Published in final edited form as:

Biochemistry. 2011 March 15; 50(10): 1651–1663. doi:10.1021/bi101736v.

Inter-monomer Electron Transfer Between the Low Potential *b* Hemes of Cytochrome *bc*₁

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Abstract

Cytochrome (cyt) *bc*₁ is a structural dimer with its monomers consisting of the Fe/S protein, cyt *b* and cyt *c*₁ subunits. Its three-dimensional architecture depicts it as a symmetrical homo-dimer, but the mobility of the head domain of the Fe/S protein indicates that the functional enzyme undergoes asymmetrical hetero-dimeric conformations. Here, we report a new genetic system for studying intra and inter-monomer interactions within the cyt *bc*₁ using the facultative phototrophic bacterium *Rhodobacter capsulatus*. The system involves two different sets of independently expressed cyt *bc*₁ structural genes carried by two plasmids that are co-harbored by a cell without its endogenous enzyme. Our results indicate that co-expressed cyt *bc*₁ subunits are matured, assorted and assembled *in vivo* into homo- and hetero-dimeric enzymes that can bear different mutations in each monomer. Using the system, the occurrence of inter-monomer electron transfer between the low potential *b* hemes of cyt *bc*₁ was probed by choosing mutations that perturb electron transfer at the hydroquinone oxidation (Q_o) and quinone reduction (Q_i) sites of the enzyme. The data demonstrate that active hetero-dimeric variants, formed of monomers carrying mutations that abolish only one of the two (Q_o or Q_i) active sites of each monomer, are produced and they support photosynthetic growth of *R. capsulatus*. Detailed analyses of the physicochemical properties of membranes of these mutants, as well as purified homo- and hetero-dimeric cyt *bc*₁ preparations, demonstrate that efficient and productive electron transfer occurs between the low potential hemes *b*_L of the monomers in a hetero-dimeric enzyme. Overall findings are discussed with respect to intra- and inter-monomer interactions that occur during the catalytic turnover of cyt *bc*₁.

Keywords

cytochrome *bc*₁; complex III; photosynthetic and respiratory electron transfer; *Rhodobacter capsulatus*

The ubihydroquinone: cytochrome (cyt) *c* oxidoreductase (also called cyt *bc*₁ or complex III) is a major multi-subunit membrane-bound enzyme that is central to respiratory and photosynthetic energy transduction pathways in various organisms (1). It converts hydroquinones (QH₂) to quinones (Q), reduces electron carrier cyts *c*, and contributes to the

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Supporting Information Available

EPR dark equilibrium redox titrations of the hemes *b*_L and *b*_H of heterodimeric cyt *bc*₁ variants using chromatophore membranes (Fig. S1A and B), and physical evidence for the production of heterodimeric cyt *bc*₁ variants using DEAE-Biogel chromatography followed by two consecutive affinity chromatography steps (Fig. S2A and B) are presented in Supplemental Information. This material is available free of charge via the Internet at <http://pubs.acs.org>

formation of membrane potential and proton gradient that are used for ATP production by ATP synthase (2, 3). In most bacteria, like the purple non-sulfur phototroph *Rhodobacter capsulatus*, cyt bc_1 is constituted of three subunits (the Fe/S protein, cyt b and cyt c_1), which are encoded by three structural genes *petA*, *petB*, and *petC*, respectively (4), located adjacent to each other to form the *petABC* operon (5). Cyt b is an integral membrane protein, whereas the Fe/S protein and cyt c_1 are membrane-anchored by their amino- and carboxyl-terminal helices, respectively. These subunits are universally conserved in all cyt bc_1 , and always carry specific cofactors essential for enzyme activity. These cofactors are the [2Fe2S] cluster with a high redox midpoint potential (E_m) in the Fe/S protein, two b -type hemes with one low (b_L) and one high (b_H) E_m in cyt b , and a high E_m c -type heme in cyt c_1 (6). The purified, native *R. capsulatus* cyt bc_1 is a dimer, but can also form tetramers upon fusion with one of its physiological electron carriers, the membrane anchored cyt c_y (7). Each monomer of the enzyme contains two Q/QH₂ binding domains, referred to as the QH₂ oxidation (Q_o) and Q reduction (Q_i) sites, located respectively on the positive (p) and negative (n) sides of energy transducing membranes. Three-dimensional structures for the cyts bc_1 of various organisms (including *R. capsulatus*) are known, and often depict the enzyme as a symmetrical homo-dimer (8–11) (Fig. 1). In each monomer (*e.g.*, **M1**), the carboxyl-terminal helix of cyt c_1 interacts with the fifth helix of cyt b to form a cyt b - c_1 core that interacts with only the head domain of the Fe/S protein, leaving the amino-terminal membrane helix of this subunit associated with cyt b of the other monomer (*e.g.*, **M2**). The Fe/S protein head domain is mobile between the cyt b and cyt c_1 , and this mobility is essential for cyt bc_1 activity (12) as mutants with immobile Fe/S protein head domains are inactive (13). How the subunits are assembled to yield a functional enzyme is not well established. Earlier works using *R. capsulatus* indicated that among the subunits and subcomplexes, the cyt c_1 subunit and the cyt b - c_1 subcomplex are the longest-lived *in vivo* (14, 15). These observations suggested that first cyts b and c_1 might assemble together to which the Fe/S protein is added, but how and when the monomers form a dimer remains unknown. Related sub-complexes, probably representing assembly intermediates of cyt bc_1 , have also been observed with yeast mitochondria (16).

