Cross-Linked Electron Transfer Complex between Cytochrome c_2 and the Photosynthetic Reaction Center of *Rhodobacter sphaeroides*†

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ABSTRACT: Electron donation from the soluble cytochrome (cyt) c_2 to the photooxidized primary donor, P^+, of reaction centers isolated from *Rhodobacter sphaeroides* was studied by using chemical zero-length cross-linking. This cross-linking stabilizes a 1:1 covalent complex between subunit M of the reaction center and cyt c_2. In 80% of the reaction centers, P^+ generated by a laser flash is reduced by covalently bound cyt c_2. Kinetics of P^+ reduction show (i) a fast phase with a half-life of 0.7 μs similar to that observed for electron transfer in the noncovalent proximal complex and (ii) a slow phase ($t_{1/2} = 60$ μs) that is attributed to a cyt c_2 bound less favorably for electron transfer. Its relationship with similar kinetic phases attributed to a distal conformation of the complex in previous studies is discussed. Both kinetic phases are slightly accelerated upon addition of glycerol. Upon addition of reduced soluble cyt c_2 to the cross-linked complex the kinetics of both phases are not affected. The kinetics of P^+ reduction following the second flash (20 ms after the first) show that a complex is formed between soluble cyt c_2 and the cross-linked complex, in which electron transfer takes place in the millisecond time domain. Cross-linked cyt c_2 in complexes which give rise to the two kinetic phases of P^+ reduction shows almost pH-independent midpoint redox potentials between pH 6 and 9.5. This behavior is at variance with that of free cyt c_2, the midpoint potential of which is affected by at least two protonable groups within this pH range. The cross-linked RC−cyt c_2 complex allowed study of the effects of temperature on the electron transfer reaction without a possible disturbance by dissociation of the complex. In the 250−300 K range, Arrhenius behavior is observed showing activation energies of 11.7 and 8.0 kJ/mol for the faster and the slower kinetic phases, respectively, which are remarkably lower than the activation energy of 20.5 kJ/mol for the fast P^+ reduction by soluble cyt c_2 [Venturoli, G., Mallardi, A., & Mathis, P. (1993) *Biochemistry 32*, 13245−13253]. Between 250 and 230 K, a fall-off in amplitude is observed for both kinetic phases indicating that intracomplex electron transfer is blocked at low temperatures.

Many *intraprotein* electron transfer reactions have been interpreted in terms of three main microscopic factors determining their rate and direction: the donor−acceptor electronic coupling, the free energy gap ($\Delta G°$), and the medium reorganization energy coupled to the electron transfer (Moser *et al.*, 1992). The knowledge of the atomic structure is of special importance for a determination of the electronic coupling. It has been quite successfully approximated by semiempirical approaches: by assuming a simple exponential distance dependence (Moser *et al.*, 1992) or by calculating dominant pathways for electron transfer (Onuchic *et al.*, 1992). Currently, more exact quantum mechanical formulations are developed that may provide a better insight and solve controversies [reviewed in Friesner (1994) and Moser *et al.* (1995)].

A common understanding is rendered more difficult for *interprotein* electron transfers (McLendon, & Hake, 1992; Moser *et al.*, 1995; Curry *et al.*, 1995; Aquino *et al.*, 1995), i.e., when only the formation of a temporary complex positions the cofactors appropriately for electron transfer. In this case, the final distance and the electronic coupling between them as well as their redox potentials that determine the driving force for the electron transfer reaction ($\Delta G°$) depend on the structural details of the docked complex.

An attractive model to study electron transfer between a small soluble protein and a multiprotein complex is offered by photosynthetic reaction centers from purple bacteria. The RC[1] is a membrane integral pigment−protein complex consisting of the three polypeptide subunits L, M, and H (Feher & Okamura, 1978). Upon excitation of its primary donor (P) by light, an electron is transferred through an acceptor chain to a first quinone acceptor (Q_A). The primary charge-separated state $P^+Q_A^-$ is stabilized on the periplasmic side by re-reduction of P^+ by secondary electron donors and on the cytoplasmic side by electron transfer to the secondary quinone molecule (Q_B). In *Rhodopseudomonas* (*Rps*) *viridis*, the RC has a bound tetraheme subunit as the secondary electron donor, whereas in the RC of *Rhodobacter* (*Rb.*) *sphaeroides*, P^+ is re-reduced by a soluble monoheme cytochrome (cyt) c_2 [for reviews see Tiede and Dutton (1993) and Mathis (1994)]. In this work, we have studied in detail the complex formation and subsequent electron transfer between cyt c_2 and the RC from *Rb. sphaeroides*.

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[1] Abbreviations: cyt, cytochrome; RC, reaction center(s); BChl, bacteriochlorophyll; LDAO, lauryldimethylamine oxide; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; NHS, *N*-hydroxysulfosuccinimide; P, reaction center primary donor; *Rb.*, *Rhodobacter*; *Rps.*, *Rhodopseudomonas*; E_m, redox midpoint potential; ΔA, absorption change.

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The kinetics of electron transfer from cyt c_2 to the RC from *Rb. sphaeroides* have been studied in intact cells (Dutton *et al.*, 1975; Overfield *et al.*, 1979; Joliot *et al.*, 1989; Sabaty *et al.*, 1994), in chromatophore preparations (Bowyer *et al.*, 1979; Overfield *et al.*, 1979), and using purified RC (Prince *et al.*, 1974; Overfield & Wraight, 1980a,b; Moser & Dutton, 1988; Tiede *et al.*, 1993; Wachtveitl *et al.*, 1993; Venturoli *et al.*, 1993; Wang *et al.*, 1994). In many cases, the P^+ reduction by reduced cyt c_2 following excitation by a short flash shows a biphasic time course. A fast phase ($t_{1/2} \approx 1-3$ μs) was attributed to an electron donation within an RC−cyt c_2 complex in a proximal conformation (Overfield *et al.*, 1979; Overfield & Wraight, 1980a,b; Moser & Dutton, 1988). The interpretation of the slow kinetic component is more controversial. Whereas in some studies, a concentration dependent half-life attributed to a reaction limited by the binding of soluble cyt c_2 to the RC was sufficient to describe the kinetics (Tiede *et al.*, 1993; Venturoli *et al.*, 1993), in several other studies, a first-order limiting half-life of 55−75 μs (Long *et al.*, 1989; Tiede *et al.*, 1993; Wachtveitl *et al.*, 1993) or 350−400 μs (Overfield *et al.*, 1979; Moser & Dutton, 1988) at high reactant concentrations was attributed to the reorientation of cyt c_2 bound in a "distal" state into the "proximal" complex. Biphasic kinetics for cytochrome oxidation *in vivo* have been attributed to a cyt c_2:RC stoichiometry <1 suggesting that the slower kinetic phase ($t_{1/2} = 100$ μs) was rate-limited by the re-reduction of cyt c_2 by the cyt bc_1 complex (Joliot *et al.*, 1989; Sabaty *et al.*, 1994).

