Cytochrome $bc_1$-$c_y$ Fusion Complexes Reveal the Distance Constraints for Functional Electron Transfer Between Photosynthesis Components*

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Photons are essential for the survival of life on Earth. Among the various photosynthetic organisms that harness the energy of sunlight, purple non-sulfur bacteria (Ps) play a crucial role. These microorganisms use light to produce essential compounds like oxygen and energy, primarily through the process of photosynthesis. In this study, the authors aimed to further characterize the cytochrome complex in Ps bacteria and to understand the constraints for functional electron transfer between photosynthesis components.

The authors used a variety of techniques, including light-activated, time-resolved kinetic spectroscopy, to analyze the electron transfer rates and distances within the Ps cytochrome complex. They demonstrated that the minimal distance allowed between the cytochromes is critical for efficient electron transfer. Furthermore, they showed that the length of the cytochrome linker, which connects the cytochromes to the membrane anchor, affects the rate of electron transfer.

This study is significant because it sheds light on the structural and functional aspects of the Ps cytochrome complex, providing insights into the efficiency of electron transfer in these organisms. Understanding these mechanisms can have implications for the development of artificial photosynthetic systems and the design of biofuel production processes.
Functional Cyt bc1-cy Fusions with Variable Length Cyt cy Linkers

S-cy) that this electron carrier needs not be membrane anchored to support Ps growth of R. capsulatus. In this work, we have exploited the functional cyt bc1-cy fusion complex that we constructed earlier by fusing genetically cyt cy to the cyt bc1 complex (19) to probe the physical proximities of the Ps components to one another in R. capsulatus. We surmised that if the native length of the cyt cy linker is optimized for efficient electronic coupling across the distance between the cyt bc1 complex and the RC, then progressively shortening it might divulge the minimal distances separating these physiological partners in Ps membranes. Characterization of the physico-chemical properties of both membrane-embedded and highly purified cyt bc1-cy fusion complex variants indicate that the shortest functional cyt cy linker is about 45 residues long. This short linker still insures rapid ET from the cyt bc1 to the RC, but exhibits decreased electronic coupling to the RC, thereby limiting cyclic ET and Ps growth. Thus, Ps cyclic ET components appear to be tightly packed together, forming membrane-embedded large structural complexes.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Bacterial strains and plasmids used in this study are listed in Table 1. R. capsulatus strains were grown at 35 °C in mineral-peptone-yeast extract-enriched media (MPYE) supplemented with antibiotics as needed (10, 2.5, and 10 μg/ml kanamycin, tetracycline, and spectinomycin, respectively) under either semiaerobic/dark (respiratory), or photoheterotrophic/light (Ps) conditions using anaerobic jars and H2 and CO2 generating gas packs (BD Biosciences), as described previously (20). Ps growth curves were obtained using screw-cap tubes, incubated at 35 °C in an aquarium filled with water and illuminated by tungsten (Lumilane, Sylvania) lamps at an intensity of 150 μE/m²/s. Culture turbidity was monitored with a Klett-Summerson photometer equipped with a red (number 66) filter as described (21).

Molecular Genetic Techniques—PCR amplification of a 0.7-kb carboxyl-terminal FLAG epitope-tagged allele of cycY on plasmid pHM7 (10) using mutagenic primers YO5EcoRV (5′-GCCGCGGAGATCTGCTGTCAAAGCAGCACATC-3′) and Yo6HindIII (5′-GCGGGCAAGCTTGCAAAGATGTGAGGCG-3′) replaced the initiating methionine (ATG) of cycY with leucine (CTG). This PCR product was then digested with EcoRV and HindIII restriction enzymes and ligated into the StuI and HindIII sites of plasmid pMTS1 with the 1.2-kb HindIII-BamHI fragment containing the gentamycin resistance gene by blunt end ligation, yielding pYO37. This pYO37 allele was then transferred using Gene Transfer Agent into the chromosome of R. capsulatus strain FJ2 (∆cycA, ∆cycY) (5) to yield the triple mutant YO2, lacking both the cyt bc1 complex and electron carrier cyts c-y and c-y.

Biochemical Techniques—Intracytoplasmic (chromatophore) membranes were prepared as described previously (19), except where noted 1 mM e-aminocaproic acid and 100 mM EDTA were added to minimize proteolysis following cell disruption. The R. capsulatus cyt bc1 complex was purified as described previously (22). Protein concentrations were determined using the bicinchoninic acid method (23) with bovine serum albumin as a standard. SDS-PAGE (15%) were run as described in Ref. 24, and prior to loading, samples were solubilized in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.1 M dithiothreitol, 25% glycerol, and 0.01% bromophenol blue with subsequent incubation at 60 °C for 10 min. Cytochromes c were visualized by their heme peroxidase activities using 3,3′,5,5′-tetramethylbenzidine (TMBZ) and H2O2 according to Thomas et al. (25).

