Membrane-Bound Cytochrome c_z Couples Quinol Oxidoreductase to the P840 Reaction Center Complex in Isolated Membranes of the Green Sulfur Bacterium *Chlorobium tepidum*†

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ABSTRACT: The reaction of quinol oxidoreductase and membrane-bound *c*-type cytochromes was studied in chlorosome-depleted membranes isolated from *Chlorobium tepidum*. Rapid oxidations of *c*-type cytochromes were detected after flash excitation. Their re-reductions occurred in parallel with the reduction of cytochrome *b*, especially in the presence of antimycin A, whereas reductions of both cytochromes *c* and *b* were suppressed by added stigmatellin. These results indicate the tight coupling between the photosynthetic reaction center and quinol oxidoreductase. Turnovers of two types of cytochromes *c* were detected. One was assigned to the monoheme-type cytochrome *c* (designated cytochrome c_z), which is known to be tightly bound to the reaction center complex. The other was a new *c*-type cytochrome, cytochrome *c*-556, which functions the same as cytochrome c_1. The steps of electron-transfer scheme, menaquinol → Rieske FeS center → cytochrom *c*-556 → cytochrome c_z → P840, are estimated to have reaction times of 20 ms and 560, 150, and 40 μs, respectively. We conclude that quinol oxidoreductase and the reaction center complex in *Chlorobium tepidum* are linked by two distinct membrane-bound cytochromes, c_z and *c*-556, with no involvement of water-soluble cytochromes.

The cooperation of quinol oxidoreductase with the reaction center (RC)[1] complex characterizes photosynthetic energy conversion. In plants and cyanobacteria, the photosystem (PS) II RC donates electrons to the quinol oxidoreductase (cytochrome b_6/f complex) via a quinone pool and then to PS I RC via water-soluble small proteins plastocyanin or cytochrome c_6 (*1*). In purple photosynthetic bacteria, the RC is partially homologous to PS II RC, providing both reducing and oxidizing powers to the quinol oxidoreductase (cytochrome b/c_1 complex) via a quinone pool and water-soluble cytochrome c_2, respectively, to form the cyclic system (*2*, *3*). Membrane-bound cytochrome c_y also functions in parallel with cytochrome c_2 in *Rhodobacter capsulatus* (*4*). However, the electron mediator or mediators that connect quinol oxidoreductase with the RC complex have not been identified in green sulfur bacteria (Chlorobiaceae) (*5*), Gram-negative, strict anaerobes that form a phylogenic group separate from the other photosynthetic bacteria (*6*). These bacteria transfer electrons from sulfide or thiosulfate to NADPH in an unidentified electron-transfer pathway that couples the quinol oxidoreductase to the PS I-like RC. Elucidation of this coupling mechanism might give insight into the origin and mechanism of the cyclic electron transfer around PS I of the oxygenic photosynthesis.

The cytochrome b/c_1 (or b_6/f) complex catalyzes oxidation of quinol by water-soluble cytochrome *c* or plastocyanin, whose reaction is coupled with proton transfer with an overall stoichiometry of 2 H^+ per electron (*7*, *8*). A remarkable feature of the complex is the "oxidant-induced reduction" of cytochrome *b*. One of the electrons liberated in the two-electron quinol oxidation reduces cytochrome *b*; and the other reduces cytochrome c_1 (*f*) [via a Rieske iron−sulfur (FeS) center] by the Q-cycle mechanism originally proposed by Mitchell (*9*) and now supported by the three-dimensional structures determined by recent X-ray crystallographic studies (*10*, *11*). The function of quinol oxidoreductase in green sulfur bacteria has been suggested by the presence of a quinone pool (*12*), by photooxidation of cytochrome *b*-562 (*13*), by antimycin A-sensitive photoreduction of cytochrome *b* (*14*), by electron paramagnetic resonance detection of a Rieske-type FeS center (*15*), and by photoreduction of cytochrome *b*-562 concomitant with the oxidation of cytochrome *c*-551 (*16*). However, the connection between quinol oxidoreductase and the RC complex has not been clearly shown in these studies, which were performed under continuous illumination using crude membrane preparations. The activity of sulfide−quinone reductase (SQR), which catalyzes the reductions of cytochrome *c*-553 and *b*-563 by

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[1] Abbreviations: RC, reaction center; PS, photosystem; SQR, sulfide-quinone reductase; BChl, bacteriochlorophyll; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TMBZ, 3,3′,5,5′-tetramethylbenzidine; MQ, menaquinone.

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menaquinol and sulfide, has also complicated the situation (*17*). Moreover, a transcription unit (*fb*-operon) that encodes the Rieske FeS protein and cytochrome *b* in *Chlorobium limicola* (*18*) lacks a region encoding cytochrome c_1 that is normally contained in the *fbc*-operons of the other proteobacterial genomes (*19*, *20*). The counterparts of cytochrome c_1 and of water-soluble cytochrome c_2 have not yet been identified in green sulfur bacteria.