The binding of cyt c_2 to the RC involves electrostatic interaction between lysine residues surrounding the heme crevice of the cytochrome and negatively charged carboxylate groups on the periplasmic surface of the RC (Hall *et al.*, 1987; Long *et al.*, 1989). However, the precise binding site and the orientation of cyt c_2 within the temporarily formed complex is controversial. Cyt c_2 has been cross-linked to the L subunit of the RC using a 10 Å cross-linker (Rosen *et al.*, 1983). In two structural models based on the maximization of the number of charge pairs, cyt c_2 was positioned either nearly symmetrically over the primary donor (Allen *et al.*, 1987) or shifted toward the M side (Tiede & Chang, 1988). A recent structural model of the RC−cyt c_2 complex derived from X-ray diffraction of co-crystals and electrostatic modeling shows the cyt c_2 docked on the M subunit (Adir *et al.*, 1994, 1996). These models differ in the resulting distance between the heme and P as well as the specific amino acids that might play a role in the electron transfer pathway (Wachtveitl *et al.*, 1993; Aquino *et al.*, 1995; Adir *et al.*, 1996).

The effects of temperature (Venturoli *et al.*, 1993) and of altered driving force (Lin *et al.*, 1994) on the rate of P^+ reduction by bound cyt c_2 have been used to analyze this reaction by means of current electron transfer theories. However, both approaches rely on estimates for several parameters, including the ΔG° under working conditions, that have to be known for a quantitative analysis. For the case of mitochondrial cyt *c*, several studies have determined the redox potential of the cyt when bound to its reaction partners. A decrease in the redox midpoint potential of cyt *c* upon binding to lipid vesicles and mitochondria (Dutton *et al.*, 1970), purified respiratory (Vanderkooi & Erecinska, 1974; Speck & Margoliash, 1984) and photosynthetic electron transport complexes (Moser & Dutton, 1988) has been observed, the magnitude of which was consistent with a five to seven times tighter binding of the oxidized form [see also Moore and Pettigrew (1990, pp 312−315)]. However, a contribution of such an effect to the efficiency of electron transfer via cyt *c* has been questioned, since both its reductase and its oxidase were found to bind preferentially the ferricytochrome (Moore & Pettigrew, 1990, p 315f). Furthermore, in most of the previous studies, the existence of multiple binding sites precluded a distinction between the effect of nonspecific binding and active site binding on the midpoint potential of cyt *c*. Whereas the binding to RC phospholipid vesicles has been found to lower the midpoint potential of horse cyt *c* (Moser & Dutton, 1988), the effect of binding on the natural reaction partner cyt c_2 has not been studied so far.

Here, we report the use of a zero-length cross-linking procedure to produce a functional complex between the RC and cyt c_2 by introducing one or more covalent bonds between complementary charged amino acids located at the actual site(s) of protein−protein interaction. This provides a system for studying the intracomplex electron transfer reaction. The results help to understand the controversial interpretations for the more complex bimolecular kinetics of the formation and dissociation of the non-covalent complex. The availability of the cross-linked RC−cyt c_2 complex offers unique opportunities to determine specifically the oxidation−reduction potential of cyt c_2 bound to the active site and to study the effect of temperature on electron transfer. A preliminary account of this work has been presented (Drepper *et al.*, 1995). These RC−cyt c_2 complexes were also used for an in-depth structural study that is presented in the accompanying article (Drepper & Mathis, 1997).

EXPERIMENTAL PROCEDURES

Chemicals. Lauryldimethylamine oxide (LDAO), *N*-hydroxysulfosuccinimide (NHS), and 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) were purchased from Franconix, Fluka, and Pierce Chemical Co., respectively. Other chemicals were purchased from Sigma.

Protein Purification. Reaction centers were isolated from *Rb. sphaeroides* 2.4.1 chromatophores according to Wachtveitl *et al.* (1993) using two successive extractions with 0.25% (w/v) LDAO. The second extract containing the solubilized RC was purified by using one DEAE column (DE-52, Whatman). RC concentration was determined using an extinction coefficient of 288 mM^{-1} cm^{-1} at 803 nm (Straley *et al.*, 1973). The purity was estimated from the absorbance ratio A_{280}/A_{803} that was between 1.4 and 1.7. The buffer was exchanged to 10 mM HEPES, 0.025% LDAO using Centricon 100 ultrafiltrators (Amicon). Cyt c_2 was isolated from a strain of *Rb. sphaeroides* overexpressing cyt c_2 (kindly provided by Dr. P. Richaud, Cadarache) as described (Meyer & Cusanovich, 1985) except that the first two columns were omitted. An extinction coefficient of 148 mM^{-1} cm^{-1} at 417 nm (Meyer & Cusanovich, 1985) was used for the reduced form. The final absorbance ratio A_{275}(no addition)/A_{417}(reduced) was always below 0.25.

Chemical Cross-Linking. The cross-linking protocol was optimized by trial and error in order to achieve a high yield of 1:1 RC−cyt c_2 complexes. The reaction mixture contained 6 μM RC, 20 μM cyt c_2, 10 mM HEPES, pH 7.5,

0.025% LDAO, 1 mM ascorbate, and 60 μM 1,4-naphthoquinone. The incubation was started by addition of NHS (adjusted to pH 7.5) and EDC to final concentrations of 12 and 6 mM, respectively. After 3 h in the dark at 22 °C, the reaction was stopped by the addition of 50 mM ammonium acetate. After 10 min the mixture was diluted 10-fold with 10 mM HEPES, pH 7.5, and 0.025% LDAO and concentrated using Centricon 100 filters (Amicon). The washing procedure was repeated twice. The filtrate contained the excess soluble cyt c_2. It was purified separately and used as a control, e.g., in redox titrations, in order to check for possible effects on the properties of cyt c_2 due to the incubation with EDC and NHS. For a control, the same procedure was used for RC without cyt c_2.

Gel Electrophoresis. The buffer system of Laemmli (1970) with a 10% polyacrylamide gel and a minigel apparatus (Bio-Rad) were used. For protein staining, gels were incubated for 30 min in solutions of Coomassie Blue. Specific staining of heme groups covalently bound to the protein using 3,3′,5,5′-tetramethylbenzidine was carried out according to Goodhew *et al.* (1986).

Flash Absorption Spectroscopy. Kinetics of flash-induced absorbance changes were measured essentially as described by Venturoli *et al.* (1993). Flash excitation with a 10 ns pulse at 595 nm was provided by a dye laser pumped by a frequency-doubled Nd:YAG laser. Kinetics of electron transfer were monitored at 1283 nm in the absorption band of P^+ using a Nicolet transient recorder with a dwell time of 100 ns. In double flash experiments, two identical ruby lasers (pulse width 20 ns, 694 nm) were used for excitation and the amplified signals were measured with a Tektronix (RTD) transient recorder. The 2048 channels of the data memory were partitioned into segments with different sampling rates in order to resolve the kinetics in the microsecond and millisecond time range induced by each of the two flashes. Unless otherwise specified, the kinetic transients are the results of single flash measurements without averaging. The absorption transients were fitted to a sum of exponentials using a modified Marquardt algorithm installed by Dr. P. Sétif.