Optical spectra were recorded on a PerkinElmer UV-visible spectrophotometer Lambda 20. Absorption difference spectra for the c- and b-type cytochromes were obtained using chromatophore membranes (0.3 mg of total protein/ml), oxidized by adding a crystal of potassium ferricyanide, and reduced by a few grains of either solid sodium ascorbate or sodium dithionite, as appropriate. Time-resolved, light-activated kinetic spectroscopy was performed on a dual wavelength kinetic spectrophotometer with chromatophore membranes resuspended in 50 mM MOPS buffer containing 100 mM KCl (pH 7.0) in the presence of the following redox mediators (with their respective midpoint redox potential, E m(z) ): 100 μM ferricyanide (430 mV), 8 μM 2,3,5,6-tetramethyl-p-phenylenediamine (260 mV), 6 μM 1,2-naphthoquinone (NQ, 145 mV), 1 μM phenazine methosulfate (80 mV), 1 μM phenazine ethosulfate (50 mV), 6 μM 2-hydroxy-1,4-naphthoquinone (HNQ, −145 mV), 6 μM benzyl viologen (−359 mV), and a membrane potential uncoupler (2.5 μM valinomycin), as described (26). The amount of chromatophore membranes used in each assay was normalized to the RC content, as determined by measuring the flash-induced optical absorbance difference between 605 and 540 nm at an E₅₃₀ of 380 mV, and using an extinction coefficient of 29.8 mm⁻¹ cm⁻¹. Transient cyt c re-reduction and cyt b reduction kinetics at an ambient potential of 100 mV, initiated by a short saturating flash (~8 μs) from a xenon lamp were followed at 550–540 and 560–570 nm, respectively. Antimycin, myxothiazol, and stigmatellin were used as indicated at 5, 5, and 1 μM, respectively.

Optical potentiometric titrations were performed with the purified cyt bc1-cy fusion complex (0.1 mg/ml) in 50 mM MOPS buffer (100 mM KCl, pH 7.0) with the following mediators: 20 μM tetrachlorohydroquinone (350 mV), 20 μM 2,3,5,6-tetramethyl-p-phenylenediamine, 20 μM 1,2-naphthoquinone 4-sulfonate (210 mV), 20 μM NQ, 10 μM phenazine methosulfate, 10 μM phenazine ethosulfate, 40 μM...
d duroquinone (5 mM), 20 μM pyocyanine (PCN, −34 mM), 6 μM indigotrisulfonate (−90 mM), 20 μM HNQ, 20 μM ant-roquinone 2-sulfonate (−225 mM). The optical changes that accompanied the $E_h$ changes were recorded in the α-band region (500 to 600 nm), and the $E_m$ values were determined by fitting the normalized absorption data to a single component $n = 1$ Nernst equation. EPR spectroscopy was performed at sample temperatures of 10 or 20 K using a Bruker ESP 300E spectrometer (Bruker Biosciences), fitted with an Oxford instruments ESR-9 helium cryostat (Oxford Instrumentation Inc.). Spectrometer settings were as indicated in the appropriate figure legends.

**Functional Cyt bc$_1$-c$_y$ Fusions with Variable Length Cyt c$_y$ Linkers**

**RESULTS**

**Design of the Cyt bc$_1$-c$_y$ Fusion Complexes with Shorter Cyt c$_y$ Linker Lengths**—The availability of a functional cyt bc$_1$-c$_y$ fusion complex (19) allowed us to probe whether the cyt c$_y$ linker could be used as a “molecular ruler” to estimate the distances between the Ps ET components. Comparison of *R. capsulatus* cyt c$_y$ with its counterparts from other species indicated that the cyt c$_y$ domain is highly similar to that from *Silicibacter pomeroyi* (71%), *Paracoccus denitrificans* (70%), and *R. sphaeroides* (65%). On the other hand, the linker regions of *P. denitrificans*, *R. sphaeroides*, and *S. pomeroyi* are about 20 to 30 residues shorter than that of *R. capsulatus* (Fig. 1A). Generation of computer-assisted hypothetical three-dimensional structures of the cyt bc$_1$-c$_y$ fusion complexes with shorter linkers directed us to the region between amino acids 65 and 90 of cyt c$_y$ to mimic its non-functional variants as Ps electron carriers (Fig. 1B). Considering that the *R. sphaeroides* cyt c$_y$ linker is 26 amino acids shorter than its *R. capsulatus* counterpart, plasmids pYO30 (with a 24-amino acid long deletion between positions Ala$^{63}$ and Pro$^{88}$ of cyt c$_y$) (Δ24-c$_y$) and pYO33 (with a 19-amino acid long deletion between the positions Ala$^{67}$ and Pro$^{88}$ of cyt c$_y$ (Δ19-c$_y$)) were constructed to determine the Ps ET of each electron carrier (Table 1). The availability of a functional cyt bc$_1$-c$_y$ fusion complex (19) allowed us to probe whether the cyt c$_y$ linker could be used as a “molecular ruler” to estimate the distances between the Ps ET components. Comparison of *R. capsulatus* cyt c$_y$ with its counterparts from other species indicated that the cyt c$_y$ domain is highly similar to that from *Silicibacter pomeroyi* (71%), *Paracoccus denitrificans* (70%), and *R. sphaeroides* (65%). On the other hand, the linker regions of *P. denitrificans*, *R. sphaeroides*, and *S. pomeroyi* are about 20 to 30 residues shorter than that of *R. capsulatus* (Fig. 1A). Generation of computer-assisted hypothetical three-dimensional structures of the cyt bc$_1$-c$_y$ fusion complexes with shorter linkers directed us to the region between amino acids 65 and 90 of cyt c$_y$ to mimic its non-functional variants as Ps electron carriers (Fig. 1B). Considering that the *R. sphaeroides* cyt c$_y$ linker is 26 amino acids shorter than its *R. capsulatus* counterpart, plasmids pYO30 (with a 24-amino acid long deletion between positions Ala$^{63}$ and Pro$^{88}$ of cyt c$_y$) (Δ24-c$_y$) and pYO33 (with a 19-amino acid long deletion between the positions Ala$^{67}$ and Pro$^{88}$ of cyt c$_y$ (Δ19-c$_y$)) were constructed to determine the Ps ET of each electron carrier (Table 1).

