Viscosity Dependence of the Electron Transfer Rate from Bound Cytochrome *c* to P840 in the Photosynthetic Reaction Center of the Green Sulfur Bacterium *Chlorobium tepidum*[†,‡]

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ABSTRACT: Anomalous high viscosity dependence was found in the rate of reaction between the bound cytochrome *c* and the primary donor bacteriochlorophyll dimer (P840) of the reaction center complex purified from the green sulfur bacterium *Chlorobium tepidum*. The cytochrome has a primary structure with the N-terminal three membrane-spanning helices connected to the extended C-terminal heme-containing hydrophilic moiety. The rate constant of the reaction decreased from 5.0×10^3 s^{-1} to 1.0×10 s^{-1} as the glycerol concentration increased from 0 to 60% (v/v) at 295 K, showing a linear dependence on the −2.4th power of the specific viscosity. The glycerol effect was fully reversible. The extraordinary high viscosity dependence cannot be explained by the simple diffusive Brownian fluctuation model and suggests that the electron transfer mechanism is dependent on the unique conformational fluctuations of the heme-containing moiety of cytochrome *c*.

The photosynthetic reaction center (RC)[1] complex of green sulfur bacteria (Chlorobiaceae) contains Fe-S clusters and resembles the photosystem I (PS I) RC of plants and cyanobacteria (Feiler & Hauska, 1995). Recently developed anaerobic purification procedures indicated that this RC complex is a so-called "homodimeric" RC made up of two identical core polypeptides that bind a number of chlorophylls and of small unpigmented subunits (Büttner et al., 1992; Kusumoto et al., 1994; Kjær et al., 1994; Oh-oka et al., 1995a; Hager-Braun et al., 1995). The photoactive RC complex of *Chlorobium* isolated by several groups comprises five discrete subunits: a core protein (65 kDa), an FMO protein (41 kDa), an F_A/F_B protein (31 kDa), monoheme cytochrome *c* (22 kDa), and an 18-kDa protein (Kusumoto et al., 1994; Oh-oka et al., 1995a; Hager-Braun et al., 1995). The RC preparation from *C. vibrioforme* contains the additional sixth subunit (9 kDa) (Kjær et al., 1995). Functions of the 18- and 9-kDa proteins are not known yet. The electron transfer sequence within the *Chlorobium* RC has been assumed to be analogous to that in PS I as shown below:

cytochrome *c* → P840 → A (Chl-670) →
(quinone-like component) → Fe-S (F_X) → Fe-S (F_A/F_B)

where a quinone-like component has been detected by EPR spectroscopy (Heathcoate & Warden, 1987) but no quinone molecule has been identified yet as an essential constituent of the *Chlorobium* RC (Frankenberg et al., 1996).

In the RC preparation isolated from *C. limicola*, Feiler et al. (1992) estimated binding of four hemes per P840. However, Okkels et al. (1992) estimated about one heme per P840 in their RC complex from *C. vibrioforme*. Oh-oka et al. (1993) determined two hemes per P840 in their RC preparations from *C. limicola*. The kinetic study by Okumura et al. (1994) in the membrane preparation from *C. tepidum* also suggested two hemes per P840. A multiflash experiment with the isolated *C. tepidum* RC seems to have given the conclusion that the two molecules of identical RC-bound monoheme cytochrome *c* function as the immediate electron donors to one $P840^+$ (Oh-oka et al., 1995a) in this species. The situation will be similar in *C. limicola* under normal culture conditions since the (PscA/cytochrome $c_{551})_2$-type RC complex purified from this species, which is made up of only core PscA and cytochrome c_{551} subunits, also gave a 2 heme/1 P840 ratio (Oh-oka et al., 1995b). The amino acid sequences of monoheme cytochromes from *C. vibrioforme* (Okkels et al., 1992), *C. limicola* (Oh-oka et al., 1995c), and *C. tepidum* are highly homologous as will be shown in this work. Therefore, there has been no definite evidence for functional tetraheme cytochrome in *Chlorobium* RC. We here studied the reaction mechanism between the bound cytochrome hemes and P840 in the isolated RC complex from *C. tepidum*.