For low-temperature measurements the cuvette was inserted into a cryostat cooled with nitrogen gas. The effect of a single flash was measured at a given temperature. At temperatures below 273 K, the sample was dark-adapted at room temperature and cooled in darkness.

Redox Potentiometry. Midpoint potentials of isolated cyt c_2 were determined using the method of mixtures as described by Pettigrew *et al.* (1975). Spectra of the α-band region of cyt c_2 (5 μM) were recorded on a Cary 5E spectrophotometer in the presence of potassium ferricyanide, a mixture of potassium ferri- and ferrocyanide, and after complete reduction by sodium dithionite. The following buffers (10 mM) were used: acetic acid/sodium acetate (pH 5.2), MES (pH 6.0 and 6.5), HEPES (pH 7 and 7.5), Tris/HCl (pH 8 and 9), or glycine/NaOH (pH 9 and 9.5). Ferri- and ferrocyanide were added to give final concentrations between 0.17 and 0.5 mM resulting in a total ionic strength between 0.010 and 0.012 M. The E_m of the cyt c_2 was calculated from the fractions of reduced and oxidized cyt in the presence of ferri- and ferrocyanide using the known E_0' for the latter couple and taking into account its ionic strength dependence (O'Reilly, 1973; Hanania *et al.*, 1967).

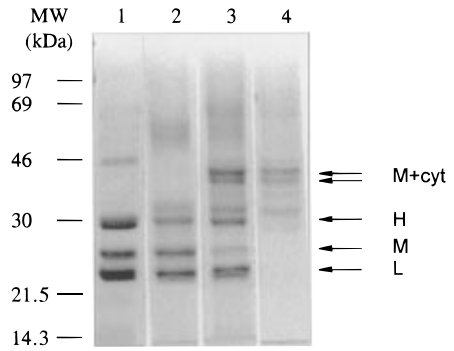

FIGURE 1: 10% SDS−polyacrylamide gel electrophoresis of RC (lane 1), RC after incubation with EDC and NHS in the absence (lane 2) and in the presence of cyt c_2 (lanes 3 and 4). Proteins were stained by Coomassie Blue (lane 1−3) or by heme staining with 3,3′,5,5′-tetramethylbenzidine (lane 4). The left scale gives the positions of molecular weight standards.

In flash titrations, the ambient redox potential was measured with a platinum electrode *vs* a calomel (saturated KCl) reference electrode in an argon purged cuvette. Reaction centers (2 μM) were suspended in 50 mM buffer and 0.025% LDAO; for pH 6.5, the LDAO was replaced by 0.1% sodium cholate. The ambient potential was changed by addition of ferricyanide and ascorbate. The following redox mediators were used: 50 μM 2,4-naphthoquinone, 100 μM TMPD, 50 μM dihydroxybenzoquinone, 10 μM as initial concentrations of each potassium ferri- and ferrocyanide. The temperature in all experiments was 25 °C. The reference electrode was calibrated *vs* SHE using saturated solutions of quinhydrone in 0.1 N HCl, 0.9 M KCl (pH 2.098), or 50 mM MOPS, pH 7.0. Values of 575 mV at pH 2.098 and 285 mV at pH 7.0 for the ambient potential of the quinhydrone solutions were used assuming $E_0 = 700$ mV at 25 °C (O'Reilly, 1973).

RESULTS

Subunit Composition of Covalent Complexes. The cross-linking products were characterized by SDS−gel electrophoresis (Figure 1). The electrophoretic profile of the isolated RC (lane 1) displays the three bands attributed to the L, M, and H subunits of the RC [see, e.g., Feher and Okamura (1978)]. Incubation of the RC with EDC and NHS leads to a very similar electrophoretic pattern (lane 2). A weak band at an apparent MW of 32 kDa may originate from a modification of the H-subunit. After incubation of the RC with EDC and NHS in the presence of cyt c_2 (lane 3) two major effects can be observed. First, the band at 25 kDa attributed to the M subunit almost disappeared, suggesting that subunit M is involved in the formation of cross-linking products. Second, a new doublet of bands appeared at apparent molecular masses of 40 and 42 kDa. These bands are also visible in the heme stain (lane 4) indicating the presence of cytochrome in these cross-linking products. From a quantitative analysis of the electrophoretic profiles we estimated that the remainder of the intensity at 25 kDa for subunit M after cross-linking was about 15% of that before cross-linking. The two new bands at 40 and 42 kDa were found to be approximately equal in intensity. A minor new band is observed at 37 kDa having an intensity of about 5% of that of the sum of the bands at 40 and 42 kDa. This band may correspond to a small amount of subunit L cross-linked to cyt c_2. Consistently, a stoichiometry of 0.8−0.9

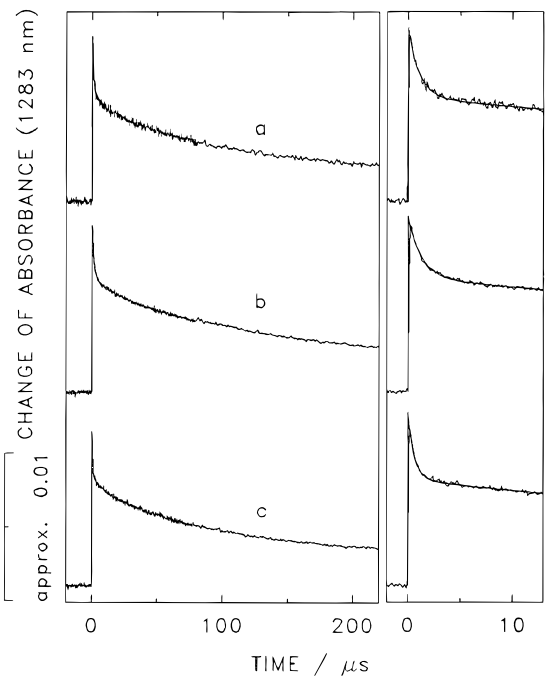

FIGURE 2: Flash-induced absorbance changes at 1283 nm of RC with cross-linked cyt c_2. Trace a: 2 μM RC in 10 mM HEPES pH 7.5, 0.025% LDAO, 100 μM TMPD, 60 μM 1,4-naphthoquinone, and 1 mM sodium ascorbate. Trace b: addition of 0.5 M NaCl. Trace c: 60% glycerol (v/v) in the buffer. On the right side the fast absorption changes are shown on an expanded time scale. P^+ reduction kinetics were fitted to a sum of two exponentials plus a constant term (solid lines).