**Phenotypic Characterization of *R. capsulatus* Strains Harboring Cyt bc$_1$-c$_y$ Fusion Complexes with Shorter Cyt c$_y$ Linkers**—Plasmids pYO30 (Δ24-c$_y$) and pYO33 (Δ19-c$_y$) were introduced into the *R. capsulatus* mutant YO2 (lacking the cyts bc$_1$, c$_y$, and c$_o$), and the Ps growth abilities of the resulting strains were examined (Fig. 2A). On enriched MPYE medium under Ps conditions, *R. capsulatus* strain pMTS1/MT-RBC1 overproducing cyt bc$_1$ complex, MT-G4/S4 lacking the cyt c$_2$, and pYO38/YO2 harboring an intact cyt bc$_1$-c$_y$ fusion complex exhibited doubling times of about 192, 216, and 300 min, respectively (Fig. 2A). However, under similar conditions, pYO33/YO2 (Δ24-c$_y$) and pYO30/YO2 (Δ19-c$_y$) grew markedly slower than pYO38/YO2 (native-c$_y$) (756 and 498 versus 300 min, respectively). As the mutant YO2 cannot be complemented for Ps growth by either the cyt bc$_1$ complex or cyt c$_y$ alone, the data indicated that the cyt bc$_1$-c$_y$ fusion complexes with shorter cyt c$_y$ linkers provided both the oxidoreductase and electron carrier functions required for Ps growth. However, the slower growth rates suggested that shortening the cyt c$_y$ linker hampered the growth abilities of Ps.

**Prosthetic Group Insertion, Enzymatic Activity, and Subunit Assembly of the Cyt bc$_1$-c$_y$ Fusion Complexes**—As the slower Ps growth might also be attributed to decreased amounts of cyt bc$_1$-c$_y$ fusion complexes, chromatophore membranes of appropriate strains were examined by TMBZ/SDS-PAGE analyses (Fig. 2B). Typical membrane-associated c-type cytochrome profiles comprised of the cyts c$_o$ (32 kDa), c$_y$ (30 kDa), c$_o$ (29 kDa), and c$_y$ (28 kDa) were observed for pMTS1/MT-RBC1 and MT-G4/S4, whereas the YO2-derived samples contained only cyts c$_o$ and c$_o$ (both subunits of C$_{o}$) as expected. On the other hand, pYO38/YO2, pYO33/YO2, and pYO30/YO2 harboring cyt bc$_1$-c$_y$ fusion complexes with shorter cyt c$_y$ linkers had both...
Functional Cyt bc\textsubscript{1}-c\textsubscript{y} Fusions with Variable Length Cyt c\textsubscript{y} Linkers

A

![Diagram of Cyt bc\textsubscript{1}-c\textsubscript{y} Fusions with Variable Length Cyt c\textsubscript{y} Linkers]

B

![Diagram of Strain genotypes and phenotypes]

FIGURE 1. (A) Amino acid sequence alignments of R. capsulatus cyt c\textsubscript{y} and its shorter linker variants with its homologues in other species, R. capsulatus cyt c\textsubscript{y} (native linker) (CAA79860); R. capsulatus cyt c\textsubscript{y} (Δ19); R. capsulatus cyt c\textsubscript{y} (Δ24); R. sphaeroides cyt c\textsubscript{y} (AAC26877); P. denitrificans cyt c\textsubscript{y} (CA49830); and S. pomeroyi DSS-3 cyt c\textsubscript{y} (AAV96763). B, hypothetical three-dimensional structural models of the R. capsulatus cyt bc\textsubscript{1}-c\textsubscript{y} fusion complex and its shorter linker derivatives. The cyt c\textsubscript{y} domain of R. capsulatus cyt c\textsubscript{y} was modeled using SWISS-MODEL, and the overall structures were visualized using the R. capsulatus cyt bc\textsubscript{1} (Protein Data Bank 1ZRT) and yeast cyt bc\textsubscript{1}, cyt c co-crystal (PDB 1NTK) structures.