The mechanism of function of cyt bc_1 is generally described by the Q cycle mechanism (17, *see also* 18). At the Q_o site, the oxidized [2Fe-2S] cluster of the Fe/S protein oxidizes QH₂ and conveys one of the two electrons emanating from QH₂ oxidation to oxidized cyt c_1 via the mobility of the Fe/S protein head domain. Cyt c_1 then reduces a mobile cyt c (in *R. capsulatus*, cyt c_y or cyt c_2), which transports electrons to the photochemical reaction center (RC) in photosynthesis (Ps) or the cyt c oxidase in respiration (Res). The second electron derived from QH₂ oxidation at the Q_o site is transferred to heme b_L of cyt b , and is then conveyed to heme b_H across the lipid bilayer, to generate membrane potential and yield a stable semiquinone (SQ) at the Q_i site. Completion of the catalytic turnover of cyt bc_1 involves a second QH₂ oxidation at a Q_o site, which proceeds via the same sequence of events as above but converting SQ in the Q_i site to a QH₂ to be released from the enzyme (Fig. 1). Whether the same Q_o site of a given monomer, or the Q_o site at the other monomer of the dimeric enzyme, catalyzes this second QH₂ oxidation is unknown. In the past, models describing the mechanism of function of cyt bc_1 using dimeric enzymes have been proposed (19, 20, *see also* 21). Recent experimental data stemming from crystallography (22), enzyme kinetics (23, 24) and spectroscopy (25) suggest that intra- and inter-monomer interactions occur in cyt bc_1 , and that during the catalytic cycle the enzyme might become asymmetrical. The functional significance of any hetero-dimeric asymmetry remains unknown.

Our earlier works indicated that the presence of different Q_i site inhibitors affected the position of the Fe/S protein at the Q_o site across the membrane to initiate QH₂ oxidation (25). These intra- and inter-monomer interactions lead us to propose a “hetero-dimeric modified Q cycle” model (26). Accordingly, the resting enzyme would initiate a turnover at

the Q_o site of any monomer, but then alternate between the Q_o sites of the two monomers to complete the initiated turnover. This model follows the Q cycle events (27, 28), but in addition, it invokes inter-monomer electron transfer between the hemes b_L of cyt *b* in each monomer to “dismutate” the two SQ accumulated at the distant Q_i sites of the dimeric enzyme to Q and QH₂ (26). More recent works indicated the occurrence of such electron transfer events (29, 30). In order to further probe our model, we sought approaches to study homo- and hetero-dimeric interactions between the monomers of cyt *bc*₁. Here we describe a new genetic system that allows modifications at will of cyt *bc*₁ subunits to induce structural asymmetry between monomers and yields architecturally hetero-dimeric enzymes. Successful production of hetero-dimeric cyts *bc*₁ indicates that the enzyme subunits are matured, assorted and assembled independently in *R. capsulatus* membranes. Using this system, we initiated dissection of intra- and inter-monomer interactions within the dimeric cyt *bc*₁, and describe a native-like hetero-dimeric enzyme that exhibits fast and efficient electron transfer between the hemes b_L of the monomers to support cyt *bc*₁-dependent photosynthetic growth of *R. capsulatus*. Overall findings are discussed in terms of the biogenesis and the mechanism of function of cyt *bc*₁ in the context of the hetero-dimeric modified Q cycle model.

Materials and Methods

Bacterial strains and growth conditions

All *R. capsulatus* strains harboring plasmids that express cyt *bc*₁ mutants were derivatives of strain MT-RBC1 [Δ (*petABC::spe*)] carrying a complete chromosomal deletion of *petABC* (5). They were grown in liquid or solid enriched (MPYE) medium, supplemented with antibiotics as needed (10, 2.5 and 10 µg/ml kanamycin, tetracycline and spectinomycin, respectively) under Res (semiaerobic/dark) or Ps (photoheterotrophic/light) conditions at 34 °C as described in (4). For Ps growth, completely filled culture vessels or plates placed in anaerobic jars with H₂+CO₂ generating gas-packs (Becton Dickinson, MD) were incubated in temperature controlled Percival light incubators.

E. coli strains harboring the plasmids used in this work (Table 1) were derivatives of HB101 (F⁻ Δ (*gpt-proA*)62 *araC14 leuB6*(Am) *glnV44*(AS) *galK2*(Oc) *lacY1* Δ (*mcrC-mrr*) *rpsL20*(Str^r) *xylA5 mtl-1 thi-1*), and grown on Luria-Bertani medium containing ampicillin, kanamycin or tetracycline (100, 50, or 12.5 µg per ml, respectively) as appropriate (4). *R. capsulatus* strains harboring a single plasmid, or co-harboring two plasmids with the same or different (*incQ/P4* or *colE1*) type of replicon, were obtained via triparental matings between appropriate *E. coli* strains as donors, and *R. capsulatus* strain MT-RBC1 as a recipient, as described earlier (5). Depending on the replicon used, the desired plasmids were introduced by either conjugation or transformation, and either step-wise or simultaneously, using appropriate selections. Strains co-harboring two plasmids were maintained using both antibiotics, and large cultures were subjected to short growth periods (~ 24 hrs). Under these conditions, cells harboring single or double plasmids produced Ps⁺ revertants at frequencies less than 10⁻⁵, and cultures that occasionally yielded larger numbers of Ps⁺ revertants (> 0.01%) were discarded not to compromise biochemical analyses.