In purple photosynthetic bacteria, RC-bound and water-soluble *c*-type cytochromes are known to function as the electron donor to the primary donor bacteriochlorophyll dimer (P). The RC-bound tetraheme cytochrome *c*, as seen in *Rhodopseudomonas viridis* RC (Deisenhofer et al., 1985;

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[‡] The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession numbers AB004459 and AB004460.

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[1] Abbreviations: RC, reaction center; PS, photosystem; P840, primary electron donor in *Chlorobium*; DTT, dithiothreitol; N-, amino-; C-, carboxyl-.

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Nitschke & Dracheva, 1995), reduces P even at cryogenic temperature, which was shown in *Chromatium vinosum* as the first example of biological electron tunneling (Chance & Nishimura, 1960). The oxidation kinetics of hemesmeasured at 7−305 K suggested heterogeneity (or several substates) in the arrangement of cytochrome and P (Ortega & Mathis, 1993). The water-soluble cytochrome c_2 mediates electron transfer from the cytochrome b/c_1 complex to the RC-bound tetraheme cytochrome, or directly to P in the RC complex that lacks the tetraheme cytochrome such as that from *Rhodobacter sphaeroides*. The reaction of cytochrome c_2 with P showed both viscosity-independent and viscosity-dependent kinetics which were derived from the proximal and the distal states, respectively (Moser & Dutton, 1988) and stopped at low temperature (Venturoli et al., 1993).

The cytochrome *c* bound to the RC complex of *Chlorobium* serves as an electron donor to P840 (Okkels et al., 1992; Okumura et al., 1994; Oh-oka et al., 1993, 1995a) and has been designated as a new class of cytochrome *c*, class V, because its amino acid sequence shows no significant homology to those of ever-known *c*-type cytochromes (Okkels et al., 1992). This protein, PscC (Bryant 1994), has three putative membrane-spanning α-helices at the N-terminus and the hydrophilic C-terminus with a single heme-attachment site. This type of electron donor with a hydrophilic tail has never been found in other types of "photosynthetic" reaction centers. On the other hand, electron carrier proteins exhibiting similar motifs that contain the redox centers in the position extruded from the membrane are rather popular in the membrane-bound oxido−reductase complexes, such as subunit II (Cu_A) in cytochrome *c* oxidase (Iwata et al., 1995) or cyotochrome c_1 (*f*) in cytochrome b/c_1 (b_6/f) complex (Malkin, 1992). The reaction mechanism of *Chlorobium* cytochrome *c*, therefore, seems to give new insight into the evolution or diversity of the photosynthetic electron transfer system, when we compare functional and structural features of *C. tepidum* cytochrome *c* with those of the tetraheme or soluble cytochromes in purple bacterial RC, or of a water-soluble plastocyanin or cytochrome c_6 in PS I RC.

In this work, we report the viscosity dependence of the electron transfer rate between the bound cytochrome *c* and P840 in the isolated RC complex from *C. tepidum*. The rate decreased dramatically with increase in the medium viscosity, suggesting a special molecular fluctuation that cannot be explained by Sumi theory (1991) which describes an inverse fractional-power dependence of the rate constant on the viscosity.

MATERIALS AND METHODS

RC complex was isolated from *C. tepidum* as described previously (Oh-oka et al., 1995a). All procedures for the preparation of RC samples and kinetic measurements were carried out under anaerobic conditions. Glycerol, which was degassed with a vacuum pump and stored for 1−2 days in an anaerobic chamber before use, was added to the RC preparation suspended in 50 mM Tris-HCl (pH 8.0), 2 mM DTT, 2 mM sucrose monolaurate, and protease inhibitors (Oh-oka et al., 1995a) to a final concentration [% (v/v)] of 0−60%.