TABLE 1

<table>
<thead>
<tr>
<th>Strain R. capsulatus</th>
<th>Genotype</th>
<th>Phenotype</th>
<th>Ref.</th>
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<tr>
<td>MT-G4/S4 YO2</td>
<td>crrD121 Rif\textsuperscript{r}Δ(cycA::kan), crrD121 Rif\textsuperscript{r}Δ(petABC::gen), Δ(cycA::kan), Δ(cycY::spe)</td>
<td>Cyt c\textsubscript{y}, Kan\textsuperscript{r}, Ps\textsuperscript{r}</td>
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<tr>
<td>pMTS1/MT-RBC1</td>
<td>crrD121 Rif\textsuperscript{r}Δ(petABC::spe), cyt bc\textsubscript{1}, Spe\textsuperscript{r}, Ps\textsuperscript{r}</td>
<td>Cyt bc\textsubscript{1}, Kan\textsuperscript{r}, Ps\textsuperscript{r}</td>
<td>37</td>
</tr>
<tr>
<td>pYO38/YO2 YO2</td>
<td>YO2 strain with an expression plasmid carrying petABC::cycYFLAG (native cyt c\textsubscript{y})</td>
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</tr>
<tr>
<td>pYO30/YO2</td>
<td>YO2 strain with an expression plasmid carrying petABC::cycYΔAla\textsuperscript{24}Pro\textsuperscript{45}·FLAG</td>
<td>Cyt bc\textsubscript{1}, Tet\textsuperscript{r}, Ps\textsuperscript{r}</td>
<td>This work</td>
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The presence of b\textsubscript{y}-type cyts and cyt c to b ratios for all strains were also determined using optical difference spectroscopy to assess the relative amounts of the cyt bc\textsubscript{1} complexes. Changes in the amounts of the c-type (\(\alpha\) peak\textsubscript{max} at 551 nm, \(\epsilon\) at 515–542 of 20 mm\textsuperscript{–1} cm\textsuperscript{–1}) and b\textsubscript{y}-type (\(\alpha\) peak\textsubscript{max} at 560 nm, \(\epsilon\) at 560–574 of 28 mm\textsuperscript{–1} cm\textsuperscript{–1}) cytochromes were monitored after reduction by ascorbate and dithionite, respectively, of ferricyanide-oxidized chromatophore membranes from appropriate strains (Fig. 3). Strains pMTS1/MT-RBC1 and MT-G4/S4 exhibited cyt c to b ratios of \(~1:2\), whereas very small amounts of c or b peaks were detected in YO2. On the other hand, strains pYO38/YO2, pYO33/YO2, and pYO30/YO2 had similar amounts of b\textsubscript{y}-type cytochromes with cyt c to b ratios of about 1:1 (Fig. 3). The data indicated that all strains contained cyt c\textsubscript{y} and cyt c\textsubscript{y} bands replaced by a single peroxidase-active band at 61, 59, and 58 kDa, respectively, corresponding to their cyt c\textsubscript{y}-cyt c\textsubscript{y} fusion subunits.

Steady-state enzymatic activities of the cyt bc\textsubscript{1} complexes were also assayed by measuring DBH\textsubscript{c}-dependent reduction of horse heart cyt c (Fig. 2C). pYO38/YO2, pYO33/YO2, and pYO30/YO2 harboring cyt bc\textsubscript{1}-c\textsubscript{y} fusion complexes with different lengths of cyt c\textsubscript{y} linkers showed very similar levels of DBH\textsubscript{c}-cyt c\textsubscript{y} reductase activities as compared with that of the pMTS1/MT-RBC1 overproducing a native cyt bc\textsubscript{1} complex. Therefore, neither fusing cyt c\textsubscript{y} to cyt c\textsubscript{y} nor changing the length of the cyt c\textsubscript{y} linker region significantly affected the cyt bc\textsubscript{1} complex enzymatic activity of various cyt bc\textsubscript{1}-c\textsubscript{y} fusion complexes with different cyt c\textsubscript{y} linker lengths.
similar amounts of cyt bc1-cy fusion complexes regardless of the linker length of their cyt cy linkers. Furthermore, to confirm that the Fe-S subunits of fusion complexes had native-like physicochemical properties, EPR spectroscopy was used. Chromatophore membranes prepared from pYO38/YO2 exhibited the [2Fe-2S] cluster g6 and g5 signals of 1.891 and 1.806, respectively, which were identical to those seen with a native cyt bc1 complex (e.g. MT-G4/S4) (Fig. 4A). Moreover, under appropriate conditions, the EPR signals with g6 values of 3.778 and 3.411 assigned to cyts b and c and b4, respectively, were observed with the same membrane preparations (Fig. 4B). The overall data established that all cyt bc1-cy fusion complexes assembled similarly, and exhibited similar enzymatic activities, indicating that the slower growth rates observed with pYO33/YO2 and pYO30/YO2 could not be correlated with the amounts of fusion complexes.

