exp[-\beta(r - r_0)] \quad (4)$$

where r_0 is the "adiabatic" separation distance and β is a decay coefficient of electronic coupling. Using $\beta = 12$ nm^{-1} (Marcus & Sutin, 1985) and an edge-to-edge distance ($r - r_0$) of 1.1 nm (Allen et al., 1987b), we obtain $\Delta S^* = 51.8$ J K^{-1} mol^{-1}, which results in $\Delta G^* = \Delta H^* - T\Delta S^* = 4.4$ kJ mol^{-1} at 298 K. The reorganization energy can now be evaluated from the Marcus relation (Marcus & Sutin, 1985):

$$\Delta G^* = \frac{\lambda}{4}\left(1 + \frac{\Delta G^\circ}{\lambda}\right)^2 \quad (5)$$

provided that the free energy difference ΔG° is known. An approximate value for ΔG° can be obtained from the redox midpoint potentials of cyt c_2 and of the primary donor measured in chromatophores [$E_m(P/P^+) = 445$ mV (Dutton & Jackson, 1972; Jackson et al., 1973); $E_m(c^{ox}/c^{red}) = 345$ mV (Dutton & Jackson, 1972; Bowyer et al., 1981; Meinhardt & Crofts, 1982)] and taking into account the electrostatic interaction of the redox species with the reduced primary acceptor Q_A^- formed upon flash excitation. In *Rps. viridis*, Q_A^- has been calculated by Gao et al. (1990) to perturb the equilibrium between P^+/P and $c_{559}{}^{ox}/c_{559}{}^{red}$ by 55 mV. Assuming this value as a reasonable estimate of the correspondent electrostatic interaction within the cyt c_2–RC complex of *Rb. sphaeroides*, from eq 5 a reorganization energy $\lambda \simeq 25$ kJ mol^{-1} is obtained. In spite of the large number of assumptions and approximations, this value, obtained from the experimentally determined activation parameters of the reaction, compares favorably with an upper limit of 27 kJ mol^{-1} calculated by Churg et al. (1983) for the reorganization energy associated with electron exchange between two cytochromes *c* immobilized with respect to translational motion.

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