The N-terminal amino acid sequence of cytochrome *c* was determined with a gas-phase sequencer (Applied Biosystems, Model 473A) after being transferred onto a PVDF membrane from SDS−PAGE. The PCR product was obtained from the cytochrome *c* gene of *C. limicola* using two oligonucleotides as reported previously (Oh-oka et al., 1995c), labeled with digoxigenin-11−dUTP (DNA Labeling and Detection Kit Non-radioactive, Boehringer Mannheim), and used as a probe for Southern blot analysis. The fragments of the genomic DNA of *C. tepidum* were digested with restriction endonucleases and were separated by agarose gel (1%) electrophoresis. The appropriate-sized DNA fragments which were hybridized with the labeled PCR product were extracted from the agarose gel, ligated into the plasmid pUC18, and transformed into *Escherichia coli* DH5α cells. Positive clones were obtained by the colony hybridization method with the labeled PCR product as a probe. These hybridization and subsequent detection procedures were carried out according to the instruction manual supplied with the labeling kit. The plasmid DNAs obtained from the positive clones were mapped and subcloned into pUC118 for sequencing by the Sanger dideoxy chain-termination method using a DNA sequencer (Applied Biosystems, Model 373A). The N-terminal sequence of cytochrome *c* was identical up to the 25th residue to that deduced from the DNA sequence determined as above.

The viscosity (η, in units of centipoise) values of the glycerol/water mixture at 283 and 295 K was calculated from those at 293, 298, and 303 K in the Handbook of Chemistry (1962, Japanese Society of Chemistry), after the exponential extrapolation using the $T \propto \log(1/\eta)$ relation (where T is temperature), as listed in Table 1 with measured rate constants.

Laser-flash photolysis was performed as described previously (Nakane et al., 1991). The probing light passing through the sample cuvette was detected by a photomultiplier through a monochromator. The sample was excited by a 10-ns, 532-nm Nd-YAG laser flash. The sample in an air-tight cuvette was maintained at the desired temperature with a water circulation system.

RESULTS

The amino acid sequence deduced from the DNA sequence of the cytochrome *c* gene in *C. tepidum* exhibited 84% homology (34 replacements in a total of 206 residues) to those in *C. vibrioforme* and *C. limicola* (note that amino acid sequences in *C. vibrioforme* and *C. limicola* were completely identical) [Figure 1(b)] (Okkels et al., 1992; Oh-oka et al., 1995c). On the nucleotide level, it showed about 80% homology to those of *C. vibrioforme* and *C. limicola* (data not shown). The sequence contained the typical heme binding motif, Cys-X-X-Cys-His, at the position of the 152−156th amino acid residues, to which a single heme *c* is attached through a thioether bond to cysteine. Our homology search of the DDBJ data base with the FASTA program indicated about 22% identity around the putative three transmembrane helices to the V, VI, and VII helices of subunit 3 of cytochrome *c* oxidase of *Paracoccus denitrificans*, whose X-ray crystal structure was determined by Iwata et al. (1995). The N-terminal membrane-spanning α-helices of the present cytochrome *c* seem to be tightly bound to the RC complex.

Figure 2 shows the typical kinetic traces of the flash-induced absorption changes at 283 K of P840 [monitored at