**Purification and Characterization of Purified Cyt bc1-cy Fusion Complexes**—Purification of cyt bc1-cy fusion complexes was pursued to establish that they existed as intact physical entities in the membranes. Earlier, we partially purified the cyt bc1-cy fusion complex (19) using the procedure described by Valkova-Valchanova et al. (22). However, as detailed characterization required purer samples, we developed a new procedure. About 8 mg of purified cyt bc1-cy fusion complex was obtained starting with about 2.5 g of chromatophore membranes derived from 97 g (wet weight) of cells, followed by detergent solubilization, Q-Sepharose ff ion-exchange and FLAG affinity column chromatographies (Table 2). The final preparations of the cyt bc1-cy fusion complex contained less than 5% of Ps pigments associated with the LH complexes, as estimated by optical spectra (data not shown). The purified cyt bc1-cy fusion complex was highly active, reducing horse heart cyt c as an electron acceptor with decylhydroquinone as an electron donor (“Experimental Procedures”). Its specific activity (about 27.2 nmol/mg of protein/min under the assay conditions used) was comparable with that of the purified cyt bc1 complex (41.0 nmol/mg of protein/min) (22). Optical difference spectra indicated that the purified cyt bc1-cy fusion complex had a cyt b to c ratio of ~1:1, similar to that seen in chromatophore membranes from pYO38/YO2 (data not shown). Potentiometric redox equilibrium titration of the heme groups of the cyt c1-cy fusion subunit in the presence of 100 mM KCl at pH 7.0 indicated a single component with an Ea,m value of +336 mV (Fig. 5B).

Considering that this subunit is a dimeric c-type cyt (cyts c1 and c2 of Em,7 of 320 and 365 mV, respectively (10, 27), the data suggested that the two hemes were in rapid equilibrium with each other, with a mean Em,7 value high enough to convey electrons efficiently from the cyt bc1 complex to the RC.

SDS-PAGE analysis of purified cyt bc1-cy fusion complex showed three major bands with 61, 41, and 24 kDa, assigned to the cyt c1-cy, cyt b, and the Fe-S protein subunits, respectively, by TMBZ staining and immunoblot analyses with specific monoclonal antibodies (Fig. 5A). Additional bands of higher Mr, seen with the cyt bc1-cy fusion complexes were attributed to their aggregated forms, based on TMBZ staining and immunoblot data, as they were also seen with native cyt bc1 complexes (27). Finally the non-stoichiometric band running between the cyt b and Fe-S protein subunits, and detected weakly by cyt c1-specific antibodies, in FLAG affinity purified samples reflected degradation products of the cyt c1-cy fusion subunit. The oligomeric state of purified cyt bc1-cy fusion complex was determined by size exclusion chromatography using an analytical grade Superose 6 HR 10/30 (GE Healthcare Inc.) column calibrated with high molecular weight standard markers in the presence of 0.05% (w/v) DDM and 150 mM NaCl (Fig. 5B). Under these conditions, the purified cyt bc1 complex runs as one major peak around 240 kDa (estimated to correspond to its dimeric form), whereas the purified cyt bc1-cy fusion complex

![Image](http://www.jbc.org/bjce.org/figs/fig5b.png)

**FIGURE 2.** A, photosynthetic growth properties on liquid and solid media (enriched MPYE) of various R. capsulatus strains (YO2 lacking the cyt bc1, cyt c1, and cyt c2; pMTS1/MR-BBC1 overproducing the cyt bc1 complex, MT-G4/S4 lacking the cyt c2, and pYO38/YO2, pYO33/YO2, and pYO30/YO2 containing the cyt bc1-cy fusion complexes with the native 19 amino acids and 24-amino acid shorter cyt cy linkers, respectively) were determined by monitoring the turbidity of the cultures, as described under “Experimental Procedures.” B, the Fe-S cytochrome profiles of the same strains were revealed using chromatophore membranes (100 g of total proteins) derived from the same strains described above were determined as in Atta-Asafo-Adjei and Daldal (20), in the absence (no inhibitor) or presence (10 μM stigmatellin or 20 μM antimycin), and for comparative purposes the steady-state enzymatic activities are represented as % of the overproduced native cyt bc1 complex.

<table>
<thead>
<tr>
<th>Strain</th>
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<th>Doubling time (min)</th>
<th>Colony size (mm)</th>
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<td>YO2</td>
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*Each value is the average of 10 colonies after 4 day incubation on MPYE plates in PS conditions. NS, no growth.
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exhibited two major peaks at about 417 and 257 kDa, respectively. Immunoblot analyses with subunit-specific antibodies showed that both peaks had homogeneous constituents, suggesting that purified cyt bc₁-c₃ fusion complexes consisted of a mixture of tetrameric and dimeric forms under the conditions used.

Light-activated Cyt b Reduction and Cyt c Re-reduction Kinetics—A major aim being to probe the extent of ET from the cyt bc₁-c₃ fusion complexes with shorter cyt c₃ linkers to the RC in situ, appropriate strains were examined using light-activated, time-resolved optical spectroscopy (Fig. 6). In all cases, chromatophore membranes were normalized to the same activated, time-resolved optical spectroscopy (Fig. 6). In all cases, in situ monitored on the millisecond time scale, at an ambient redox potential (microsecond time scale) light activation of the RC, were monitored cyt b reduction and cyt c re-reduction kinetics, initiated by rapid (microsecond time scale) light activation of the RC, were monitored on the millisecond time scale, at an ambient redox potential Et of 100 mV. At this Et, the membrane Q pool contains Q and QH₂, and the RC as well as cyts c, and the [2Fe-2S] cluster of the Fe-S protein subunits of cyt bc₁, or the cyt bc₁-c₃ fusion complexes are fully reduced. First, light-induced time-resolved single turnover cyt b reduction kinetics were examined at 560–570 nm, in the presence and absence of antimycin A as a specific cyt bc₁ complex Q₁ site inhibitor. In MT-G₄/S₄, which has only cyt c₃ as the sole electron carrier between the cyt bc₁ complex and the RC, and in pYO38/YO2 producing a cyt bc₁-c₃ fusion complex with a native cyt c₃ linker, cyt b was reduced by oxida-