Three water-soluble *c*-type cytochromes, *c*-551, *c*-553, and *c*-555, are known to be contained in cells of *C. limicola* f. *thiosulfatophilum* (*21*−*23*). Cytochrome *c*-551 (a dimer of 27-kDa cytochrome *c*) is an electron acceptor for a thiosulfate−cytochrome *c* reductase and does not seem to be contained in some species of Chlorobiaceae (e.g., *C. vibrioforme*), which cannot use thiosulfate. Cytochrome *c*-553 is a flavo-cytochrome *c* (11-kDa cytochrome *c* plus 47-kDa flavoprotein subunits) and serves as a sulfide−cytochrome *c* reductase. Cytochrome *c*-555 (cytochrome *c*-554 in *C. tepidum* used in this paper), molecular mass 10 kDa, gives a characteristic asymmetrical α-absorption peak, as does algal water-soluble cytochrome c_6, which is an electron donor to PS I RC (*24*). Cytochrome *c*-555 seems to mediate electron transfer between the RC complex and cytochromes *c*-551 and *c*-553 in intact cells, as shown by Okumura et al. (*25*), who reported photooxidation of this cytochrome when it was added to isolated membranes. Albouy et al. (*26*) reported the flash-induced oxidations of cytochromes *c*-552, *c*-554/555, and *c*-553 and assumed their oxidation times to be <7 ms and ∼70 and 30 μs, respectively, in intact cells of *C. limicola* f. *thiosulfatophilum*. However, coupling of the reactions between quinol oxidoreductase and the RC complex has never been clearly identified.

Apart from the water-soluble cytochromes listed above, monoheme-type cytochrome *c*-552 with a molecular mass of 23 kDa—designated hereafter "cytochrome c_z" to characterize its functional similarity to the membrane-bound cytochrome c_y in *R. capsulatus* (*4*)—is tightly bound to the RC complex of *C. tepidum* (*27*, *28*), *C. limicola* f. *thiosulfatophilum* (*29*), or *C. vibrioforme* (*30*, *31*). Each of the two molecules of cytochrome c_z donates an electron to P840 in a reaction time of 150 μs (*28*) and shows an anomalously high dependency on the viscosity of the medium so that the heme-containing moiety of cytochrome c_z seems to fluctuate on the RC surface by anchoring to the membrane via its NH_2-terminal three transmembrane helices (*32*). Here, we report the mediation of the electron transfer between quinol oxidoreductase and the RC complex by cytochrome c_z and by the newly identified cytochrome *c*-556, which serves a function like that of cytochrome c_1.

MATERIALS AND METHODS

Chlorobium tepidum was cultured as described by Wahlund et al. (*33*) in 20 L of medium. Cells 2 days old were harvested by centrifugation and treated immediately to isolate their membranes to avoid damage to the cytochrome activity.

Chlorosome-depleted membranes were prepared according to a previously reported method (*34*, *35*) with some modifications as follows. All the procedures were carried out under anaerobic conditions at room temperature (*28*). The harvested cells (14−15 g wet weight) were suspended in 50 mL of buffer A (50 mM Tris-HCl, pH 8.0, containing 5 mM EDTA and 10 mM cysteine) with added protease inhibitors as used previously to isolate the RC complex (*28*) and were disrupted by four passages through a French pressure cell at 20 000 psi (138 MPa). Residual cells and large membrane fragments were removed by a series of differential centrifugations at 23 500*g* for 20 min and at 40000*g* for 60 min. The resulting supernatant (∼50 mL) enriched in bacteriochlorophyll (BChl) *a* content was laid on top of the discontinuous sucrose gradients [40, 42.5, 45, 47.5, and 50% (w/w) sucrose in buffer A] and centrifuged in an Hitachi RP 50−2 angle rotor at 200000*g* for 17−19 h. Broad green bands spread around the 45% layer were collected with a Pasteur pipet. The chlorosome-depleted membranes were obtained by centrifugation at 37500*g* for 1 h after three fold dilution of the collected fraction with Buffer B (50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and 2 mM dithiothreitol). Before the centrifugation, a solution of the glucose oxidase/catalase system (*28*) had been added to the diluted suspension and left for 20−30 min in an anaerobic chamber to completely remove any residual oxygen. After centrifugation, the membranes were resuspended in 4 mL of buffer B, giving a final absorbence of ∼17 *A* at 810 nm.

Water-soluble cytochromes were isolated basically according to the method of Meyer et al. (*21*). The asymmetrical α-absorption peak of low-molecular-mass (10-kDa) cytochrome *c* was at 554 nm in *C. tepidum* instead of at 555 nm as found in *C. limicola*.

We performed sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) by the method of Schägger et al. (*36*) and stained the separated protein bands with Coomassie brilliant blue. We stained heme with 3,3′,5,5′-tetramethylbenzidine (TMBZ) according to the method of Thomas et al. (*37*). Samples for SDS−PAGE were solubilized as described previously (*38*) except the concentration of β-mercaptoethanol was reduced to 1%. A kit of molecular-mass markers was purchased from Bio-Rad Laboratories.

Absorption and chemically oxidized-minus-reduced difference absorption spectra at 290 and 77 K were measured by a home-built spectrometer equipped with an image intensifier-coupled diode array and cryostat (*39*). Samples were suspended in buffer B containing 60% (v/v) glycerol. Flash photolysis was performed as previously described, at room temperature (*40*). Samples were placed in airtight cuvettes to avoid oxidative degradation of the redox centers.

Antimycin A and stigmatellin were purchased from Sigma and Fluka, respectively.