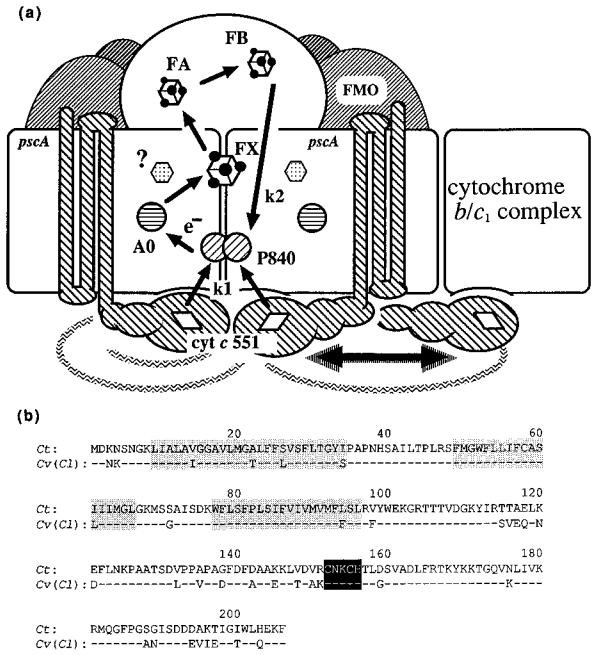

FIGURE 1: (a) A schematic structure of the *Chlorobium tepidum* RC with two cytochromes. k_1 and k_2 are rate constants for the electron transfer reactions from cytochrome *c* to P840$^+$ and from Fe-S$^-$ to P840$^+$, respectively. (b) Comparison of amino acid sequences of cytochrome *c* deduced from the DNA sequences of the *pscC* gene in *C. tepidum* (*Ct*) and *C. vibrioforme* (*Cv*) or *C. limicola* (*Cl*). Identical amino acid residues are indicated by dashes (−). The putative membrane-spanning regions are shadowed, and the heme binding site is highlighted by inverse contrast.

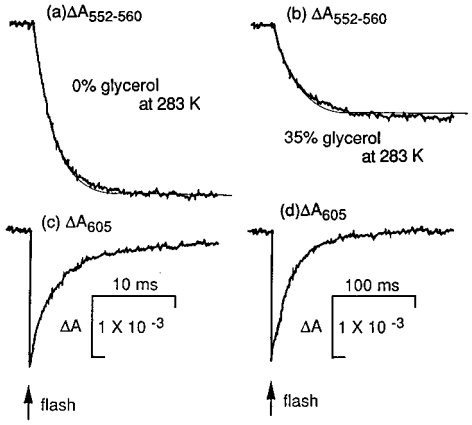

FIGURE 2: Kinetics of flash-induced absorption changes of cytochrome *c* and P840 at 283 K. (a) and (b), oxidation of cytochrome *c* monitored as the difference between kinetics at 552 and 560 nm; (c) and (d), oxidation and re-reduction of P840$^+$ monitored at 605 nm. (a) and (c), 0% glycerol; (b) and (d), 35% glycerol. Sample concentrations were adjusted to be $A_{812} = 1.5$.

605 nm, (c) and (d)] and cytochrome *c* [difference between kinetics at 552 and 560 nm, (a) and (b)]. At 0% glycerol, P840$^+$ formed by flash excitation was re-reduced concomitant with the oxidation of cytochrome *c*, indicating the direct oxidation of the latter by P840$^+$. The electron transfer rate from cytochrome *c* to P840$^+$ decreased markedly on addition of glycerol. At 35% glycerol, the extent of cytochrome *c* oxidation was approximately half, while the bleaching of P840$^+$ recovered almost completely. This indicates that the re-reduction rate of P840$^+$ by cytochrome *c* became slower than that by the reduced acceptors (F_A^- or F_B^-).

At 200 K in the presence of 60% glycerol, the absorption change at 830 nm rose immediately after the flash (P840$^+$

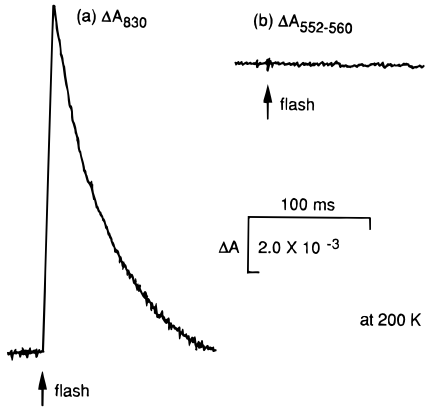

FIGURE 3: Flash-induced absorption changes monitored at (a) 830 nm (P840) and (b) 552−560 nm (cytochrome *c*) in *C. tepidum* RC. Measurements were carried out at 200 K in the presence of 60% glycerol as in Figure 2. Sample concentration was adjusted to be $A_{812} = 1.5$.

formation) and decayed (P840$^+$ re-reduction) exponentially with the time constant $t_{1/e} = 50$ ms (Figure 3). The 50 ms re-reduction of P840$^+$ could be ascribed to charge recombination between a reduced terminal electron acceptor (probably F_A^- or F_B^-) and P840$^+$ because no absorption change due to cytochrome *c* was observed [Figure 3(b)]. In the presence of 60% glycerol, cytochrome *c* was not involved in the fast electron transfer to P840$^+$ at temperatures up to 273 K (data not shown), as reported previously (Oh-oka et al., 1995b).