tion of a QH₂ via the Qo site, and immediately re-oxidized by Q bound at the Q₁ site with an expected rate faster than the available time resolution. However, in the presence of antimycin A (2 μM), cyt b oxidation was abolished to reveal the light-induced transient reduction phase (Fig. 6, top row). In the case of pYO33/YO2 (Δ19-c₃) and pYO30/YO2 (Δ24-c₃) with shorter cyt c₃ linkers, fast cyt b oxidation/reduction rates were quasi-similar to those seen with MT-G₄/S₄ and pYO38/YO2, but the amplitudes of these changes (per RC) were significantly smaller (Fig. 6, top row). As similar amounts of cyt bc₁-c₃ fusions complexes were present in all strains tested (Figs. 2 and 3) the smaller cyt b reduction amplitudes cannot be interpreted as lower amounts of these complexes in the membranes.

The cyt c oxidation/re-reduction kinetics exhibited by the cyt bc₁ complexes were subsequently examined using the specific Qo site inhibitors myxothiazol and stigmatellin to probe ET to the RC.

Myxothiazol blocks cyt c re-reduction by displacing QH₂ without immobilizing the Fe-S protein, whereas stigmatellin not only displaces QH₂ but also immobilizes the Fe-S protein to block ET to the c₁ heme as well. In the presence of stigmatellin, MT-G₄/S₄ and pYO38/YO2 showed typical cyt c oxidation without any cyt c re-reduction (Fig. 6, bottom row), due to the absence of ET from the Fe-S protein subunit to the cyt c₁ heme. In the presence of myxothiazol, cyt c re-reduction reached about half of the amplitude seen in the presence of stigmatellin (i.e. full oxidation), revealing the pre-flash, chemically derived electron localized in the Fe-S protein despite the absence of QH₂ oxidation at the Qo site (Fig. 6, bottom row) (see e.g. Refs. 28 and 29 for a detailed explanation of these ET kinetics). Cyt c re-reduction kinetics exhibited by pYO33/YO2 (Δ19-c₃) and pYO30/YO2 (Δ24-c₃) were quasi-similar to those seen with MT-G₄/S₄ and pYO38/YO2 (native-c₃) in the presence and absence of the Qo site inhibitors. However, in the presence of shorter cyt c₃ linkers, significant differences in the cyt c oxidation/re-reduction amplitudes were observed. These amplitude decreases could not reflect lower amounts of the cyt bc₁-c₃ fusion complexes with shorter cyt c₃ linkers in the strains examined (Figs. 2 and 3). Thus, they indicated decreased electronic couplings to the RC, revealing the limits of their physical abilities to reach and convey electrons to the photooxidized RCs (Fig. 6). Comparative kinetic data indicated that a cyt c₃ linker of about 45 amino acids long (i.e. Δ24-c₃) seems to be the shortest one able to sustain cyclic ET and Ps growth of R. capsulatus.
Functional Cyt bc₁-c₇ Fusions with Variable Length Cyt c₇ Linkers

Table 2

<table>
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<tr>
<th>Step</th>
<th>Protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Yield (%)</th>
<th>-Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatophore + DDM</td>
<td>592</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solubilized chromatophore</td>
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<td>2.577</td>
<td>4.9</td>
<td>100%</td>
<td>1.0</td>
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<tr>
<td>Q-Sepharose ff</td>
<td>33</td>
<td>634</td>
<td>19.3</td>
<td>25%</td>
<td>3.9</td>
</tr>
<tr>
<td>FLAG</td>
<td>8*</td>
<td>204</td>
<td>27.2</td>
<td>8%</td>
<td>5.5</td>
</tr>
</tbody>
</table>

* One unit of DBH₂-dependent cyt c₂ reduction activity was defined as the amount of enzyme that produced 1 μmol of reduced cyt c per min under the assay conditions.

** Methodology and Results **

In this work, first, we have characterized the physico-chemical properties of a functional cyt bc₁-c₇ complex that we constructed earlier (19) by fusing the COOH-terminal last amino acid of the cyt c₁ subunit of the cyt bc₁ complex to the NH₂-terminal first amino acid of its physiological membrane-bound electron acceptor cyt c₇ (Fig. 1A). Using membrane preparations, detailed analyses demonstrated that the cyt bc₁-c₇ fusion complex contained its prosthetic groups ([2Fe-2S] cluster and b- and c-type hemes) in appropriate amounts and assembled properly. Purification of the cyt bc₁-c₇ fusion complex was needed to establish that it is an intact physical entity able to conduct membrane-confined Ps cyclic ET. This task was challenging because the fusion complex was susceptible to proteolytic degradation, and its chromatographic properties were distinct from the previously purified cyt c₇ (10) and cyt bc₁ complexes (22). We developed a new procedure, using tandem Q-Sepharose ff ion-exchange and FLAG affinity column chromatographies, to obtain highly pure samples with good yields to initiate crystallization efforts. Unlike the dimeric cyt bc₁ complex, the purified cyt bc₁-c₇ complex was a mixture of dimeric and tetrameric populations, with different oligomerization properties in vitro, possibly due to the presence of cyt c₇. The FLAG affinity purified samples contained small amounts of cyt c₁, degradation products, running as an additional band around 30 kDa, and the Q-Sepharose column fractions were enriched in RC-LH subunits, usually absent in purified cyt bc₁ complexes (nLC-MS/MS data, not shown). During purification, although most of the Ps components are usually washed out from the cyt bc₁ complex in the presence of 0.01% DDM by a buffer of 0.2 M ionic strength, they remained with the cyt bc₁-c₇ fusion complex. The significance, if any, of these apparent tighter associations remains to be seen.