Molecular genetic techniques

Molecular genetic techniques were performed using standard procedures (31). All constructs were verified using DNA sequencing, and sequence analyses conducted using MacVector (Accelrys, San Diego, CA). Carboxyl-terminally epitope-tagged derivatives of cyt *b* were constructed using the “QuickChangeTM Site-Directed Mutagenesis kit” (Stratagene Inc., La Jolla, CA), with plasmid pPET1 carrying the wild type *petABC* operon (5) as a template, and

the primers pairs Flag F1: 5'-TCCGGCCGAG **GACTACAAGGACGACGACGACAAG** **TGAGGAAAGGAACCG**-3' and FlagR1: 5'-CCTTTCCTCA **CTTGTCGTCGTCGTCCTTGTAGTC** CTCGGCCGGATTGCCG-3' encoding the Flag (-**DYKDDDDK**-COOH) (F) tag; or StrepF1: 5'-TCCGGCCGAG **TGGTCGCACCCGCAGTTCGAAAAG** **TGAGGAAAGGAACCG**-3' and StrepR1 5'-CCTTTCCTCA **CTTTTCGAACTGCGGGTGCGACCA** CTCGGCCGGATTGCCG-3' encoding the Strep (-**WSHPQFEK**-COOH) (S) tag; or HAF1: 5'-TCCGGCCGAG **TACCCGTACGACGTCCCGGACTACGCG** **TGAGGAAAGGAACCG**-3' and HAR1: 5'-CCTTTCCTCA **CGCGTAGTCCGGGACGTCGTACGGGTA** CTCGGCCGGATTGCCG-3' encoding the HA (-**YPYDVPDYA**-COOH) (H) tag; or cMycF1: 5'-TCCGGCCGAG **GAGCAGAAGCTTATCTCGGAGGAGGACCTG** **TGAGGAAAGGAACCG**-3' and 5'-CCTTTCCTCA **CAGGTCCTCCTCCGAGATAAGCTTCTGCTC** CTCGGCCGGATTGCCG-3' encoding the cMyc (-**EQKLISEEDL**-COOH) (M) tag, yielding the plasmids pPET1-F, or pPET1-S, or pPET1-H, or pPET1-M, respectively. The *XmaI*-*StuI* fragment of pMTS1 was then exchanged with its counterparts from appropriately tagged plasmid pPET1 derivatives to yield pMTS1-F, pMTS1-S, pMTS1-H and pMTS1-M (Table 1). Similarly, pED2 (Kan^R) carrying a mutant allele of *petABC* with *cyt b*-S:Y147A mutation that inactivates Q_o site electron transfer was obtained by exchanging the *XmaI*-*StuI* fragments between pPET1-S and pMTS1-*b*:Y147A plasmids (32). Plasmid pPET1-H212N* was obtained by exchanging the *StyI*-*SphI* fragments between pPET1-F and pPET1-H212N plasmids, and then the *BstEII* fragment carrying *cyt b*-F:H212N mutation of pPET1-H212N* was substituted with that of pRKpPET1-bH212N (28) to yield pED3 (Tet^R). To construct plasmid pPHY101, which is a pRK415 Tet^R derivative carrying a mutant *petABC* with *cyt b*-F:H212N mutation, pED3 was digested with *HindIII*, ligated and transformed into HB101 selecting for Amp^R, to yield pPHY100 that carries *petABC*. This plasmid was digested with *TthIII*, blunt ended with T4 polymerase and digested with *HindIII* to generate a DNA fragment carrying *petABC* with *cyt b*-F:H212N mutation, which was ligated to the Tet^R plasmid pRK415 digested first with *XbaI*, blunt ended with T4 polymerase, and then digested with *HindIII* to yield pPHY101 (Table 1). An Amp^R Tet^S derivative of plasmid pBR322 (33) was constructed by digestion with *EcoRV* and *AvaI*, religation and transformation into HB101 to yield pPHY102. Ligation of plasmids pPHY102 and pED2 carrying *petABC* with *cyt b*-S:Y147A mutation using their unique *HindIII* sites yielded plasmid pPHY103. The characteristics of the plasmids used are summarized in Table 1.

Biochemical techniques

Intra-cytoplasmic (chromatophore) membranes were obtained from frozen washed cells after two passages through a French press, as described earlier (5). Protein concentrations were determined using the bicinchoninic acid method (34) with bovine serum albumin as a standard. SDS-PAGE (12.5%) were conducted as described in (35), and prior to loading, samples were solubilized by incubation for 10 min room temperature in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.1 M dithiothreitol, 25% glycerol, and 0.01% bromophenol blue. Immunoblot analyses were carried out as in (5) using polyclonal antibodies against *R. capsulatus* *cyt b*, or anti-Flag (Sigma Inc.) and anti-Strep antibodies (Novagen Inc.). Steady-state *cyt bc*₁ activity was measured using decylbenzohydroquinone (DBH₂) as electron donor and horse heart *cyt c* as electron acceptor at 25 °C (5). The reaction was initiated by enzyme addition, monitored at 550 nm for 2 min, and the portion of the initial rate that is stigmatellin-sensitive was taken as enzyme activity.

Spectroscopic techniques

Optical spectra were recorded on a Hitachi U3210 UV-visible spectrophotometer. Absorption difference spectra for the *c*- and *b*-type *cyts* were obtained using chromatophore

membranes (0.3 mg of total protein/ml), oxidized with potassium ferricyanide, and reduced with sodium ascorbate or sodium dithionite, as appropriate. Potentiometric dark titrations using optical or EPR spectra were performed with chromatophore membranes (1.5 or 10 mg/ml, respectively) or purified cyt *bc*₁ (0.5 mg/ml) preparations, as described earlier (36). A buffer (50 mM MOPS, 100 mM KCl, pH 7.0) containing 20 μ M tetrachlorohydroquinone (TCHQ, $E_{m,7}$ = 350 mV), 20 μ M 2,3,5,6-tetramethyl-1,4-phenylenediamine (DAD, 260 mV), 20 μ M 1,2-naphthoquinone 4-sulfonate (NQS, 210 mV), 20 μ M 1,2-naphthoquinone (NQ, 145 mV), 10 μ M *N*-methyl-dibenzopyrazine methyl sulfate (PMS, 80 mV), 10 μ M *N*-ethyl-dibenzopyrazine methyl sulfate (PES, 50 mV), 40 μ M duroquinone (DQ, 5 mV), 20 μ M pyocyanine (PCN, -34 mV), 6 μ M indigotrisulfonate (ITS, -90 mV), 20 μ M 2-hydroxy-1,4-naphthoquinone (HNQ, -145 mV) and 20 μ M anthraquinone 2-sulfonate (AQS, -225 mV) as redox mediators was used. 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 (given with respect to the standard hydrogen electrode) were determined by fitting the normalized absorption data to a single component $n = 1$ Nernst equation. The mediator cocktail was tested for the absence of any optical absorbance contribution during the titrations. EPR measurements were performed on a Bruker Elexsys E500 spectrometer. X-band EPR spectra were recorded using a standard rectangular Bruker cavity (ER 4102ST) fitted to an Oxford Instruments Helium flow cryostat (ESR900). Spectrometer settings are indicated in figure legends.