The scheme in Figure 1(a) was assumed by postulating the rate constant k_1 for electron transfer from cytochrome *c* to P840 and k_2 for the one from the terminal acceptor (F_A or F_B) to P840. The extent of oxidized cytochrome *c* can be expressed by eq 1, provided that an equilibrium between the two hemes *c* is slower than the present experimental time scale (Oh-oka et al., 1995a):

$$[\mathrm{cyt}_{ox}] = [k_1/(k_1 + k_2)][1 - \mathrm{e}^{-(k_1+k_2)t}] \qquad (1)$$

k_2 was assumed to be constant [(50 ms)$^{-1}$], and k_1 was varied to fit the kinetics at varied glycerol concentrations. In Figure 2(a),(b), values for the time constant (t_1) of 2.5 and 50 ms, respectively, were assumed to fit the experimental data at 0 and 35% glycerol as well as the kinetics of P840$^+$. About 80% of P840$^+$ was re-reduced by the forward electron transfer from cytochrome *c* within 10 ms at 0% glycerol [Figure 2(c)], suggesting almost full establishment of the equilibrium between P840 and cytochrome *c* (Oh-oka et al., 1995a), and then the electron flow from the reduced terminal acceptor (F_A^-/F_B^-) seemed to fully re-reduce the residual P840$^+$.

The glycerol effect was completely reversible as demonstrated in Figure 4. The slow oxidation kinetics of cytochrome *c* observed at 60% glycerol were simulated with t_1 and t_2 of 80 and 50 ms, respectively [Figure 4 (a)]. It changed into the kinetics with $t_1 = 0.8$ ms and $t_2 = 50$ ms after the dilution of the sample to 15% glycerol concentration (note that the temperature is 295 K). The re-reduction of P840$^+$ was complete in both cases, showing a constant $t_2 = 50$ ms ascribable to the electron transfer from the acceptor side (F_A^-/F_B^-) to P840$^+$, so that the components on the acceptor side seem to be intact even after treatment with a high concentration of glycerol.

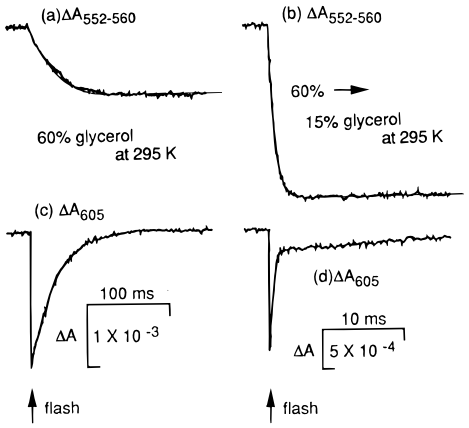

FIGURE 4: Reversibility of the glycerol effect on the kinetics of flash-induced absorption changes of cytochrome *c* and P840 at 295 K. (a) and (b), cytochrome *c* monitored at 552−560 nm; (c) and (d), P840 monitored at 605 nm. (a) and (c), 60% glycerol (A_{812} = 1.5); (b) and (d), 15% glycerol (A_{812} = 1.3) after 4-fold dilution of the 60% pretreated sample.