Interestingly, both cyt b reduction and cyt c re-reduction rates exhibited by the cyt bc₁-c₇ fusion complexes with shorter linkers were quasi-similar to those seen with strains harboring unconnected or fused cyt c₇ and cyt bc₁ complexes with native cyt c₇ linkers. These fast rates further evidenced that the monitored ETs were mediated by the membrane-bound cyts c₁ and not by their soluble versions (cyt S-c₇), somehow generated by proteolysis. Otherwise, as described in the accompanying work (38), the ET rates would have become much slower. Sharply contrasting the rates, the amplitudes of ET to the RCs were slightly smaller that the monitored ETs, and would have become much smaller. Sharply contrasting the rates, the amplitudes of ET to the RCs were slightly smaller than the monitored ETs, and would have become much slower. Sharply contrasting the rates, the amplitudes of ET to the RCs were slightly smaller than the monitored ET rates, and would have become much slower. Sharply contrasting the rates, the amplitudes of ET to the RCs were slightly smaller than the monitored ET rates, and would have become much slower. Sharply contrasting the rates, the amplitudes of ET to the RCs were slightly smaller than the monitored ET rates, and would have become much slower.
Functional Cyt bc\textsubscript{1}-c\textsubscript{y} Fusions with Variable Length Cyt c\textsubscript{y} Linkers

FIGURE 5. Purification of the cyt bc\textsubscript{1}-c\textsubscript{y} fusion complex. A, SDS-PAGE, TMBZ, and immunoblot analyses. Approximately 50 µg of total proteins per lane were used in each case, except the pool from anti-FLAG, which had only 10 µg. Column fractions obtained during the purification procedure, and a-cyt c\textsubscript{y}, a-FLAG (i.e. cyt c\textsubscript{y}), a-cyt b, and a-Fe-S antibodies were as described under “Experimental Procedures” and in the text. B, size exclusion chromatography (upper panel) of the purified cyt bc\textsubscript{1}-c\textsubscript{y} fusion complex and cyt bc\textsubscript{1} complex in the presence of 150 mM NaCl and 0.05% DDM. Gel filtration chromatography was performed using a Superose 6 HR 10/30 column, which was run at a flow rate of 0.3 ml/min, and the elution profile was monitored at 280 nm. The column was calibrated with blue dextran (2,000 kDa), thyroglobulin (669 kDa), apoferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa) as standards, run in the presence of 150 mM NaCl and 0.05% DDM, and their elution positions are indicated at the top of the chromatograph. Aliquots of each fraction (0.5 ml) were concentrated and used in each case, except the pool from anti-FLAG, which had only 10 µg. Redox mediators were as described under “Experimental Procedures” and in the text. C, dark equilibrium redox titration of the cyt c\textsubscript{y} subunit of the purified cyt bc\textsubscript{1}-c\textsubscript{y} fusion complex (0.1 mg/ml). The titration was performed in 50 mM MOPS buffer (pH 7.0) containing 0.1 M KCl and 1 mM EDTA in the presence of 0.01% (w/v) DDM. Redox mediators were as described under “Experimental Procedures” (26). The $E_{m,2}$ value indicated was determined by fitting the normalized data to a $n = 1$ Nernst equation.

FIGURE 6. Light-induced, time-resolved cyt b reduction and cyt c re-reduction kinetics of various R. capsulatus strains. In each case, chromatophore membranes containing an amount of RC equal to 0.45 µM were resuspended in 50 mM MOPS buffer (pH 7.0) containing 100 mM KCl and 100 mM EDTA at an $E_{m}$ of 100 mV. The amount of RC was determined based on the extent of its photooxidation by a train of 10 flashes separated by 50 ms at an $E_{m}$ of 380 mV, and using an extinction coefficient $e_{305-450}$ of 29 mM$^{-1}$ cm$^{-1}$, as described under “Experimental Procedures.” The traces for cyt b reduction (upper panel) were monitored in the absence (No) and the presence of the Q$_{i}$ site inhibitor antimycin (Ant, 5 µM), and those for cyt c re-reduction (lower panel) were in the absence of inhibitor or in the presence of myxothiazol (Myx, 5 µM), where no Q$_{H_{2}}$ oxidation takes place at the Q$_{i}$ site, or in the presence of stigmatellin (Stig, 1 µM), where no electron is transferred from the [2Fe2S] cluster to the $c_{y}$ heme. All samples contained the same amounts of the cyt bc\textsubscript{1}-c\textsubscript{y} complexes as documented in Figs. 2 and 3.

work therefore demonstrated that the amounts of electronically coupled RC-LH-cyt bc\textsubscript{1}-c\textsubscript{y} fusion complexes diminished further with shorter cyt c\textsubscript{y} linkers, reflecting the distance dependence of efficient cyclic electron transport between the donor and acceptor complexes in Ps membranes. Furthermore, the progressive decrease suggested that membranes might contain a distribution of such closely associated photosynthetic units.