Time-resolved, light activated kinetic spectroscopy was performed on a dual wavelength kinetic spectrophotometer with chromatophore membranes resuspended in 50 mM MOPS/100 mM KCl buffer (pH 7.0) supplemented with 100 μ M ferricyanide (FeCN, 430 mV), 8 μ M DAD (260 mV), 6 μ M NQ (145 mV), 1 μ M PMS (80 mV), 1 μ M PES (50 mV), 6 μ M 2 HNQ (-145 mV), 6 μ M benzyl viologen (BV, -359 mV) as redox mediators and 2.5 μ M valinomycin as a membrane potential uncoupler (37). The amounts of chromatophore membranes used in each assay were normalized to their reaction center (RC) content, as determined by measuring the flash induced optical absorbance difference at 605 - 540 nm (E_h = 380 mV), and an extinction coefficient of 29.8 mM⁻¹cm⁻¹. Transient cyt *c* re-reduction and cyt *b* reduction kinetics at an E_h of 100 mV, initiated by a short saturating flash (~8 μ s) from a xenon lamp were followed at 550 -540 nm and at 560 - 570 nm, respectively. Antimycin, myxothiazol and stigmatellin were used at 10 μ M each, as needed.

Protein purifications

R. capsulatus wild type, homo- or hetero-dimeric mutant, epitope-tagged or untagged cyts *bc*₁ were purified using appropriate derivatives of MT-RBC1 (Table 1). Washed chromatophore membranes were resuspended to a final protein concentration of 10 mg/ml in 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl, 20% glycerol, 1 mM PMSF. Dodecyl maltoside (DDM, 20% w/v stock solution) was added drop wise to a final concentration of 1 mg detergent per mg of total protein. The mixture was stirred for 1 h at 4 °C, then ultra-centrifuged (120,000 $\times$ g for 1 h) to eliminate non-dispersed membranes. The supernatant was loaded onto a DEAE-Biogel column (5.2 $\times$ 15 cm) pre-equilibrated with 50 mM Tris-HCl (pH 8.0) containing 20% glycerol, 0.01% (w/v) DDM, and 100 mM NaCl (Buffer A). The column was washed with three column volumes (CV) of Buffer A, followed by five CV of the same buffer supplemented with 150 mM NaCl. With the cyt *bc*₁ variants derived from cells harboring pMTS1, pMTS1-S, pMTS1-F and pED2, the column was washed step-wise between 150 and 350 mM NaCl until a red band adsorbed on top of the column became visible, which was then eluted with 350 mM NaCl. However, with the cyt *bc*₁ variant with the cyt *b*-F:H212N mutations produced by cells harboring pED3, or pED2+pED3, this red band started to elute at salt concentrations above 150 mM. In all cases, fractions were monitored for their absorption at 280 and 420 nm, and those with a

reddish color dithionite-reduced *minus* ferricyanide-oxidized optical difference spectra at 520–580 nm were recorded. Fractions containing the highest concentrations of *c*- and *b*-type cyts were pooled, concentrated using an Amicon Diaflo apparatus equipped with a PM30 membrane, and stored at -80°C . Concentrated cyt *bc*₁ preparations from cells carrying pMTS1-F or pED3 were passed twice through an anti-FLAG M2 affinity column (1 ml), pre-equilibrated with 15 CV of TBS (50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, 20% glycerol and 0.01% (w/v) DDM). The column was washed with 10 CV of the same buffer, and eluted with 6 CV of a solution containing 100 $\mu\text{g/ml}$ FLAG peptide in TBS. Fractions containing cyt *bc*₁ were pooled, concentrated using Amicon Ultra (50,000 M_r cut off) centrifugal filter devices (Millipore Inc.), and stored at -80°C in the presence of 20% glycerol until further use. In the case of cyt *bc*₁ variants derived from cells carrying pMTS1-S or pED2, concentrated sample were loaded twice onto a Strep-Tactin affinity column (1 ml) pre-equilibrated with 5 CV of TBS buffer. The column was washed with 10 CV of the same buffer, and eluted with 6 times 0.5 CV of a solution containing 1mM hydroxy-azophenylbenzoic acid (HABA) in TBS. Fractions containing cyts *bc*₁ were pooled, concentrated using Amicon Ultra (50,000 M_r cut off) centrifugal filter devices (Millipore Inc.) and stored at -80°C in the presence of 20 % glycerol until further use. When affinity purified cyt *bc*₁ samples were subjected to a second affinity chromatography using a different epitope (*i.e.*, Flag to Strep or Strep to Flag), they were first concentrated and then processed as described above.

Chemicals

The cyt *bc*₁ inhibitors antimycin A, myxothiazol and stigmatellin as well as duroquinone were purchased from Sigma Inc. Dodecyl maltoside was from Anatrace Inc., and DEAE-BioGel A from Bio-Rad. All other chemicals were of reagent grade or of highest quality commercially available.