RESULTS

Determination of Cytochromes c Bound to the Isolated Membranes. Chlorosome-depleted membranes with a low content of water-soluble cytochromes were isolated from broken cells of *C. tepidum* by a series of differential centrifugations and discontinuous sucrose-density gradient centrifugation. Membrane-associated *c*-type cytochromes were analyzed with SDS−PAGE followed by heme staining, as shown in Figure 1. Three TMBZ-stained bands with apparent molecular masses of 23, 21, and 17 kDa were detected (Figure 1, lanes 2). The most prominent staining was given by the 23-kDa band, which migrated to the same

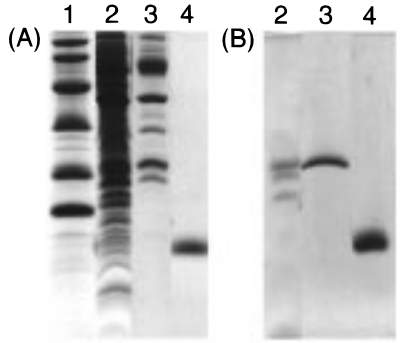


FIGURE 1: SDS−PAGE analysis of membrane-bound *c*-type cytochromes stained with (A) Coomassie brilliant blue or (B) TMBZ. Lane 1, molecular-mass markers: phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.5 kDa); lane 2, chlorosome-depleted membranes of *C. tepidum* (equivalent to 0.4 μg of BChl *a*); lane 3, RC complex of *C. tepidum* (equivalent to 0.9 μg of BChl *a*); lane 4, water-soluble cytochrome *c*-554 of *C. tepidum* (equivalent to 1.3 μg of protein).

position as the cytochrome c_z (Figure 1, lanes 3) that was contained in the purified RC complex (*28*). Water-soluble cytochrome *c*-554 with a molecular mass of 10 kDa was detected by neither SDS−PAGE analysis (Figure 1, lanes 4) nor optical analysis (discussed below). The other heme-containing bands detected at 21 and 17 kDa in Figure 1 seem to indicate thus-far-unidentified *c*-type cytochromes because their molecular masses differed from those of all other *c*-type cytochromes reported in this organism. Moreover, these two bands do not seem to be degradation products of the 23-kDa cytochrome c_z because the three TMBZ-stained bands can obviously be assigned to the three *c*-type cytochromes optically discriminated at 77 K (see next section).

Chemically Induced Difference Absorption Spectra of Cytochromes. The absorption spectra of cytochromes contained in the isolated membranes were measured at room and cryogenic temperatures. In the presence of ferricyanide, no α-peaks of cytochromes were detectable at 290 K (Figure 2A, lower panel, trace a). Addition of ascorbate produced a peak centered at 553 nm with an 11-nm half-bandwidth (Figure 2A, lower panel, trace b). Exactly the same spectrum was obtained in the hydroquinone-reduced membranes (not shown). Addition of dithionite (Figure 2A, lower panel, trace c) increased the absorbance at ∼553 nm and induced additional bands in the 560-nm region.

The heme species were further analyzed by measurement at 77 K (*41*, *42*). The 553-nm peak of the ascorbate-reduced membranes split into two peaks at 548.5 and 553.5 nm at 77 K (Figure 2A, upper panel). The position and the shape of the 548.5-nm peak were almost identical to those of cytochrome c_z, measured as the ascorbate reduced-minus-ferricyanide oxidized difference spectrum in the purified RC complex of *C. tepidum* (*28*) (Figure 2B). Cytochrome c_z, on the other hand, showed a peak at 552 nm with a 7-nm bandwidth at 290 K. Therefore, the *C. tepidum* membrane must contain an unknown high-potential *c*-type heme in addition to cytochrome c_z. The dithionite-minus-ascorbate difference spectrum at 77 K in Figure 2B further indicated a shoulder at 550 nm and two peaks at 557 and 561 nm, respectively, suggesting the reduction of low-potential *c*- and *b*-type hemes.

As shown in Figure 2B, the amplitude of the 548.5-nm peak of cytochrome c_z in the RC preparation was nearly equal to that of the 548.5-nm peak in the ascorbate-minus-ferricyanide difference spectrum of the membranes if we adjusted the concentration of the latter to give a 10% higher peak at 820 nm for BChl *a*. By subtracting the ascorbate-minus-ferricyanide difference spectrum of cytochrome c_z from that of the membranes, we obtained a spectrum for a *c*-type heme with the longer α-peak wavelength at 77 K (Figure 3, upper panel). Using the same ratio, we also substrated the spectrum of cytochrome c_z from a rather flat peak at 553 nm of the membranes at 290 K (Figure 3, lower panel). The spectrum of the additional high-potential heme thus obtained showed a peak at 556 and 553.5 nm at 290 and 77 K, respectively, the height of the peak being about half that of cytochrome c_z. These results allow us to interpret the more-intensive heme-staining band of heme c_z observed in the SDS−PAGE analysis (Figure 1) and suggest that the content of heme *c*-556 in the membranes is less than that of heme c_z.

Cytochrome c_z is known to serve as an immediate electron donor to P840 (the primary electron donor in *Chlorobium*) with a redox midpoint potential (E_m) of $+170 \pm 10$ mV (*25*, *43*). Preliminary measurements of the membranes poised at several moderate redox potentials gave almost invariant spectra with peaks at 553 nm, even though the peak heights differed (data not shown). This suggested, therefore, that both hemes c_z and *c*-556 have similar redox potentials of 150−200 mV.

The results in Figures 1−3 indicate the presence of three *c*-type and two *b*-type hemes in the isolated membranes of *C. tepidum*. Cytochromes c_z and *c*-556 were reduced by ascorbate, whereas the cytochrome *c* that absorbs at 552 or 550 nm at 290 or 77 K, respectively, was reduced by dithionite. However, both of the *b*-type hemes which absorb at 560 or 557 and 563 or 561 nm at 290 or 77 K, respectively, reduced only by dithionite.