Table 1: Values for the Viscosity (η, cP) and the Rate Constant (k_1, s^{-1}) for Electron Transfer from Cytochrome *c* to P840

glycerol concn % (V/V)	viscosity (η, cP) vs rate constant (k_1, s^{-1}) at 283 and 295 K			
	283 K		295 K	
	η^a	$k_1{}^b$	η^a	$k_1{}^b$
0	1.32	4.0×10^2	0.96	6.7×10^3
5	1.51	3.3×10^2	1.12	3.3×10^3
10	1.80	2.9×10^2	1.31	2.0×10^3
15	2.20	2.0×10^2	1.58	1.3×10^3
20	2.70	1.4×10^2	1.91	1.1×10^3
25	3.37	1.0×10^2	2.35	5.0×10^2
30	4.20	3.3×10	2.86	2.5×10^2
35	5.28	2.0×10	3.51	2.0×10^2
40	7.08	1.4×10	4.60	1.0×10^2
45	9.38	7.7	5.92	4.0×10
50	12.6	5.3	7.80	2.5×10
55	18.0	4.0	10.6	1.7×10
60	24.6	2.0	14.1	1.3×10

[a] Calculated as described in the text. [b] Experimentally obtained by fitting the kinetics using eq 1 as described in the text.

The k_1 values at varied glycerol concentrations are listed in Table 1 along with the calculated viscosity (η). Logarithms of k_1 values in Table 1 were plotted against log (η) as shown in Figure 5 according to Asano et al. (1994). The linear relationship with slopes of −1.9 and −2.4 were obtained at 283 and 295 K, respectively. Frequency factors (A, s^{-1}) and activation energies (E_a, kJ·mol^{-1}) were calculated by the Arrhenius equation at three discrete viscosities (log η = 0, 0.5, and 1), based on the best fit lines in Figure 5 (Table 2). The different slopes at two temperatures in Figure 5 indicate the smaller activation energy at the higher viscosity. The marked decrease of the frequency factor at the high viscosity would suggest a collisional nature of the reaction as expected for a second-order reaction. However, the relevant reaction should be in a first-order one because the reaction occurs between P840 and the cytochrome *c* bound to the same RC complex.

DISCUSSION

Reaction Mechanism of Bound Cytochrome c and P840 in C. tepidum RC Complex. The two membrane-bound molecules of identical cytochrome *c* serve as immediate

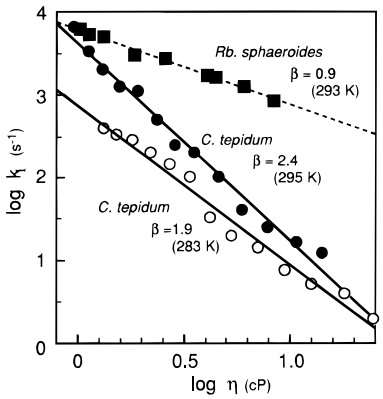

FIGURE 5: Viscosity dependence of the electron transfer rate from cytochrome *c* to P840. Temperatures were at 283 K (open circles) and 295 K (closed circles), respectively. The rate constants for the reaction between P860 and soluble cytochrome c_2 in *Rb. sphaeroides* (Overfield et al., 1979) were replotted as described in the text (closed squares).

Table 2: Frequency Factors (A, s^{-1}) and Activation Energies (E_a, kJ·mol^{-1}) Calculated from k_1 Values at Different Viscosities (log η = 0, 0.5, and 1)

log η	A (s^{-1})	E_a (kJ·mol^{-1})
0	3.88×10^{21}	101
0.5	5.06×10^{14}	69.4
1	9.18×10^7	38.0

electron donors to P840 in the RC complex of *Chlorobium* (Okkels et al., 1992; Okumura et al., 1993; Oh-oka et al., 1993, 1995a). The strong glycerol effect on the electron transfer rate from cytochrome *c* to P840, as demonstrated here, suggests the involvement of thermodynamic motions in its relevant reaction. In the RC complex of the purple bacterium *Rps. viridis*, glycerol (up to 60%) did not influence the oxidation kinetics of the tetraheme cytochrome *c* (Nitschke & Dracheva, 1995) which is rigidly bound to the RC core protein (Lancaster et al., 1995). In *Chromatium vinosum*, the electron transfer rate from the bound cytochrome *c* slows down as the temperature decreases and becomes temperature-independent below 120 K (DeVault & Chance, 1966). The reaction rate gave apparent activation energies (E_a) of 13.8 kJ·mol^{-1} for *C. vinosum* and 3.6−8.6 kJ·mol^{-1} for *Rps. viridis* (DeVault & Chance, 1966; Ortega & Mathis, 1993). The low E_a values show a clear contrast with the high E_a value (101 kJ·mol^{-1}) observed in the *C. tepidum* RC complex (see Table 2). The low-frequency factor at high viscosity suggests a lower probability of collision between the reaction sites on cytochrome *c* and the RC surface. It is unlikely that glycerol affected the redox properties of heme *c* since little effect of glycerol has recently been demonstrated in horse heart cytochrome *c* (Sanctis et al., 1996).