Results

A genetic system to study intra- and inter-monomer interactions in cyt *bc*₁

A new genetic system that produces heterodimeric variants of cyt *bc*₁ was developed to study intra- and inter-monomer interactions in *R. capsulatus* enzyme. In this system, alleles of *petA*, *petB* and *petC* encoding cyt *bc*₁ subunits were carried by different plasmids marked with distinct antibiotic resistance markers (Kan^R and Tet^R) to insure co-selection (Fig. 2A), and co-expressed in a strain devoid of its endogenous chromosomal-encoded enzyme (5). We reasoned that co-expression of genetically distinct copies of cyt *bc*₁ structural genes might yield homo-dimeric and hetero-dimeric enzyme variants if the assembly and dimerization of different subunits occur randomly during cyt *bc*₁ biogenesis (Fig. 2B). In addition, different epitope tags (Strep, Flag, HA and cMyc) were added to cyt *b* subunit of cyt *bc*₁ to physically distinguish the homo- and hetero-dimeric variants upon purification. Extension of the carboxyl terminal end of a wild type cyt *b* with any one of the four epitope-tags tested yielded functional cyts *bc*₁ that fully complemented for Ps growth a cyt *bc*₁[−] strain of *R. capsulatus*. The Strep- and Flag- tagged cyt *bc*₁ derivatives were chosen for further experiments based on their relative stabilities during subsequent purification procedures.

A hetero-dimeric cyt *bc*₁ to probe inter-monomer electron transfer between its hemes *b*_L

A hetero-dimeric cyt *bc*₁, with one inactive Q_i site in one monomer, and one inactive Q_o site in the other monomer was sought to investigate inter-monomer electron transfer between the two hemes *b*_L of cyt *bc*₁ (Fig. 2B). Earlier, we had reported that cyt *b*:Y147A mutation abolishes electron transfer from oxidized QH₂ to heme *b*_L without affecting the binding of Q/QH₂ to the Q_o site, the electron transfer from heme *b*_L to *b*_H (32), or the mobility of the

Fe/S protein head domain between cyt *b* and cyt *c*₁ (12). Similarly, we had found that cyt *b*:H212N mutation caused loss of heme *b*_H without prohibiting enzyme assembly, but enhanced the Fe/S protein head domain mobility at the Q_o site by decreasing intra- and inter-monomer regulatory controls (25). These two mutations together with the Flag- and Strep-tags were opted to produce “two Q_o site defective homo-dimer” (cyt *b*-S:Y147A + cyt *b*-S:Y147A), “two Q_i site defective homo-dimer” (cyt *b*-F:H212N + cyt *b*-F:H212N), and “one Q_o site + one Q_i site defective hetero-dimer” (cyt *b*-S:Y147A + cyt *b*-F:H212N) cyt *bc*₁ variants (Fig. 2B). Moreover, earlier works indicated that the amounts of membrane proteins produced in *R. capsulatus* cells under different growth conditions depended on the plasmids used. A broad host-range plasmid of pRK-type (*i.e.*, pMTS1) overproduced cyt *bc*₁, whereas a “composite” plasmid (*i.e.*, pMTS0) obtained by fusing a pRK-type plasmid (*i.e.*, pRK404) with a ColE1-type plasmid (*i.e.*, pPET1) yielded variable levels of overproduction in different cases (5, 38). Thus, variants with cyt *b*-S:Y147A and cyt *b*-F:H212N substitutions carried by pRK-type (Kan^R pED2 and Tet^R pHY101, respectively) or composite (Kan^R pHY103 and Tet^R pED3, respectively) plasmids were constructed (Table 1), conjugated into a cyt *bc*₁⁻ mutant under Res growth conditions using appropriate Kan^R and Tet^R selections, and yielded cells harboring one or two different kinds of plasmids and producing homo- and hetero-dimeric cyts *bc*₁ variants.

Characterization of *R. capsulatus* strains producing homo- and hetero-dimeric cyts *bc*₁

R. capsulatus strains harboring a single plasmid with wild type *petABC* (pMTS1, pMTS1-S and pMTS1-F) were Ps⁺, and those with mutant *petABC* (pED2, pED3, pHY101 and pHY103) were Ps⁻ (Fig. 3A). Interestingly, cells harboring two plasmids in any of the four combinations tested, (A=pED2+pED3; B=pED2+pHY101; C=pHY103+pED3; and D=pHY103+pHY101) exhibited slow Ps (*i.e.*, Ps^{+/-}) growth (Table 2), suggesting that concurrent expression of genetically distinct *petABC*s yielded functional hetero-dimeric cyts *bc*₁, which is a prerequisite for Ps growth of *R. capsulatus*. Indeed, use of SDS-PAGE/immunoblots with antibodies against cyt *b* as well as the Strep or Flag epitopes indicated that chromatophore membranes from the two plasmids harboring cells grown under Res conditions contained both Strep- or Flag-tagged cyt *bc*₁ variants (Fig. 2C). Semi-quantitative estimations suggested that among the four combinations, those containing two pRK-type (pED2+pHY101) or two composite (pHY103+pED3) plasmids produced comparable amounts of Strep- or Flag-tagged cyts *b*, and revealed that this subunit was matured and assorted independently during cyt *bc*₁ biogenesis. Cells harboring pED2+pED3 (combination A, cyt *b*-S:Y147A + cyt *b*-F:H212N) were retained for further studies. Under Ps growth conditions, cells harboring pED2+pED3 formed smaller colonies than a wild type strain, suggesting that they exhibited lower than normal levels of cyt *bc*₁ activity, while those containing either pED2 or pED3 remained Ps⁻ (Fig. 3A). Cells harboring either pED2 or pED3, or both pED2+pED3 yielded Ps⁺ revertants with similar frequencies of about 10⁻⁵ (data not shown). Inspections of cell populations harboring pED2+pED3 indicated that over 99.99 % cells exhibited slow Ps^{+/-} growth, which was distinct from the vigorous Ps⁺ growth of rare revertants (< 0.01%) that occurred in the population (Fig. 3A, top).