Flash-Induced Absorption Changes of Cytochromes. Flash-induced absorption changes of the *c*-type heme(s) were monitored at 553−540 nm (Figure 4A) in the isolated membrane preparation. Flash excitation induced a rapid bleach of the *c*-type heme followed by rapid re-reduction. The re-reduction rate slowed a little in the presence of antimycin A (Figure 4A, trace b), a compound known to inhibit cytochrome *b* oxidation at the Q_i-site of quinol oxidoreductase (*7*, *44*). Upon addition of stigmatellin, which inhibits quinol oxidation at the Q_o-site (*7*, *44*), the re-reduction was substantially suppressed (Figure 4A, trace c), as has been reported previously by Okumura et al. (*25*). In contrast, reduction of the *b*-type heme monitored at 560 nm was markedly increased by the addition of antimycin A (Figure 4B, trace b) and was fully suppressed by stigmatellin (Figure 4B, trace c). The fast re-reduction rate of *c*-type heme and a small reduction/oxidation of *b*-type heme observed in the absence of the inhibitors also suggested the activity of quinol oxidoreductase (Figure 4, trace a). These measurements were performed in the presence of 2 mM dithiothreitol, which fully reduced cytochromes c_z and *c*-556 but not the low-potential cytochromes *c* and *b*; ascorbate had the same effects. The reduction rates of cytochromes,

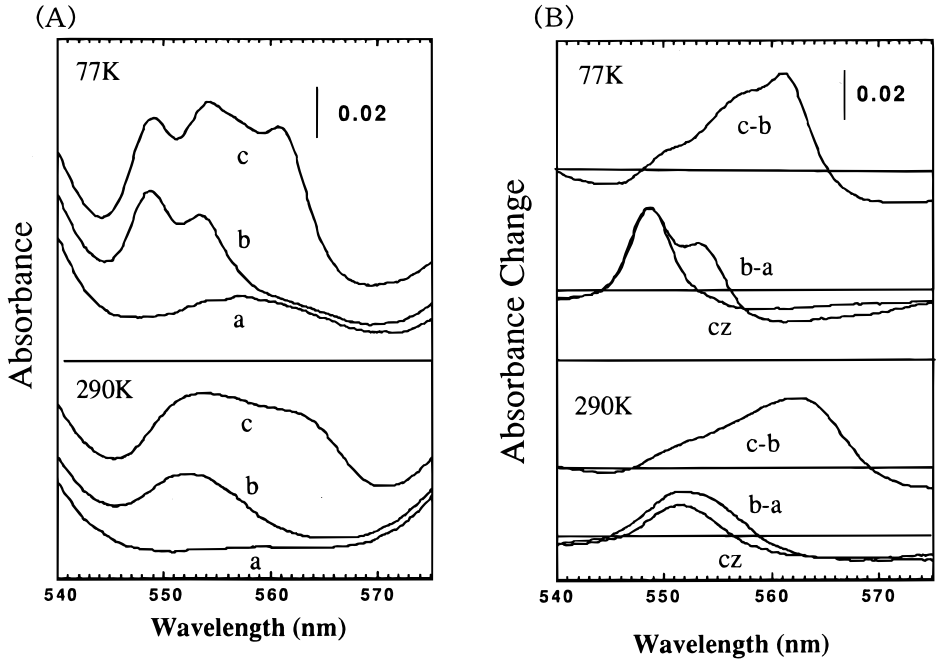


FIGURE 2: (A) Chemically induced absorption spectra of *c*- and *b*-type hemes in the membranes of *C. tepidum* at 290 (lower panel) and 77 K (upper panel). The sample (A_{810} = 1.6), suspended in buffer B containing 60% glycerol (v/v), was oxidized by the addition of a small amount of potassium ferricyanide (trace a) and then reduced by an excess amount of sodium ascorbate (trace b), followed by further reduction with sodium dithionite (trace c). (B) Chemically reduced-minus-oxidized difference absorption spectra of *c*- and *b*-type hemes at 290 (lower panel) and 77 K (upper panel). Shown are the ascorbate-minus-ferricyanide (b − a) and dithionite-minus-ascorbate difference spectra (c − b). The spectrum of cytochrome c_z in the purified RC complex (A_{810} = 1.6) is also shown (see text for details).

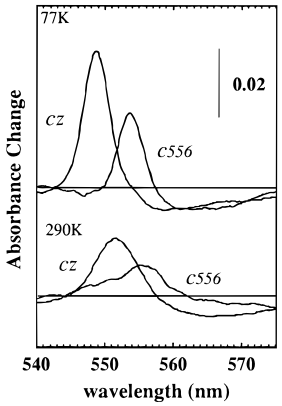


FIGURE 3: Comparison of the difference absorption spectra of cytochromes c_z and *c*-556 at 290 (lower panel) and 77 K (upper panel). The spectrum of cytochrome *c*-556 was obtained as described in the text.

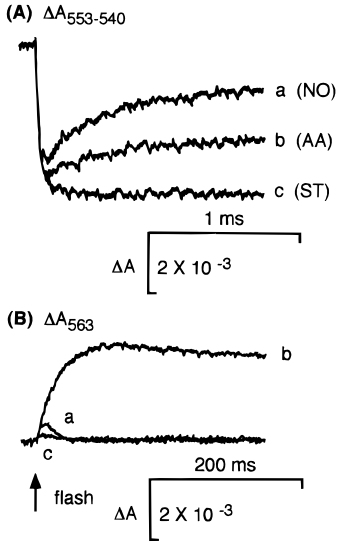


FIGURE 4: Flash-induced absorption changes monitored at (A) 553−540 nm (cytochrome *c*) and (B) 563 nm (cytochrome *b*) in membranes of *C. tepidum*. Traces a, b, and c represent no addition (NO) or additions of 20 μM antimycin A (AA) or 20 μM stigmatellin (ST). Measurements were carried out at 295 K. Concentrations of samples were adjusted to be A_{810} = 2.0.

however, were accelerated more by dithiothreitol than by ascorbate, which suggests the electron donation from the quinol pool (discussed later).