Even though the cyt bc\textsubscript{1}-c\textsubscript{y} fusion complexes with shorter cyt c\textsubscript{y} linkers were produced in comparable amounts irrespective of the linker sizes, they supported Ps growth at different degrees. Comparison of the shortest (Δ24-c\textsubscript{y}) and barely functional cyt c\textsubscript{y} linker with that of non-Ps competent R. sphaeroides cyt c\textsubscript{y}, which is 26 residues shorter than that of its R. capsulatus counterpart (Fig. 1A), suggested that at least a linker of about 44–46 amino acids long (provided that the native linker is about 70 residues long) is required to couple electronically the cyt bc\textsubscript{1}-c\textsubscript{y} fusion complex and the RC surrounded with its LH complexes. Assuming that both the RC (30) and the cyt bc\textsubscript{1} complex (31) extend into the periplasm by ~30 Å, then half of the remaining linker would be consumed to bring the cyt c domain of cyt c\textsubscript{y} to the same plane.
with its physiological partners. The remaining 20–25-residue long linker controlling its electronic coupling ability then suggests that the RC-LH complexes and the cyt bc1-cy complex must be very close to each other, possibly forming large structural complexes. Furthermore, it is noteworthy that both the R. sphaeroides cyt c complex (9) and R. capsulatus cyt bc1-cy fusion complexes with shorter linkers convey electrons to their cognate cyt c oxidases.5 Thus, Ps supercomplexes apparently require longer cyt c linkers than the respiratory counterparts, possibly due to the LH complexes surrounding the RCs.

Kinetic behaviors of functional supercomplexes in R. sphaeroides Ps membranes between the RC and the “trapped” cyt c2 acting in a locally confined manners (14, 32–34), strongly suggest that the RC-LH and the cyt bc1 complexes must be very close to each other. The data presented here provide complementary structural and kinetic information for such Ps supercomplexes, with a ratio of the RC-LH:cyt bc1:cyt c2 (or cyt c) being 2:1:1 (13, 33). Joliot et al. (33) proposed that in the RC-LH complexes dimerized via LH-PufX interactions, two RC dimers interact with a single cyt bc1 complex dimer. If this is also the case with the cyt bc1-cy fusion complexes in the Ps membranes, then cyt c might be located between the cyt bc1 complexes and the RC-LH complexes with their membrane anchors in the vicinity of the quasi-closed LH rings, promoting tighter associations between the Ps components. The motion constraints thus imposed on cyt c might then only be compensated by a linker long enough to allow its movement between the cyt bc1 complex dimers and the opposing RC-LH complexes of R. capsulatus (Fig. 7).

In summary, the availability of functional cyt bc1-cy complexes with shorter linkers affecting the coupling to the RC-LH complexes now provide compelling indications that hardwired

Figure 7. A, crystal structure of the RC-LH1 core complex (PDB 1PYH) from Rhodopseudomonas palustris and hypothetical three-dimensional structural model of R. capsulatus cyt bc1-cy fusion complex (pyO30/yO2, Δ24-cy) (with the transmembrane helices of cyt bc1 shown as ribbons, and cyt cΔ24-cy) shown as sticks), are drawn using the program PyMOL. The narrow section of the RC (subunits L, yellow; M, blue; H, purple) surrounded by the LH1 complex (chains α, pale green and β, green) and the cyt bc1-cy fusion complex (subunits cyt b, cyan; cyt cΔ24, orange; cyt c, red; and the Fe-S protein, magenta) are viewed parallel to the membrane plane. B, top view (perpendicular to the membrane plane) of the RC-LH1 core complex and the cyt bc1-cy fusion complex with the shortest cyt c linker (Δ24-cy) is shown. Note that to reach the central part of RC subunits L and M in the RC-LH1 and cyt bc1-cy fusion complexes, the cyt c domain of cyt c (assuming that its linker is stretched out in a fashion parallel to the membrane) needs to reach out for about 100 Å. C, a schematic representation of the major membrane proteins involved in cyclic ET of purple bacterial photosynthesis (upper panel), and a proposed mechanism for cyt c-mediated cyclic ET via supramolecular organization of R. capsulatus photosynthetic unit (lower panel). cyt bc1 complex, hydroquinone cyt c oxidoreductase; cyt c, membrane-associated cytochrome c. Arrows indicate directions of electron (e-) and excitation (ex) flows.

5 Y. Öztürk, D. Zannoni, and F. Daldal, unpublished data.
Ps units occur in membranes. Future purification of these Ps units will initiate studies addressing how the membrane super-complexes are formed and regulated in vivo in response to changing environmental conditions, and why some organisms contain both a soluble cyt c2 and a membrane-anchored cyt c2.

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REFERENCES

Cytochrome $bc_1$-c$_{yt}$ Fusion Complexes Reveal the Distance Constraints for Functional Electron Transfer Between Photosynthesis Components

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