In agreement with the Ps phenotypes, chromatophore membranes of cells harboring pMTS1, pMTS1-S and pMTS1-F plasmids had cyt *bc*₁ activities of about 1.74, 1.85 and 1.39 μmoles of cyt *c* reduced/min/mg total membrane proteins, whereas those with pED2 and pED3 yielded only 0.23 and 0.16 μmoles of cyt *c* reduced/min/mg total membrane proteins (~ 13 and 9 % of activity seen with wild type pMTS1). Cells harboring two plasmids exhibited higher, but somewhat variable (~ 22 to 15 % of wild type, partly due to enzyme lability) cyt *bc*₁ activities (Table 2), which could not be attributed to the rare revertants (at best forming 0.01% of the populations used). These data indicated that independent assortment of the two

different variants of cyt *b* occurred in cells harboring two plasmids to yield functional hetero-dimeric cyts *bc*₁ variants in line with their slow Ps growth.

Additional analyses of chromatophore membranes by SDS/PAGE-immunoblots established that cells harboring pED2+pED3 contained both cyt *b*-S and cyt *b*-F variants, and that the amounts of cyt *b* and cyt *c*₁ subunits detected using appropriate antibodies were similar to those observed with the corresponding wild type strains (Fig. 3B). Ascorbate (dotted lines) or dithionite (solid lines) reduced *minus* ferricyanide oxidized optical difference spectra (Fig. 3C) of chromatophore membranes were used to assess the amounts of total *c*-type and *b*-type cyts in different strains. As expected, the wild type parent (pMTS1) and the cyt *b*-S:Y147A (pED2) mutant had more 560 nm than 550 nm absorbing materials attributed to the *b*- and *c*-type cyts, respectively. The cyt *b*-F:H212N (pED3) mutant showed the opposite pattern, in agreement with its missing heme *b*_H. Comparatively, membranes of cells harboring pED2+pED3 had intermediate amounts of *b*- and *c*-type cyts, seemingly producing cyts *bc*₁ variants with different amounts of heme *b*_H (Fig. 3C and D). Dark equilibrium redox titrations using membranes derived from appropriate strains verified the thermodynamic properties of the hemes *b*_L, *b*_H and *c*₁ cofactors of cyt *bc*₁ (Table 2). In these experiments, wild type cyt *bc*₁ (pMTS1) displayed *E*_{m,7} values of -133, +67 and +310 mV for the hemes *b*_L, *b*_H and *c*₁ cofactors, respectively. We note that these values appear slightly higher than those routinely observed, possibly due to the experimental conditions used here. The homo-dimeric cyt *bc*₁ mutant variants cyt *b*-S:Y147A and cyt *b*-F:H212N resulted in -137, +70 and +305 mV and -145, +62 and +300 mV for the hemes *b*_L, *b*_H and *c*₁ cofactors, respectively. Similar *E*_{m,7} values were obtained with membranes of cells harboring pED2+pED3 (-147, +73 and +300 mV for the hemes *b*_L, *b*_H and *c*₁, respectively). We note that the reported *E*_{m,7} values should be considered approximate, especially in the light of heterogeneous cyt *bc*₁ subpopulations in the membranes of these strains. The identity of cyt *b* species exhibiting an *E*_{m,7} value of around +60 mV in membranes of cells harboring pED3 (cyt *b*-F:H212N) is unknown, but it does not correspond to heme *b*_H, which is missing in this mutant (see EPR studies below and Supplemental information). In any event, no major difference was seen between the *E*_{m,7} values of the hemes *b*_H (when present), *b*_L and *c*₁ cofactors of the homo- and hetero-dimeric cyt *bc*₁ variants (Table 2). However, note that these experiments could not decide whether or not the two hemes *b*_L of a heterodimeric cyt *bc*₁ variant have identical or different *E*_{m,7} values.

The cofactors associated with cyt *bc*₁ were also analyzed using EPR spectroscopy, especially to monitor the hemes *b*_L and *b*_H, and the [2Fe2S] cluster of cyt *bc*₁ in various mutants (Fig. 3D and E). The EPR resonance lines 3.78 and 3.43 corresponding respectively to the *g*_z features of oxidized hemes *b*_L and *b*_H were detected in membranes derived from cells producing wild type (pMTS1) and cyt *b*-S:Y147A variant (pED2) (39). Cells with cyt *b*-F:H212N variant (pED3) lacked the *g*_z feature at 3.43, while those harboring pED2+pED3 had both of the *g*_z at 3.78 and 3.43. However, the amplitudes and shapes of the latter EPR spectra were distinct from those of a wild type strain (Fig. 3D). EPR dark equilibrium redox titrations of the hemes *b*_L and *b*_H of cyt *bc*₁ were carried out to avoid any overlap with interfering component(s) of similar optical properties (Fig. S1A and B, Supplemental Information). These measurements indicated that the hemes *b*_L and *b*_H detectable in membranes from cells harboring pED2+pED3 producing hetero-dimeric cyt *bc*₁ variants had *E*_{m,7} values comparable to those determined by optical titrations (Table 2 and Supplemental Information). The EPR spectra of the Fe/S protein subunit of cyt *bc*₁ were also monitored. The Fe/S protein was present in similar amounts in all cases, as deduced from the *g*_y transition amplitudes of the reduced [2Fe2S] clusters (Fig. 3E). The shape and *g*_x (1.804) values of the Fe/S protein [2Fe2S] cluster signals were identical in all mutants, indicating that the Q_o site Q/QH₂ occupancies of all cyt *bc*₁ variants were comparable. Overall, the

data established that all strains, including those harboring pED2+pED3, produced the expected cyt *bc*₁ mutant variants at similar amounts.