In Figure 5, the kinetics were measured over a wider time range, between 1 μs and 1 s, with a time resolution of 7 μs. Note that the kinetics in the shorter time ranges are expanded, given the logarithmic expresion of time (*x*-axis). The absorption changes of P840 were also monitored at 540 nm (panel A). The $P840^+$ formed by the flash excitation was re-reduced with a time constant ($t_{1/e}$) of 40 μs, parallel to the oxidation of heme *c*. The reaction time was faster than that ($t_{1/e}$ = 150 μs) observed in the solubilized RC preparations (*28*, *32*). The incomplete re-reduction of $P840^+$ after the full oxidation of heme *c* indicates a small difference between their redox midpoint potentials (*28*). As expected, these kinetics were not affected by the addition of inhibitors.

In the absence of inhibitors (closed circles in Figure 5), the re-reduction of cytochrome *c* was simulated as a combination of three exponential phases with $t_{1/e}$ values of 560 μs and 20 and 500 ms, whose estimated contributions to the total amplitude were 78, 12, and 10%, respectively. Small absorption changes at 563 nm were also detected at ∼0.5 ms and over a longer time range. The kinetics in the presence of antimycin A (open circles in Figure 5) was fitted by the 41% ($t_{1/e}$ = 560 μs), 44% ($t_{1/e}$ = 20 ms), and 15% ($t_{1/e}$ = 500 ms) contributions of each phase. The reduction

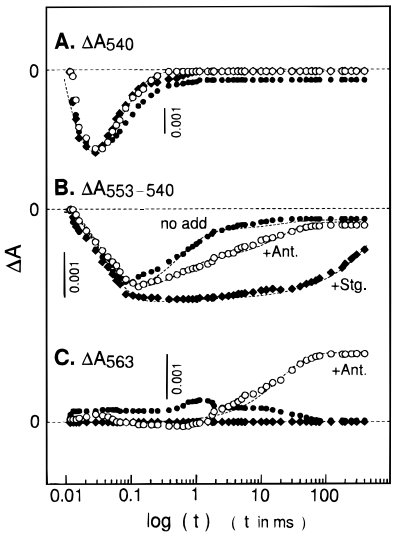


FIGURE 5: Redox changes of P840 and cytochromes *c* and *b* after the flash excitation, monitored by absorption changes at 540 nm (inverted) (A), 553−540 nm (B), and 563 nm (C) between 1 μs and 1 s in membranes of *C. tepidum*. Each kinetics represents no addition (closed circles) or additions of 20 μM antimycin A (open circles) or 20 μM stigmatellin (closed squares). The other experimental conditions were similar to those in Figure 4.

of the *b*-type heme proceeded in parallel with the re-reduction phase of the *c*-type heme. In the presence of stigmatellin (closed squares in Figure 5), ~90% of the total amplitude decayed in the slow phase ($t_{1/e}$ = 500 ms) with no absorption change of cytochrome *b*.

Spectral Changes of Cytochromes. Time-resolved difference absorption spectra were measured after flash excitation. In the absence of inhibitors, a difference spectrum with a sharp peak at 552 nm was observed after 125 μs (Figure 6A). The narrow spectrum indicated the fast oxidation of cytochrome c_z. A shoulder at ~556 nm then increased (see the spectrum at 250 μs and later), suggesting the subsequent oxidation of cytochrome *c*-556. Re-reduction afterward was not accompanied by any significant spectral changes.

In the presence of antimycin A, a spectrum just after the flash excitation also showed a narrow bleaching at 552 nm (Figure 6B). At 625 μs after the flash, the peak height decreased without significant changes in the 560-nm region. Its further decrease accompanied a parallel increase in the absorption at 563 nm, showing the concomitant reduction of *b*-type cytochrome. The maximal amplitudes of the absorption peaks at 552 and 563 nm were nearly equal to each other, suggesting a one-to-one stoichiometric reaction between *c*- and *b*-type hemes. This clearly demonstrates the oxidant (oxidized cytochrome *c*)-induced reduction of cytochrome *b* expected from the Q-cycle model (*9*) and clearly indicates the tight coupling of quinol oxidoreductase with the RC complex.

Time-resolved spectra obtained in the presence of stigmatellin indicated the sequential oxidations of two *c*-type hemes more clearly (Figure 6C). The difference spectrum at 125 μs showed a narrow peak at 552 nm. The 556-nm shoulder then developed by 1 ms, after which the spectral shape did not change substantially until 38 ms. However, the spectrum at 125 μs gave a bandwidth somewhat wider than that of cytochrome c_z (the dotted curve in Figure 6C), suggesting that some part of the heme *c*-556 had already been oxidized even at this time.

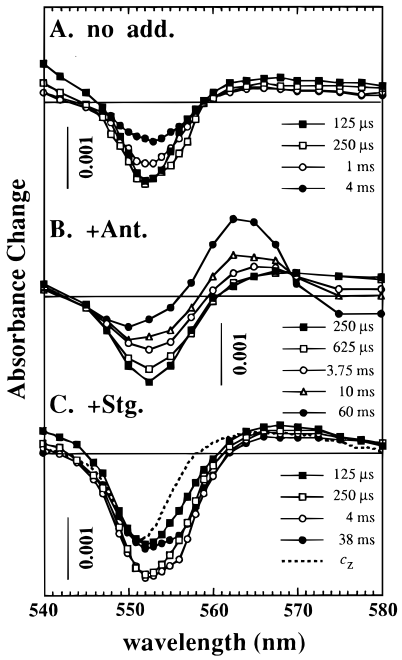


FIGURE 6: Time-resolved flash-induced difference absorption spectra of *c*- and *b*-type cytochromes in the absence of inhibitors (A) or in the presence of antimycin A (B) or stigmatellin (C) in membranes of *C. tepidum*. Times shown are those after the flash excitation. Each data point was obtained from the kinetics as measured in Figure 5. The spectrum of cytochrome c_z in the purified RC complex is also shown (dotted curve).