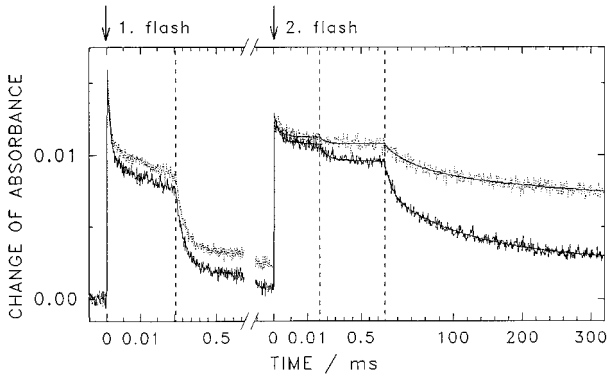

FIGURE 3: Absorbance changes at 1283 nm of RC with cross-linked cyt c_2 induced by two laser flashes spaced at 20 ms. 3 μM RC were suspended in 10 mM HEPES, pH 7.5, 0.025% LDAO, 60 μM 1,4-naphthoquinone, and 0.35 mM sodium ascorbate without (dotted traces) or with (solid traces) additional 80 μM reduced cyt c_2. Small variations in the signal amplitudes are corrected by normalization to equal initial amplitudes after the first flash. Absorbance transients are the average of two individual signals recorded with a dark interval of 5 min. A DC-coupled amplifier was used. The vertical dashed lines separate intervals recorded with different sampling rates: 50 ns and 2 μs/point (first flash) and 50 ns, 8 μs and 0.8 ms/point (second flash). Results of curve fitting of a sum of five exponentials to the absorbance transients induced by the second flash are shown by solid lines (see text for more explanation).

cyt c_2 per RC was determined from optical absorption spectra of the purified cross-linked complexes (not shown) indicating that a small fraction of RC have no cross-linked cyt c_2. Furthermore, the position of the cross-linking products in the gel at 40 and 42 kDa suggests the presence of 1 cyt c_2 (14 kDa) bound to subunit M. The small difference in the apparent molecular weight between these two bands may be due to a heterogeneity in the number or the position of covalent bonds leading to different mobilities in the gel. In summary we may conclude that about 85% of the RC were cross-linked with one molecule of cyt c_2 per RC attached to subunit M. The apparently heterogenous cross-linking products may indicate two different positions being almost equally occupied.

Kinetics of Electron Transfer. The function of the RC−cyt complexes was investigated by measuring the flash-induced kinetics of P^+ reduction in conditions where cyt c_2 was reduced before flash excitation. We observed two major kinetic phases (Figure 2, trace a) with half-lives of 0.7 μs ($\pm$0.1 μs) and 60 μs ($\pm$10 μs) under standard conditions, each of them accounting for about 40% of total photooxidized P. About 20% of total P^+ decayed in the millisecond domain; it was attributed to RC devoid of cross-linked cyt c_2 undergoing back-reaction from acceptor side quinones to P^+. The kinetics of these slow components were not correctly resolved in the traces shown in Figure 2 due to high-pass filtering of the amplifier AC coupling. Figure 2 (trace b) shows the P^+ reduction in the presence of 0.5 M NaCl. Both kinetic phases are almost identical to those without added NaCl (Figure 2, trace a). By contrast, the reaction between the RC and soluble cyt c_2 under these conditions showed no detectable contribution of the 0.7 μs phase and a bimolecular reaction slowed down by almost 2 orders of magnitude [data not shown, in agreement with well-established results; see, e.g., Overfield and Wraight (1980a) and Wachtveitl *et al.* (1993)]. In the presence of 60% glycerol, the half-lives of the two kinetic phases of P^+ reduction are slightly decreased to 0.4 and 40 μs whereas their amplitudes are not modified (Figure 2, trace c). Since both phases are even slightly accelerated at increased viscosity, their rates cannot be limited by a diffusional reorientation but should be attributed to an electron transfer within covalently stabilized complexes.

Accessibility to Soluble cyt c_2. In order to investigate the accessibility of cross-linked RC−cyt c_2 complexes to soluble cyt c_2, we measured the kinetics of P^+ re-reduction following one or two turnovers of the RC in the absence and in the presence of reduced soluble cyt c_2. The first question that can be answered by this kind of experiment is whether soluble cyt c_2 can change the kinetic behavior of RC displaying the 60 μs phase, e.g., convert it into the fast electron transfer associated with the proximally bound state. On the left side of Figure 3, the kinetics of P^+ re-reduction induced by the first laser flash are shown for RC with cross-linked cyt c_2 in the absence (dotted trace) and in the presence (solid trace) of 80 μM soluble cyt c_2, a concentration at which at least 80% of RC display a 0.7 μs phase when they are not cross-linked. Upon addition of soluble cyt c_2, the amplitudes of both the 0.7 μs and the 60 μs phase slightly increased by about 6% and 2%, respectively, compared to the control. The half-life of the intermediate phase was not changed significantly by the addition of soluble cyt c_2. However, the very small increase in the amplitude of this phase prevented an exact determination of the kinetics and concentration dependence for the additional signal.

The increased amplitude of the two kinetic phases is attributed to a reaction of soluble cyt c_2 with RC that have no cross-linked cyt. These results can be rationalized assuming that most of the RC that have no cross-linked cyt, but are still accessible to soluble cyt c_2, are able to produce

the proximal complex at high concentrations of soluble cyt c_2. There is no conversion of the 60 μs phase of cross-linked RC−cyt c_2 complexes into faster kinetics, even at the highest concentration of soluble cyt c_2 tested in our experiments, i.e., 260 μM.

As a control we measured the P^+ reduction kinetics (data not shown) in RC that were incubated with EDC and NHS in the absence of cyt. We obtained the following results: in the absence of soluble cyt c_2, a stable charge separation was observed and P^+ decayed in the 100 ms time domain attributable to the back reaction from acceptor side quinones (see also below). Upon successive addition of soluble cyt c_2, for 75−80% of the RC the P^+ reduction kinetics were similar to those of untreated RC. The bimolecular reaction was slightly slowed down (by a factor of 1.5−2) and the dissociation constant for the proximal complex was increased by the same factor compared to the kinetics of untreated RC. The half-life for the fast phase was the same as in untreated RC. About 20% of the RC showed a bimolecular reaction of P^+ reduction by cyt c_2 slowed down about 10-fold compared to control RC. The fraction of RC that were not accessible to cyt c_2 was almost not altered compared to control RC (<3%).

The P^+ reduction kinetics following a second laser flash were measured in order to check the accessibility of cross-linked ferricyt c_2 generated by the first flash and of P^+ generated by the second flash to be reduced by soluble cyt c_2. Figure 3 (right side) shows absorbance transients induced by a second flash given 20 ms after the first flash, measured in the absence (dotted trace) or in the presence of 80 μM soluble cyt c_2 (solid trace). Under these conditions, the initial absorbance change induced by the second flash indicates the generation of P^+ to a level of 75−80% of that observed at the first flash. The decrease in amplitude at the second flash can be attributed to a fraction of RC where Q_A was not reoxidized between the two flashes. Since the interval between the first and the second flash of 20 ms was long compared to the time for electron transfer from Q_A^- to Q_B, typically 100 μs, apparently 20−25% of RC have no functional Q_B site quinone. In the absence of soluble cyt c_2 (Figure 3 right, dotted trace), the P^+ reduction following the second flash shows small contributions from the fast and the intermediate phase with relative amplitudes of about 10% and 5% of total P^+ generated in the second flash, respectively, indicating that most of the cross-linked cyt c_2 was still oxidized. The remaining P^+ decayed with half-lives of 50−100 ms (∼15%) or 1−1.5 s (∼70%, not resolved in Figure 3) consistent with RC undergoing back-reaction between P^+ and Q_A^- or Q_B^-, respectively.