Purification and characterization of epitope-tagged homo-dimeric cyts *bc*₁

The properties of cells harboring pED2+pED3 enticed us to seek for direct physical evidences for the occurrence of active hetero-dimeric cyts *bc*₁ under appropriate conditions. A two-step purification method, starting with anionic exchange chromatography with a DEAE-Biogel column (15) followed by epitope tag affinity chromatography, yielded large quantities of purified, epitope-tagged wild type (pMTS1-S and pMTS1-F) and single mutation (pED2 and pED3) cyt *bc*₁ homo-dimers for biochemical characterizations (Table 2, *bc*Fig. 4A, and not shown). Purified wild type Strep-(pMTS1-S) or Flag-tagged (pMTS1-F) cyt *c* had activities of 8.3 and 7.7 micromoles of cyt *c* reduced/min/mg of total proteins, respectively. In contrast, purified mutant cyt *b*-S:Y147A (pED2) and cyt *b*-F:H212N (pED3) variants had about ten fold lower activities of 0.8 and 0.5 micromoles of cyt *c* reduced/min/mg of total proteins, respectively. SDS-PAGE/immunoblots with cyt *b* and epitope tag antibodies, as well as optical and EPR spectral analyses (Fig. 4B and C), indicated that the properties of purified, epitope-tagged homo-dimeric cyt *bc*₁ variants were comparable to those of their membrane-embedded versions. Only the cyt *b*-F:H212N (pED3) variant lacking heme *b*_H was unstable, and aggregated rapidly. This mutant enzyme did not bind tightly to DEAE-Biogel column at salt concentrations above 150 mM NaCl, rendering its purification difficult, in contrast to the other variants that remained bound up to 350 mM salt concentrations,

Physical evidence for the production of a heterodimeric cyt *bc*₁ in *R. capsulatus*

Despite the difficulties encountered with the cyt *b*-F:H212N mutant, we purified epitope-tagged hetero-dimeric cyt *bc*₁ variants from cells harboring pED2+pED3 by DEAE-Biogel chromatography followed by two consecutive affinity chromatography steps. DEAE-Biogel chromatography yielded partially purified and active epitope-tagged cyt *bc*₁ (~ 2 micromoles of cyt *c*/min/mg of total proteins) with expected optical and EPR spectra (Fig. 4). These preparations were expected to contain both homo-dimeric (called SS and FF for cyt *b*-S:Y147A and cyt *b*-F:H212N, respectively) and hetero-dimeric (called FS for cyt *b*-S:Y147A + cyt *b*-F:H212N) cyt *bc*₁ populations. They were further fractionated into homo- (FF and SS) and hetero- (FS) dimeric cyt *bc*₁ variants by two consecutive affinity chromatography steps (Flag- followed by Step-binding columns, and Strep- followed by Flag-binding columns) (Fig. S2A, and S2B, Supplemental Information). In both cases, final elution fractions contained highly enriched hetero-dimeric cyt *b*-S:Y147A + cyt *b*-F:H212N (FS) variants of cyt *bc*₁, which had no enzymatic activity. None the less, the successful isolation of FS subpopulations by two consecutive affinity chromatography steps in either order, confirmed the occurrence of hetero-dimeric cyts *bc*₁ in cells harboring pED2+pED3 plasmids (Supplementary Information).

Light activated, single turnover cyt *c* and cyt *b* reduction kinetics of homo and heterodimeric *R. capsulatus* cyt *bc*₁

The slow Ps growth ability of cells harboring pED2+pED3 plasmids indicated that they conducted photosynthetic cyclic electron transport upon light activation. The cyt *c* and cyt *b* reduction kinetics were monitored using chromatophore membranes derived from cells harboring homo- or hetero-dimeric cyt *bc*₁ variants using light activated, time resolved optical kinetic spectroscopy (Fig. 5). Chromatophore membranes of various strains were normalized per amount of photochemical reaction center (RC) that they contained (Fig. 5, upper panel). Millisecond time scale cyt *c* (Fig. 5, middle panel) and cyt *b* (Fig. 5, lower panel) reduction kinetics were monitored at an ambient potential (*E*_h) of 100 mV with the wild type (pMTS1) as well as the homo-dimeric and hetero-dimeric cyts *bc*₁ variants (pED2,