The *c*-type cytochromes are known to undergo structural changes depending on the heme redox state (Rackovsky & Goldstein, 1984; Berghuis & Brayer, 1992). Conformational change or dehydration of cytochrome *c* induced by the high concentration of glycerol may affect the reaction rate. Another possibility could be that the high viscosity of glycerol itself disturbs the structural change of the cytochrome *c* as proposed in the reaction of tetraheme cytochrome *c* in *Rps. viridis* RC, which exhibited several substates depending on temperature (Ortega & Mathis, 1993).

These effects were rather small and seem to be insufficient to explain fully the extremely high amplitude of glycerol effects detected in *C. tepidum* RC.

In the isolated membranes of *Rb. sphaeroides*, soluble cytochrome c_2 shuttles between cytochrome b/c_1 and RC complexes and donates electrons directly to P^+. Overfield et al. (1979) proposed the three-state model which described the cytochrome reaction as 'off', 'distal', and 'proximal' states, respectively. Although the electron transfer through the proximal state ($t_{1/2} = 1\ \mu s$) is viscosity-independent, the conversion from the distal to the proximal state is viscosity-dependent (Moser & Dutton, 1988). This suggests that the distal cytochrome c_2 undergoes rotational and/or translational diffusion to the proximal state. The E_a values were recently calculated to be 20.6 and 27.3 kJ·mol^{-1} for the fast and slow kinetic components (probably corresponding to the proximal and distal states), respectively, at 60% glycerol (Venturoli et al., 1993). It is of interest that the reaction of cytochrome c_2 which was cocrystallized with the RC complex of *Rb. sphaeroides* exhibits only a first phase kinetic component ($t_{1/2}$ = approximately 1 μs) (Adir et al., 1996). The high E_a values observed in *C. tepidum* RC therefore indicate the loose binding of the C-terminal heme moiety of the two cytochromes to the reaction site around P840. This suggests that each heme moiety on the RC surface may move to search for the coordination appropriate for the reaction with $P840^+$, respectively, although its N-terminal transmembrane helices are bound to the RC core [Figure 1(a)].

Anomalous Viscosity Dependency of the Reaction of C. tepidum Cytochrome c. Since the early 1980s, a large number of works have indicated protein reactions sensitive to the viscosity of the solvent. The observed rate constant k_{obs} has been shown to be inversely proportional to the fractional power of the viscosity η in most cases (eq 2).

$$k_{obs} = B\eta^{-\beta} \text{ or } (0 < \beta < 1) \qquad (2)$$

For example, biochemical reactions such as catalysis by carboxypeptidase A (Gavish & Werber, 1979) and the photodissociation rate of oxygen from myoglobin (Beece et al., 1980) and hemerythrin (Lavalette & Tetreau, 1988) gave $\beta = 0.4-0.6$. Other reactions have also been known such as spin transition in camphor-bound ferric cytochrome P-450 (β = approximately 1) (Marden & Hui Bon Hoa, 1982), proton exchanges in lysozyme ($\beta = 0.6$) (Somogyi et al., 1988), RNase T1 ($\beta = 1$), and Cod PA III ($\beta = 0.63$) (Rosenberg et al., 1989), and ester hydrolysis catalyzed by subtilisin BPN′ ($\beta = 0.65$) (Ng & Rosenberg, 1991). These data give β values less than unity and suggest that the Brownian motion of the solvent drives the viscosity-dependent conformational changes and/or structural fluctuations.