Upon addition of 80 μM soluble cyt c_2 (Figure 3 right, solid trace), only a small increase in amplitude is observable for the fast and the intermediate phases following the second flash. The concentration dependence for the amplitude increments of these two kinetic phases (not shown) confirms that the effect of soluble cyt c_2 resembles the behavior observed in the first flash experiment. These effects are consistent with about 10% of RC that have no cross-linked cyt c_2 and retained their ability to interact with soluble c_2. For these centers, the re-formation of the functional complex with reduced cyt c_2 after the first turnover is expected to be complete in less than 1 ms, taking as an estimate the upper limit determined for the analogous reaction between cyt c_2 and the RC from *Rb. capsulatus* (Venturoli *et al.*, 1990).

The millisecond kinetics following the second flash are strongly affected by the presence of soluble cyt c_2 (Figure 3 right, solid trace). Curve fitting analysis of the P^+ reduction kinetics measured in the presence of soluble cyt c_2 yields the following results. In addition to the fast ($t_{1/2}$ = 0.7−1 μs) and the intermediate kinetic phases ($t_{1/2}$ = 50−60 μs), in general, three slower phases were needed for the fit of the signals following the second flash for all concentrations of cyt c_2 tested. A "6 ms phase" with a constant half-life (5.5−8.0 ms), a variable ms phase, and a phase with a half-life of 1.5 s. The 6 ms phase increased in amplitude with increasing concentrations of cyt c_2 up to 25−30% of P^+ generated in the second flash (not shown). As to the variable phase, its relative amplitude increased with increasing cyt c_2 concentration reaching a limit of 35% and its half-life decreased from about 200 ms at 5 μM to a limiting value of 50 ms for concentrations greater than 50 μM cyt c_2 (not shown). The very slow phase with a half-life of 1.5 s associated with the back-reaction from $P^+ Q_B^-$ decreased in amplitude from 70% in the absence to 20% in the presence of 80 μM soluble cyt c_2 (see Figure 3, right traces).

At cyt c_2 concentrations greater than 50 μM, the two kinetic phases in the millisecond time range following the second flash represent the two major phases of P^+ reduction with half-lives of 6 and 50 ms. These kinetics were virtually unchanged upon addition of 30% (v/v) glycerol whereas both kinetic phases were drastically slowed down in the presence of NaCl (data not shown). Therefore, these two phases are attributed to electron transfer processes from cyt c_2 bound to other binding sites on the RC surface in addition to the cross-linked cyt c_2.

Effects of Temperature. The temperature dependence of the electron transfer reaction from covalently bound cyt c_2 to P^+ was studied in a buffer containing 60% glycerol. At room temperature, the decay of P^+ recorded under these conditions can be accurately fitted to a sum of three exponential components with half-lives of 400 ns, 45 μs, and a millisecond component. As mentioned above, it is noticeable that the two fast phases of P^+ reduction by cross-linked cyt c_2 are slightly accelerated due to the presence of 60% glycerol (compare Figure 2, traces a and c). At temperatures below 260 K, the quality of the fit was slightly improved by including a fourth component with a half-life between that of the fast and the intermediate phase. However, if its half-life was tentatively kept at a constant value between 10 and 15 μs, its amplitude was always below 5% of the total generated P^+. Under conditions where Q_A can be expected to be reduced before the flash, i.e., after several flashes given at low temperature, no significant amplitude of a ^3P triplet decay with a half-life in the μs time range was observable, presumably because of very fast triplet energy transfer to the carotenoid molecule present in the RC from *Rb. sphaeroides* 2.4.1. Thus, if significant, such an intermediate component should correspond to a minor fraction of centers with a different rate of electron transfer at low temperatures. This could originate, e.g., from different conformations on the time scale of the electron transfer reaction. For our analysis of the kinetic traces this additional component was omitted, since no meaningful results were obtained without restraining the rate of such a component at a fixed value. Therefore, the data for the fast component at temperatures below 250 K may give average values including a minor fraction of centers with a slightly smaller

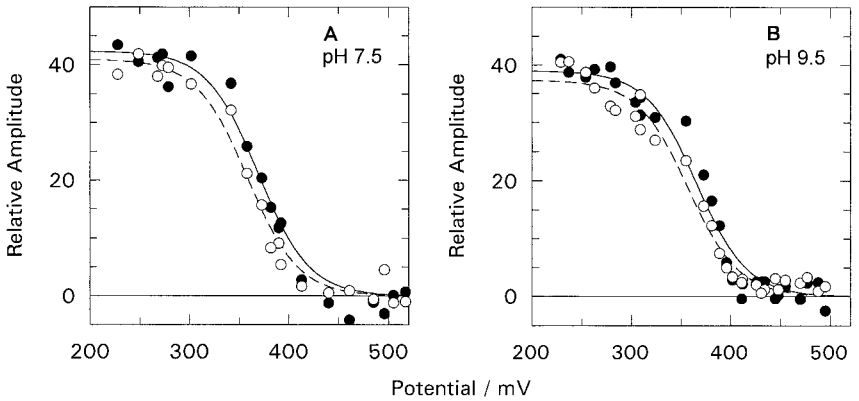

FIGURE 4: Effect of ambient redox potential on the amplitude of the fast (●) and the intermediate (○) kinetic components of P^+ reduction in cross-linked RC−cyt c_2 complexes. A: pH 7.5. Fit of Nernst's equation to the data points (solid and dashed lines) yields E_m values of 369 and 358 mV for the fast and the intermediate components, respectively. B: pH 9.5. E_m values are 366 and 357 mV for the fast and the intermediate components, respectively.

rate of electron transfer. For that reason, they were not taken into account for the estimation of activation energies.

In the temperature range between 295 and 250 K, the rate of electron transfer for the fast and the intermediate kinetic components can be described by an Arrhenius behavior with apparent activation energies of 11.7 and 8.0 kJ/mol, respectively [not shown; for details of data analysis, see Venturoli *et al.* (1993)]. Below 250 K the amplitude of the fast phase decreases rather drastically with a characteristic temperature of about 240 K where the amplitude appears to have 50% of its maximum value. Similar behavior is observed for the intermediate phase. In parallel, the slow millisecond component increases in amplitude, suggesting that no cytochrome oxidation occurs at lower temperatures. These effects of temperature are qualitatively similar to the results reported previously for the electron donation from spontaneously bound cyt c_2 to P^+ (Venturoli *et al.*, 1993).