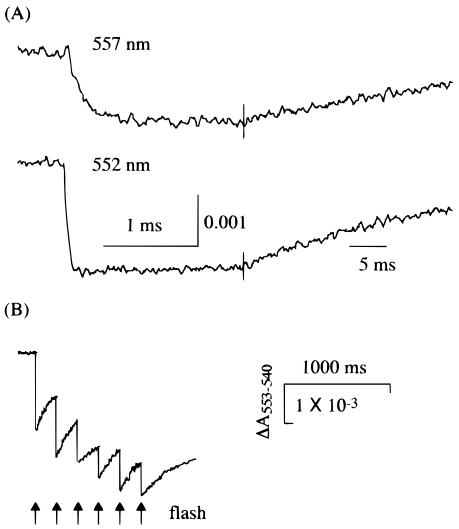


FIGURE 7: (A) Time courses of the absorption changes monitored at 552 and 557 nm in the presence of stigmatellin after flash excitation. (B) Multiflash-induced absorption changes monitored at 553−540 nm in the presence of stigmatellin. Absorption changes were induced by six laser flashes at 200-ms intervals. The other experimental conditions were similar to those in Figure 4.

To determine the reaction times of the two *c*-type cytochromes in the faster time ranges, we measured the absorption changes at 552 and 557 nm in the presence of stigmatellin (Figure 7A). Their absorbances decreased with $t_{1/e}$ values of 55 μs at 552 nm and 155 μs at 557 nm. On the other hand, Figure 3 indicates the peak and isosbestic wavelengths for cytochrome c_z to be at 552 and 545/557 nm, respectively; those of cytochrome *c*-556 are 556 and 545/562 nm, respectively. The absorption change at 552 nm is thus expected to result from a mixture of cytochromes c_z

and *c*-556 and that at 557 nm to be almost exclusively from cytochrome *c*-556. The actual oxidation time of cytochorme c_z could be estimated as 40 μs from the kinetics at 552 nm, when we eliminated the contribution of cytochrome *c*-556 at 552 nm by using the time course at 557 nm (not shown). In the absence of the inhibitors, both cytochromes c_z and *c*-556 were re-reduced in parallel, with $t_{1/e}$ values of 500 μs (Figure 6A).

We also examined the number of photooxidizable heme *c* molecules per P840 by using multiple flash excitations in the presence of stigmatellin. The total extent of the absorption change monitored at 553 nm after a train of six flashes was nearly twice (1.8-fold) that after the first flash (Figure 7B). Similar experiments in the presence of sucrose monolaurate detergent, which is expected to dissociate membrane-bound protein complexes and to decouple their mutual linkage, also showed a 2.2-fold absorption change after the six flashes (data not shown). These results indicate that 2−3 molecules of *c*-type hemes can be oxidized by P840.

DISCUSSION

Oxidant-Induced Reduction of Cytochrome b. In the present work, we have demonstrated the reduction of the *b*-type cytochrome coupled with the re-reduction of the flash-oxidized *c*-type hemes (the oxidant-induced reduction of cytochrome *b*). The results indicate the tight coupling of electron transfer between quinol oxidoreductase and the RC complex in *C. tepidum*.

The effects of antimycin A and stigmatellin on the kinetics of the *c*- and *b*-type cytochromes observed in this study are very similar to those reported in the typical cytochrome b/c_1 complex in mitochondria or purple photosynthetic bacteria (*7*, *45*) and are well interpreted by the Q-cycle mechanism (*9*). Antimycin A is known to inhibit the quinone reduction at the Q_i-site adjacent to heme b_H, and stigmatellin inhibits the quinol oxidation at the Q_o-site adjacent to heme b_L (*44*), as shown in the three-dimensional structures of the cytochrome b/c_1 complexes (*10*, *11*). The low extent of *b*-type heme reduction detected in the absence of inhibitors may also be interpreted as the concomitant oxidation of the *b*-type heme (heme b_H) on the Q_i-site coupled to the oxidant-induced reduction of the *b*-type heme (heme b_L) on the Q_o-site (*9*).

The amino acid sequences of *Chlorobium* cytochrome *b* and Rieske protein show some homologies to those of their counterparts in the cytochrome b_6/f complexes of chloroplasts and cyanobacteria (*18*). The high sensitivity of quinol oxidoreductase in *C. tepidum* to antimycin A, however, contrasts with the low sensitivity of the cytochrome b_6/f complex to this inhibitor, suggesting different structures around the Q_i-sites as revealed by the comparison between their amino acid sequences (*18*). The features presented here rather resemble those of the cytochrome b/c_1 complex (*7*, *45*). The most remarkable feature of the quinol oxidoreductase in the isolated membranes of *C. tepidum* seems to be the tight coupling with the RC complex, which is mediated only by the membrane-bound cytochromes c_z and *c*-556.

Reaction Scheme from Quinol to P840. The results in the present study suggest the following electron-transfer sequence and reaction times (see also Figure 8).