A new theoretical framework for the viscosity effect was given by Sumi (1991) as the introduction of a solvent-fluctuation-controlled mode to the transition-state-theory (TST). The TST established in the 1940s, which is useful to understand the mechanism of chemical reaction, assumes a priori that the distribution of population in the reactant state is always in thermal equilibrium in the course of the reaction. Therefore, viscosity does not appear in the formula describing a first-order rate constant ($\beta = 0$). However, the TST cannot be applied to a highly viscous solvent system in which the thermal fluctuations (Brownian motion) of solvent molecules are not fast enough to realize thermal equilibrium among the reactant states due to the friction between the reactant and the solvent. As the friction is usually proportional to the solvent viscosity (η), Kramers (1940) predicted that the rate constant is inversely proportional to the viscosity as the case with $\beta = 1$ in eq 2. This prediction ($\beta = 1$) of Kramers, however, has rarely been realized as mentioned above. Sumi (1991) assumed a two-dimensional reaction coordinate and proved the value of β in eq 2 to be $0 < \beta < 1$. In his theory, the observed rate constant k_{obs} can be described as

$$1/k_{obs} = 1/k_{TST} + 1/k_f \qquad (3)$$

where k_{TST} and k_f represent the TST-expected and the fluctuation-limited rate constants, respectively. In the low-viscosity solvents, where the solvent fluctuations are so fast ($k_f >> k_{TST}$), the reaction becomes TST-controlled: $1/k_{obs} = 1/k_{TST}$. On the other hand, in the extremely viscous solvents ($k_f << k_{TST}$), the reaction turns solvent-fluctuation-controlled: $1/k_{obs} = 1/k_f \propto \eta^{-\beta}$. In fact, the thermal *Z*/*E* isomerization of the stilbene derivatives was shown to fit to eq 3 over a wide range of viscosity under varied pressure in viscous liquids (Asano et al., 1994; Sumi, 1995).

The three-state model for the reaction between the RC complex and soluble cytochrome c_2 in *Rb. sphaeroides* (Overfield et al., 1979) seems to be useful to understand the cytochrome *c* reaction in the present study. The transition from the distal to proximal state, which is ascribable to a first-order reaction, was shown to be the reciprocal viscosity dependence (Moser & Dutton, 1988; Wachtveitl et al., 1993). The data reported by Wachtveitl et al. (1993) were replotted against η to test the Sumi theory also in Figure 5. The exponent β of this reaction was found to be 0.9. This indicates the reaction to be the solvent-fluctuation-controlled process.

In the case of cytochrome *c* oxidation in *C. tepidum* RC, on the other hand, the exponent β was found to be 1.9 and 2.4 at 283 and 295 K, respectively. The values are significantly higher than that ($\beta = 0.9$) observed in the distal−proximal change of cytochrome c_2 in *Rb. sphaeroides* RC. The *Chlorobium* cytochrome *c* has three hydrophobic, putative membrane-spanning helices in the N-terminal half. The helices seem to be bound to the RC complex and to function as an anchor for the C-terminal region with a heme *c* which may fluctuate on the RC surface searching for the reaction site(s) with P840. The cytochrome *c* might also mediate electrons to other components, such as cytochrome b/c_1 complex, situated in the vicinity of the RC.

The apparent activation energy of electron transfer from cytochrome *c* to P840 becomes smaller as the solvent viscosity η increases (see Table 2). This indicates that the reaction proceeds in the framework of the Sumi model because the theory predicts that the slow Brownian process without remarkable loss of energy determines the overall reaction rate at the high-viscosity region (Sumi & Asano, 1997). There is, however, no rational or theoretical explanation for the abnormally high β value at present. This might be related to the unique structure of the cytochrome *c* or to the existence of two cytochromes which react with one P840 (Oh-oka et al., 1995a). The cytochrome *c* might undergo structural changes or conformational substates with freedoms

in an order higher than that defined by the simple diffusive Brownian fluctuations postulated by Sumi theory. The high-viscosity effect on the electron transfer from cytochrome *c* to P840 in *Chlorobium* RC will be further studied.

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