Oxidation−Reduction Potential of Free and Bound cyt c_2. Redox titrations of the flash-induced absorbance changes in cross-linked RC−cyt c_2 complexes have been carried out at different pH values. Figure 4 shows the amplitude of the fast (0.7 μs) and the intermediate (60 μs) component of P^+ reduction as a function of the ambient redox potential at pH 7.5 (Figure 4A) and pH 9.5 (Figure 4B). Redox midpoint potentials of 360 and 370 mV for the intermediate and the fast kinetic phase, respectively, were determined at both pH values. We assume that these midpoint potentials determined in the flash titrations reflect titrations of cross-linked cyt c_2. By contrast, the E_m of cyt c_2 in solution shows a strong pH dependence between pH 7 and 10 (Figure 5). A similar pH dependence has been described previously and attributed to an influence of two ionizable groups on the midpoint potential of the heme (Pettigrew *et al.*, 1975). The solid line in Figure 5 is a curve calculated according to the equation used by Pettigrew *et al.* (1975) assuming one ionizable group, the pK of which changes from pK_{o1} to pK_r upon reduction of the heme, a second group that is ionizable in the oxidized state (pK_{o2}) but not in the reduced state, and the maximum midpoint potential from extrapolation to pH 0 ($E_{m,0}$). In order to check for a possible effect of the cross-linking reagents on the E_m of cyt c_2, we determined in parallel the E_m for cyt c_2 that was recuperated from the cross-linking incubation. No difference between the EDC-incubated and the control cyt c_2 was found within our measuring accuracy (Figure 5, open diamonds). In conclusion, the drastically

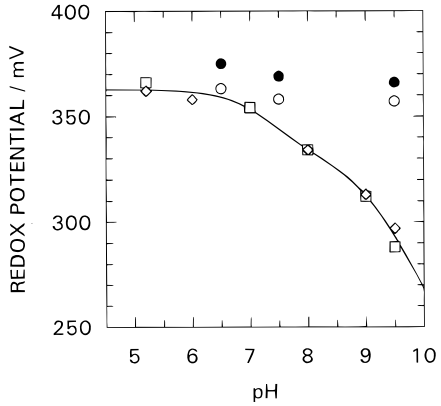

FIGURE 5: pH dependence of midpoint potentials for cross-linked cyt c_2 monitored by the amplitude of the fast (●) and the intermediate (○) kinetic components of P^+ reduction and for cyt c_2 in solution (□). Midpoint potentials measured for cyt c_2 recuperated after incubation for cross-linking are also indicated (◇). The solid line gives a theoretical curve assuming two ionizable groups in the oxidized state, pK_{o1} = 7.1 and pK_{o2} = 9.0, and one in the reduced state, pK_r = 7.6, and a maximum midpoint potential in the protonated form of $E_{m,0}$ = 363 mV.

changed redox properties of the heme, with respect to the pH dependence, for cross-linked cyt c_2 compared to free cyt c_2 are apparently caused by the (covalently stabilized) association to the RC surface.

From the total amplitude of formation of P^+ by a flash as a function of the ambient redox potential we determined an E_m for the primary donor in the cross-linked complex of 520 mV at pH 7.5. This is to be compared to a value of 500 mV in free RC (Drepper *et al.*, 1995). At increasing pH values the E_m for P^+/P in the cross-linked RC−cyt c_2 complex slightly decreased to an E_m of 490 mV at pH 9.5 (data not shown), a pH dependence which is similar to that reported for free RC (Maróti & Wraight, 1988).

DISCUSSION

The interaction between cyt c_2 and the RC from *Rb. sphaeroides* was studied using zero-length chemical cross-linking. A comparison of gel electrophoresis and of flash absorption data leads us to conclude that, after cross-linking, ca. 80% of the RC had a covalently bound cyt c_2 in a fully active, one-to-one complex. About 20% of the RC had no cross-linked cyt c_2. Cyt c_2 was cross-linked selectively to subunit M of the RC, in agreement with two proposed

structural models for the RC−cyt c_2 complex in which the cyt was positioned toward the M-side of the periplasmic RC surface (Tiede & Chang, 1988; Adir *et al.*, 1996). A minor band on the SDS-gel that could be attributed to cyt c_2 covalently bound to subunit L indicates that such a complex may amount to about 5% of that between subunit M and the cyt. These covalent complexes provide a system for studying functional parameters of the electron transfer reaction without disturbance from the bimolecular process of formation and dissociation of the complex.

In a previous cross-linking study (Rosen *et al.*, 1983), cyt c_2 was cross-linked only to subunit L, however by bifunctional agents as dithiobis(propionimidate) and dimethyl suberimidate, which introduce a bridging chain of about 10 Å between two lysines. By comparison, EDC and NHS used in the present study cross-link more specifically by introducing an isopeptide bond between an amino and a carboxyl side chain which are likely to form a salt-bridge in the non-cross-linked sample [see, e.g., Grabarek and Gergely (1990)]. In the presence of EDC as the only cross-linking agent, the carbodiimide-catalyzed formation of amide bonds involves as an intermediate step the formation of an *O*-acylisourea derivative. The rapid hydrolysis ($t_{1/2}$ less than 1 s) of this intermediate, competing with the reaction with an amino group, reduces the yield of cross-linking (Hoare & Koshland, 1967). Upon addition of NHS, a *N*-succinimidyl ester of a carboxyl group is formed as intermediate, the half-life of which is drastically increased to about 1 h even at neutral pH (Anjaneyulu & Staros, 1987; Grabarek & Gergely, 1990). Under these conditions, the reactions of EDC with tyrosines or SH groups are likely to be much slower than that with solvent-exposed carboxyl groups (Carraway & Koshland, 1968; Carraway & Triplett, 1970; Grabarek & Gergely, 1990). In addition to the cross-linking between two proteins, however, intramolecular cross-linking can occur if there are interactions between amino and carboxyl groups within the protein. Such cross-links of amino−carboxyl pairs on the periplasmic surface of the RC might explain the slight decrease of the cyt c_2 binding affinity of RC incubated with cross-linkers in the absence of cyt.

Kinetics of Electron Transfer. Two major features were observed for the electron transfer from cross-linked cyt c_2 to P^+, a fast and an intermediate phase with half-lives of 0.7 and 60 μs, respectively. It is of note that also two major cross-linking products of about equal quantity have been observed in the SDS-gel which were both attributed to a covalent complex between subunit M and cyt c_2. Both structural and functional heterogeneity may reflect the same two populations of the complex. Although at first sight these biphasic kinetics appear to be similar to those observed for the electron transfer from non-cross-linked cyt c_2, a closer look reveals also some important differences.