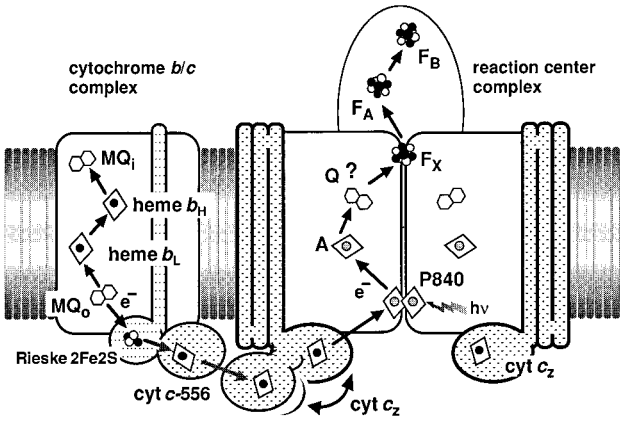
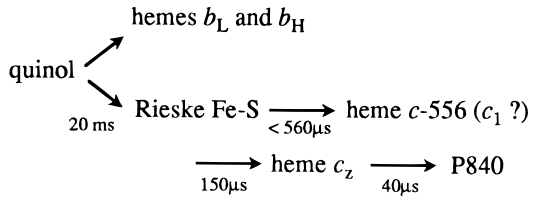


FIGURE 8: Schematic illustration of quinol oxidoreductase (cytochrome *b*/*c*) and the RC complex in *C. tepidum*. Conformational gating of membrane-bound cytochrome c_z mediates electron transfer between the two complexes.



The flash excitation oxidized P840 and then heme c_z with $t_{1/e} = 40$ μs. The hemes c_z and *c*-556 equilibrated to each other with $t_{1/e} = 150$ μs. The oxidized hemes were partially re-reduced, presumably by the Rieske FeS center without changes in the redox state of hemes *b* ($t_{1/e} < 560$ μs); this was followed by their complete re-reductions, which occurred in parallel with the reduction of cytochrome *b* as in the presence of antimycin A ($t_{1/e} = 20$ ms). Apparently, hemes c_z and *c*-556 sequentially mediate the electron transfer between $P840^+$ and quinol oxidoreductase. Therefore, heme *c*-556 functions as a counterpart to heme c_1 in the ordinary cytochrome b/c_1 complex (although it is still possible that heme *c*-556 is on more of a side path). The two *b*-type hemes that absorb at 560 and 557 and at 563 and 561 nm at 290 and 77 K, respectively (see Figure 2), will thus serve as hemes b_H and b_L, respectively, or vice versa. The rate of the electron flow from quinol also depends on the reduction level of the quinone pool. The function of low-potential heme *c*-552, which is reducible only with dithionite (see Figure 2), remains to be studied.

What then is the role of water-soluble, low-molecular-mass (10-kDa) cytochrome *c* in green sulfur bacteria? The cytochrome *c*-554 in *C. tepidum* (*c*-555 in *C. limicola*) might operate between the quinol oxidoreductase and the RC complex in parallel with cytochrome c_z or c_{556} (c_1) or both, or it might be specifically involved in the metabolism of sulfide or thiosulfate oxidation(s). Kjærulff et al. (*46*) reported difficulty in obtaining transformants that lack cytochrome c_z in *C. vibrioforme*, which suggests an indispensable function for this cytochrome in the electron-transfer pathway. It is not clear, however, how the reaction scheme described above helps to interpret the recently postulated cytochrome reactions in intact cells, which support somewhat different reaction times and absorption peaks (*26*).

Stoichiometries of c-Type Hemes to P840. The contents of *c*-type hemes in the membranes can be estimated from the chemically induced difference absorption spectra at 290 K (see Figures 2 and 3) by assuming the (difference) absorptivities as follows; $\Delta\epsilon = 20$ mM^{-1} cm^{-1} for the α-peak of each heme *c*, $\Delta\epsilon_{850} = 52$ mM^{-1} cm^{-1} for P840, and $\epsilon_{810} = 100$ mM^{-1} cm^{-1} for BChl *a*. We recalculated the value of $\Delta\epsilon_{850}$ from the P840 difference absorption spectrum obtained in the isolated RC complex by using $\Delta\epsilon_{830} = 100$ mM^{-1} cm^{-1} to minimize any error caused by the overlapping bleach or shift of antenna BChl *a* around a 810-nm region observed in membranes (data not shown). The present membrane preparation was estimated to contain (per mole of BChl *a*) 0.043 and 0.026 mol of high-potential hemes c_z and *c*-556, respectively, and 0.028 mol of low-potential heme *c*-552. The molar ratios of the hemes c_z, *c*-556, *c*-552, and total BChl *a* to the P840 were then calculated to be 2.1, 1.3, 1.4, and 55, respectively. Because the purified RC complex of *C. tepidum* has two identical molecules of cytochrome c_z per P840 (*28*), we could therefore expect almost equal amounts of quinol oxidoreductase (cytochrome b/c_1 complex) and the RC complex when we considered the stoichiometry to be ∼2 cytochromes c_z per 1 cytochrome *c*-556.