As to the fast phase, its half-life being identical to that observed for electron transfer from spontaneously bound cyt c_2 under similar conditions [data not shown; compare, e.g., Venturoli *et al.* (1993) and Tiede *et al.* (1993)] suggests that the cross-linked and the spontaneously formed RC−cyt c_2 complexes have an equivalent geometry. Addition of glycerol accelerates the reaction. A similar effect was evident from previous measurements of the fast electron transfer from non-cross-linked cyt c_2 (Venturoli *et al.*, 1993). In the case of soluble cyt c_2, such an acceleration at increased viscosity could be rationalized in the following way. If bound cyt c_2 giving rise to the fast kinetic phase interconverts rapidly between two different conformations, one of which allows optimal electron transfer, the reaction will be rate-limited by the interconversion process. At increased viscosity, the interconversion may be hindered and, for a fraction of the RC, electron transfer within the optimal conformation of the complex would be monitored. However, this explanation is rendered unlikely by the observation of a similar effect for cross-linked cyt c_2 for two reasons. First, at a decreased rate of interconversion, the apparent acceleration of the reaction should be accompanied by a decrease in amplitude reflecting the fraction of molecules being in the optimal configuration on the time scale of the electron transfer. However, the amplitude for the fast phase in the cross-linked complex was not altered by the addition of glycerol. Second, rapid interconversion that could change the rate of electron transfer should be affected or hindered by the cross-linking, whereas the effect of glycerol on the apparent rate of electron transfer was the same with and without cross-linking. We propose instead that the presence of glycerol increases the driving force, e.g., by decreasing the midpoint potential of cyt c_2. The amplitude of such a change may be estimated from the classical limit at zero driving force, i.e., a factor of 10 in rate per change of 120 mV in $\Delta G°$ (Marcus & Sutin, 1985). Thus, a change of −30 mV for the midpoint potential of cyt c_2 is sufficient to account for the 1.8-fold increase in rate at 60% glycerol.

The intermediate phase with a half-life of 60 μs resembles at first sight the slower phase for electron transfer from soluble cyt c_2 attributed to a second-order bimolecular reaction. In some studies, a first-order limiting half-life for this kinetic component of 55−75 μs (Long *et al.*, 1989; Tiede *et al.*, 1993; Wachtveitl *et al.*, 1993) or 350−400 μs (Overfield *et al.*, 1979; Moser & Dutton, 1988) at high cyt concentrations was observed, and its viscosity sensitivity (Moser & Dutton, 1988) led to its interpretation in terms of a reorientation of the cyt c_2 from a "distal" into a "proximal" bound state. By contrast, the 60 μs phase in the cross-linked RC−cyt c_2 complex is even slightly accelerated in the presence of glycerol. Thus, this process does not involve a viscosity sensitive spatial reorientation, but represents an electron transfer reaction with a relatively low activation energy, similar to that for electron transfer within the proximal RC−cyt c_2 complex. Since both the midpoint potential and the activation energy for the cyt c_2 giving rise to the 60 μs phase are very similar to those observed for the 0.7 μs phase, the difference in the electron transfer rate cannot be explained by changes in $\Delta G°$ or in the reorganization energy λ. Thus, we may tentatively attribute the slower rate of electron transfer for the intermediate phase to a RC−cyt c_2 complex providing a longer distance between the heme and the primary donor than in the proximal complex. Using the average distance dependence obtained by Moser *et al.* (1992) for a number of intraprotein electron transfer reactions after correction for effects from $\Delta G°$ and λ, i.e., a factor of 10 in rate per 1.7 Å, the 85 times slower rate would translate into a distance being longer by about 3.2 Å. As to the position of the binding sites on the RC surface, we note that the amplitude ratio of the fast and the intermediate kinetic phases and their half-lives remained essentially unchanged upon addition of soluble cyt c_2 to cross-linked RC−cyt c_2 complexes (Figure 3, left). Thus, cross-linked cyt c_2 in the configuration that gives rise to the 60 μs phase prevents the

binding of soluble c_2 to a proximal configuration. This suggests that the fast and the intermediate phases involve the same exclusive site for binding of cyt c_2 on the RC, resulting, however, in two complexes of different geometry.

Although the 60 μs phase in the cross-linked complex clearly does not represent a "distal" to "proximal" reorientation, it may be related to such a mechanism. It seems likely that the zero-length cross-linking traps the cyt c_2 in bound states that are populated also before cross-linking. Although the cross-linking yield is not a quantitative measure for the binding affinity, the equal amplitudes for the fast and the intermediate kinetic phases suggest roughly similar occupancies of the two bound states before cross-linking. This view is also supported by the appearance of only two well-defined kinetic phases, indicating specific cross-linking rather than formation of a number of different complexes. Since reorientation is hindered in the cross-linked complex, electron transfer from a distally bound cyt c_2 may become observable. We will give a more detailed interpretation of this bound state in the accompanying article on the basis of additional data on the cyt orientation.

Binding Sites for a Second cyt c_2. Two new cyt c_2-dependent kinetic phases were observed in the millisecond time range following the second flash by monitoring P^+ reduction in RC having a cross-linked ferricyt c_2. A phase with a constant half-life of 6 ms is clearly identified at concentrations of cyt $c_2 \geq 20$ μM. Since this kinetic component exhibits a first order behavior and is not changed by glycerol, it is attributed to an electron donation to P^+ from cyt c_2 bound in some manner to the RC. It is of note that this identifies a direct electron transfer to P^+ from added cyt c_2 bound to the RC surface rather than a serial electron transfer via the cross-linked cyt c_2. The latter possibility would have manifested itself as a re-reduction of cross-linked cyt c_2 by soluble cyt c_2 during the 20 ms time interval between the two flashes in experiments as shown in Figure 3. That re-reduction was not observed.

The interpretation of the 50 ms phase is less straightforward, since a minor phase with a similar half-life of 50−80 ms, accounting for about 15% of the centers in the absence of added cyt c_2 (Figure 3 right, dotted trace), is associated with the back-reaction from $P^+Q_A^-$. However, at increasing concentrations of cyt c_2, the contribution of this latter reaction will be diminished due to the competitive reduction of P^+ by cyt c_2. The dominant, cyt dependent process having a half-life of 50 ms and representing the limiting first-order reduction for about 35% of total P^+ at high concentrations of cyt c_2 is therefore attributed to an electron donation to P^+, in RC having a cross-linked ferricyt c_2, from a peripherally bound molecule of cyt c_2, presumably bound in a different position than in the complex displaying the 6 ms phase.

In conclusion, we have shown that (i) no electron transfer from soluble cyt c_2 to the RC *via* cross-linked oxidized cyt c_2 is observable in the ms time domain and (ii) the electron transfer to P^+ from cyt c_2 bound to the RC surface in addition to the cross-linked cyt is 50−500-fold slower than the estimate for the dissociation rate of oxidized cyt c_2 ($t_{1/2} \approx 115$ μs, see discussion in the accompanying article). At least for isolated RC, these results indicate a reaction mechanism involving one functional binding site from which the oxidized cyt c_2 has to unbind before a new reduced cyt can bind and a second electron transfer can take place. The additional sites on the RC surface having an about 10-fold lower cyt binding affinity, and from which electrons can be donated to P^+ in the ms time range, may be related to previous observations of two (Dutton *et al.*, 1975; Moser & Dutton, 1988) or three (Pachence *et al.*, 1983) cyt binding sites on the RC. Whether these peripheral binding sites may play a role under some physiological conditions is not known and has to be addressed in experiments using more intact systems.

Midpoint Potential of Bound cyt c_2. At pH 6.5 and 7.5, *i.e.*, within the physiological pH range, the E_m of cross-linked cyt c_2 was found to be slightly higher (+5 to +25 mV) than that for cyt c_2 in solution ($E_{m,7} = 355$ mV, see Figure 5). Larger, negative shifts of −20 to −50 mV have been observed in previous studies for cyt *c* upon binding to its reaction partners in mitochondria [see Moore and Pettigrew (1990, p 313)]. A similar shift of −40 mV was reported by Moser and Dutton (1988) for the binding of cyt *c* to the RC from *Rb. sphaeroides* reconstituted into neutral phospholipid vesicles. For cyt c_2 in chromatophores of *Rb. sphaeroides*, E_m values of 295 mV (Dutton *et al.*, 1975) and 330 mV have been reported, the lower value in the former study being probably caused by interference with cyt c_1 (Bowyer *et al.*, 1979).

However, cyt *c* and cyt c_2 differ in their net charge (*e.g.*, +6 and −2 at neutral pH, calculated for the reduced form of horse cyt *c* and *Rb. sphaeroides* cyt c_2, respectively) and in the orientation of their electrostatic dipole (Tiede *et al.*, 1993). Indeed, although cytochromes *c* and c_2 compete for the same binding site on the RC surface (Rosen *et al.*, 1980), when they are bound to the RC, linear dichroism spectra revealed differences in the orientation of their heme plane (Tiede, 1987). It has been suggested that the lowering of the E_m for bound cyt *c* may be predominantly due to nonspecific binding to lipids or peripheral sites rather than to the active site [Nicholls, 1974; see also Moore and Pettigrew (1990, pp 314−316)]. This view was suggested by the observation that similar shifts in the E_m of cyt *c* were observed upon binding to its reductase and to cytochrome oxidase, and thus no contribution to the efficiency of electron transfer was apparent. It is clear from the results of the present study that for cyt c_2 in each of two bound states that promote electron transfer to the primary donor, the midpoint potential is equal or slightly higher than that of free cyt c_2.

The pH dependence for the midpoint potential of bound cyt c_2 appears to differ significantly from that for free cyt c_2, the behavior of which suggests that at least two ionizable groups perturb the E_m (Figure 5), in agreement with a previous study (Pettigrew *et al.*, 1975). The pronounced decrease in E_m at alkaline pH with an apparent p*K* value of about 9 (pK_{o2} in Figure 5) has been found to be a common feature of class I cytochromes *c* (Pettigrew *et al.*, 1975, 1978; Moore & Pettigrew, 1990, p 193; Caffrey & Cusanovich, 1994). A similar apparent p*K* has been observed for the drop in the midpoint potential and the loss of the 695 nm band for *Rb. sphaeroides* cyt c_2 (Pettigrew *et al.*, 1975). The transition has been associated with a perturbation in the ligand field of the iron, most probably a replacement of the sixth (methionine) ligand (Caffrey & Cusanovich, 1994). It seems likely that for bacterial cyt c_2, the apparent p*K* of about 9 for the alkaline transition can be attributed to the ionization of a group with a p$K \approx 11$ coupled to a conformational equilibrium with $K_c \approx 100$ as shown for mitochondrial cyt *c* [Davis *et al.*, 1974; Pettigrew *et al.*, 1975; compare Moore and Pettigrew (1990, pp 190−196)].

Although the ionizable group that triggers the replacement of the sixth ligand has not been identified, we may anticipate that interactions of cyt c_2 surface charges with the RC have a minor influence on its p*K*. It seems more likely that the conformational equilibrium for the replacement of the methionine ligand is affected by the association of cyt c_2 to the RC. The cross-linking may play (i) a minor role by fixing the cyt c_2 in a position such that the inherent electrostatic interactions with the RC surface stabilize the correct folding of the cyt c_2 or (ii) a major role, *i.e.*, by fixing different segments of the cyt covalently to the RC surface. Stabilization of cyt c_2 by internal cross-linking between different parts of the protein is unlikely, since incubation with the cross-linking reagents had no major effect on the pH dependence for the E_m of soluble cyt c_2 (Figure 5).

The second ionizable group, the p*K* of which differs for the ferri- and ferrocytochrome (pK_{o1} and pK_r in Figure 5, respectively), has only a minor influence on the pH dependence of the E_m of soluble *Rb. sphaeroides* cyt c_2. For analogous class I cytochromes *c*, the buried heme propionate and its surrounding protein environment have been identified as the most likely location of this group (Moore & Pettigrew, 1990, pp 348−354). The group has not been determined for *Rb. sphaeroides* cyt c_2. For cyt c_2 from *Rps. viridis* showing a similar pH dependence of its E_m [compare Moore and Pettigrew (1990, pp 349−351)] this effect has been attributed to protonation of histidine 39 (Moore *et al.*, 1984). However, the corresponding residue is a threonine in *Rb. sphaeroides* cyt c_2 [see Ambler *et al.* (1979)].

Temperature Dependence of Electron Transfer. The observed decrease of the rate of electron transfer from cross-linked cyt c_2 to P^+ with decreasing temperature is consistent with an Arrhenius behavior, at least within the temperature range between 295 and 250 K. Further lowering of the temperature to 235 K appears to inhibit the electron transfer reaction. A similar fall-off in amplitude has been observed for the fast phase of electron donation from soluble cyt c_2 to the isolated RC from *Rb. sphaeroides* R26, either in detergent or reconstituted in liposomes (Venturoli *et al.*, 1993). In that study, it has been tentatively interpreted as inhibition of the electron transfer reaction rather than originating from a dissociation of the complex. This interpretation was suggested by the finding that in *Rps. viridis* reaction centers, under conditions where only the highest potential heme of the tightly bound tetraheme subunit was reduced, inhibition of electron transfer occurred within about the same narrow temperature interval (Ortega & Mathis, 1993). Our observation of a similar effect in the cross-linked complex further supports the proposal that it is the intra-complex electron transfer that becomes impossible in the cyt c_2−RC complex of *Rb. sphaeroides* at low temperatures.

In the high-temperature range, the apparent activation energy for the fast electron transfer in the cross-linked complex, $E_A = 11.7$ kJ/mol, is smaller than that found for the complex with soluble cyt c_2 of 20.5 kJ/mol (Venturoli *et al.*, 1993). Even considering only the data from the previous study within the temperature interval used in our present work for determination of the slope in the Arrhenius plot, it appears that the apparent activation energy is still significantly lower in the cross-linked than in the noncovalent complex. On the other hand, it is of interest to compare the present results with observations on the RC from *Rps. viridis* with a tightly bound tetraheme cytochrome subunit. In this latter case, still smaller activation energies (6.7−8.6 kJ/mol) for the electron donation have been measured under conditions where only the highest potential heme was reduced (Ortega & Mathis, 1993). Thus, due to cross-linking of the cyt c_2 to the *Rb. sphaeroides* RC, the activation energy is decreased compared to that for the soluble cyt c_2 and approaches the values found for the tightly bound cyt subunit. According to the semiclassical theory of electron transfer, a lower activation energy could be straightforwardly explained by either an increased $-\Delta G°$ or a decreased reorganization energy λ (Marcus & Sutin, 1985). Since we do not observe an increased $-\Delta G°$ for the electron transfer from the cross-linked cyt c_2—there might even be a small decrease of $-\Delta G°$ (see Figure 4)—it seems more likely that it is λ which is lowered upon covalently stabilizing the RC−cyt c_2 complex.

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