The experiments in Figure 7B indicated the 1.8-fold larger absorption change at 553 nm after the series of six-flash excitations. This suggests the existence of 2−3 molecules of photooxidizable *c*-type hemes per P840 (note that heme *c*-556 has lower absorption at 553 nm), as was estimated by Okumura et al. (*25*) in the *C. tepidum* membranes. Franken and Amesz (*47*) also detected the turnover of 2 hemes *c* per RC in the *Prosthecochloris aestuarii* membranes. They interpreted the finding as showing the sequential electron transfer between 2 hemes with similar E_m values but with slightly different peak wavelengths in tetraheme-type cytochrome *c*. However, their results might be better understood as the sequential function of the high-potential hemes c_z and *c*-556. In fact, the anomalously high viscosity dependence of the oxidation rate of heme *c* they reported is a unique feature of the reaction of cytochrome c_z (*32*). These results are thus consistent with the conclusion of the present study that >2 hemes—at least 1 or presumably 2 hemes c_z and 1 heme *c*-556—are linked to P840. The precise stoichiometry of the cytochromes that actually function in the coupling between quinol oxidoreductase and the RC complex is now under investigation.

In other species of green sulfur bacteria, the membrane-bound cytochromes as well as their coupling to quinol oxidoreductase or to the RC complex (or both) have not yet been clearly shown. The isolated RC complex of *C. vibrioforme* was reported to contain 0.8-1.2 molecules of cytochrome c_z (*30*, *31*), which shows 84% homology of amino acid sequences to the cytochrome c_z of *C. tepidum* (*32*). The 32-kDa, tetraheme-type cytochrome *c* recently isolated from the membranes of *C. limicola* (*48*) was not, however, detected in the present membranes of *C. tepidum* (see Figure 1).

Architecture of Quinol Oxidoreductase in C. tepidum. A typical cytochrome b/c_1 complex contains 1 heme on cytochrome c_1, a high-potential [2 Fe−2 S] center on Rieske protein, and high- and low-potential hemes on cytochrome *b* (see Figure 8). They are encoded on genes designated *petA*, *B*, and *C* (or *fbcF*, *B*, and *C*), respectively, which are organized in this sequence as one transcriptional unit (*fbc*-operon) in most proteobacteria (*19*, *20*). In *Chlorobium*, the *petC* gene could not be identified with a sequential gene cluster of *petA* and *B* (*fb*-operon) (*18*). A crude fraction rich in cytochrome *b* from the membranes of *C. limicola* also lacked cytochrome c_1 and contained a small amount of cytochrome c_z (*49*). However, the present results suggest that cytochrome *c*-556 plays the role of cytochrome c_1, although its estimated molecular mass of 21 or 17 kDa is somewhat smaller than those of the ordinary cytochrome c_1 molecules (30−35 kDa) (*19*, *20*). Recently, an unusual cytochrome c_1 (29 kDa), showing an NH_2-terminal portion similar to the subunit IV of b_6/f complexes, has been reported in *Bacillus subtilis* (*50*). The primary structure of cytochrome *c*-556 is therefore of interest and now under investigation.

The electron-transfer scheme in Figure 8 seems to constitute a reasonable energy conversion system in *C. tepidum*. The E_m values for P840 and cytochrome c_z are +235 ± 10 mV and +170 ± 10 mV, respectively (*25*, *43*). Although the E_m value of heme *c*-556 is unknown, it should be close to the reported +160 mV E_m value of a Rieske FeS center (*15*), judging from the partial reduction by the Rieske FeS center detected in this study. The time-resolved difference spectra observed at longer times seem to indicate a mixture of hemes c_z and *c*-556 in the ratio of 1.0 to 0.7−1.0. This suggests that the E_m of heme c_z is 0−10 mV more positive than that of heme *c*-556. The E_m values of −70 and −140 mV were reported for hemes b_H and b_L, respectively (*17*), values 100−150 mV more negative than those of their counterparts in plant chloroplasts, cyanobacteria, and purple bacteria. However, menaquinone-7 (MQ-7) with an $E_m = -81$ mV (*12*) in the *Chlorobium* membrane has a reducing power strong enough to operate the Q-cycle. Another major quinone in the same membrane, 1-oxo-MQ-7 (*Chlorobium* quinone), with a more positive E_m (+35 mV), might be involved only in the SQR activity (*51*). The membrane-bound cytochromes c_z and *c*-556, which are situated close to each other, seem to efficiently connect the Rieske FeS center and P840, even with small energy gaps. The connecting system is apparently very different from that between cytochrome b_6/f complex and PS I RC in the oxygenic photosynthetic organisms (*1*), although the connection on the reducing side in *Chlorobium* membranes still remains to be studied.

The coupling of quinol oxidoreductase and the RC complex has also recently been demonstrated by flash-induced turnovers of *b*- and *c*-type hemes in whole cells of *Heliobacillus mobilis* (*52*). Similar membrane-bound cytochromes might be involved in the coupling reaction because the heliobacteria (Heliobacteriaceae), which are Gram-positive photosynthetic bacteria containing the PS I-type RC complex (*53*), are considered to have no water-soluble cytochromes (*54*).

The properties of cytochrome c_z resemble those of the membrane-bound cytochrome c_y that functions in parallel with cytochrome c_2 in *R. capsulatus* (*4*). Cytochrome c_y, which has 199 amino acid residues (*55*) and a single membrane-spanning helix in the NH_2-terminus, donates an electron to the RC complex ($t_{1/2} \leq 100$ μs) and is re-reduced concomitantly with the reduction of cytochrome *b*. That their functions are analogous seems to be supported by the

common structural features of their NH_2-termini anchored to the membranes, although their amino acid sequences show no meaningful similarities to each other. In summary, we have shown that the efficient electron-transfer system mediated by the cascade of membrane-bound cytochromes so far reported in *R. capsulatus* is also distributed in a different phylum of photosynthetic bacteria, Chlorobiaceae. This system might represent the one of the early forms in the evolution of energy-coupling between membrane-bound supermolecular complexes.

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