PED3 and pED2+pED3). As expected, chromatophore membranes of the wild type strain exhibited sub-msec time scale oxidation, followed by cyts *c* re-reduction kinetics in the msec range (40). Addition of myxothiazol or stigmatellin inhibited the cyt *c* re-reduction kinetics partly or completely, respectively (Fig 5, left column, middle row). Stigmatellin revealed the full extent of cyt *c* oxidation by inhibiting the Q_o site catalysis and immobilizing the Fe/S protein head domain at this site, while myxothiazol inhibited only QH₂ oxidation leaving the Fe/S head domain mobile (8). Fast cyt *c* re-reduction kinetics were not seen in membranes derived from cells carrying either pED2 or pED3, even in the absence of inhibitor, in agreement with their lacking an active cyt *bc*₁ (Fig. 5, middle two column, middle rows). Membranes of cells harboring cyt *b*-S:Y147A (pED2) variant of cyt *bc*₁ exhibited slightly faster reduction kinetics than those seen with cyt *b*-F:H212N (pED3), and these kinetics were fully inhibited upon addition of inhibitors, as reported earlier (32). The extents of cyt *c* oxidation seen with inactive cyt *bc*₁ mutants in the absence of inhibitor were almost similar to those observed with wild type cyt *bc*₁ in the presence of inhibitors. Thus, these mutants did not contain active cyts *bc*₁ in agreement with their Ps⁻ phenotype. Interestingly, membranes of cells harboring pED2+pED3 exhibited cyt *c* re-reduction kinetics that were faster than those carrying pED2 or pED3, but slower than those seen with membranes from wild type cyt *bc*₁ producing cells (Fig. 5, right column, middle row). In all cases the cyt *c* re-reduction kinetics were sensitive to myxothiazol and stigmatellin (38), indicating that they reflected cyt *bc*₁ specific activities. Kinetic patterns similar to cyt *c* re-reduction kinetics were also observed with cyt *b* reduction kinetics (Fig. 5, lower panel). Upon addition of antimycin A, which eliminates electron transfer from cyt *b*_H to Q at the Q_i site, rapid cyt *b* reduction was seen with membranes derived from cells producing wild type cyt *bc*₁, but not with those that have a defective enzyme lacking heme *b*_H (pED3), or an extremely slow Q_o site catalysis (pED2). In the presence of antimycin A, membranes from cells harboring pED2+pED3 exhibited readily detectable cyt *b* reduction kinetics, although these kinetics were slower, and had amplitudes smaller than those seen with membranes from cells producing wild type cyt *bc*₁ (Fig. 5, right versus left columns, lower row). In all cases, the cyt *b* reduction kinetics were sensitive to the Q_o site inhibitors myxothiazol or stigmatellin, confirming that they originated from Q_o site catalysis. The cyt *c* re-reduction and cyt *b* reduction kinetics seen with membranes of cells harboring the cyt *b*-S:Y147A + cyt *b*-F:H212N variants of cyt *bc*₁ (pED2+pED3) established that the hetero-dimeric enzymes were active and able to sustain cyclic electron transport between the RC and cyt *bc*₁ to support Ps growth. As the homo-dimeric enzymes are inactive, we concluded that hetero-dimeric cyts *bc*₁ variants effectively carry out electron transfer between the hemes *b*_L of their two monomers (Fig. 2B, middle panel).

The extent of cyt *b* reduction depends on the amount of the hetero-dimeric cyt *bc*₁ present in cells

As different plasmids with different replication properties yielded different amounts of hetero-dimeric cyt *bc*₁ based on SDS-PAGE/immunoblot data, we examined membranes derived from cells harboring the four different combinations (A, B, C and D) of plasmids (Fig. 2C) producing hetero-dimeric cyts *bc*₁. The extents of cyt *b* reduction were monitored in the presence of antimycin A using membrane preparations normalized per amounts of RC (Fig. 6). The data indicated that membranes from cells harboring identical plasmids (*i.e.*, pRK-type pED2+pHY101, or composite pED3+pHY103) exhibited more pronounced cyt *b* reduction and cyt *c* re-reduction kinetics. Under identical light activation conditions, the detection of higher extents of cyt *b* kinetics per amount of RC suggested that higher amounts of hetero-dimeric cyts *bc*₁ were electronically coupled to the RCs. These experiments documented that the new genetic system used here has the ability to produce different amounts of hetero-dimeric cyt *bc*₁ variants, changing the coupling ratios between the physiological Ps electron transport partners in chromatophore membranes.

Discussion

Static three-dimensional structural models often depict cyt bc_1 as a dimer of two identical monomers (8–11, 22) (Fig. 1). However, the mobility of the Fe/S subunit during hydroquinone oxidation at the Q_o site indicates that the enzyme undergoes large conformational changes during its catalytic turnover (41). These considerations led us to the “hetero-dimeric modified Q cycle” model (26), which was reviewed recently by Cooley (42). In the present work, we developed a new genetic system to examine intra- and inter monomer interactions in cyt bc_1 , and used it to test the occurrence of inter-monomer electron transfer between the hemes b_L of this enzyme. We thought that if a hetero-dimeric cyt bc_1 with only one active Q_o site in one monomer and one active Q_i site in the other monomer is obtained by co-expression of two *petABC*, then inter-monomer electron transfer between the hemes b_L could be probed. However, neither the degree of recombination *in vivo* that might occur between homologous copies of *petABC* carried by two plasmids, nor which specific mutation(s) to use to inactivate the Q_o and Q_i sites, were known (6). We found that the Ps^+ reversion rates observed in cells harboring two similar plasmids, co-maintained using two antibiotic resistance markers, were not exorbitantly high as compared with those carrying only one plasmid. In order to avoid the mutations that affect the occupancy of the Q binding sites or those that immobilize the Fe/S protein head domain, we opted for the cyt *b*:Y147A and cyt *b*:H212N mutations that we had characterized earlier (12, 32). To our knowledge, the hetero-dimeric cyt bc_1 obtained in this work (Fig. 7) is the first example of such an enzyme that is active enough to sustain *Ps* growth. This cyt bc_1 variant remains active, and conserves its subunit stoichiometry upon detergent solubilization and DEAE-Biogel chromatography, but loses its activity during subsequent affinity purification. Matrix-induced inactivation observed with wild type *R. capsulatus* cyt bc_1 (15) might account for the loss of activity. However, as Strep tagged wild type and cyt *b*-S:Y147A enzymes were readily purified without loss of activity using the same columns, the observed instability and the poor DEAE-Biogel binding properties of the hetero-dimeric cyt *b*-S:Y147A + cyt *b*-F:H212N variant is probably due to the intrinsic instability of cyt bc_1 in the absence of its heme b_H . The four helices-bundle that coordinates the hemes b_L and b_H defines the interface between the two monomers, and is a key feature for the dimeric architecture of cyt bc_1 (11). While embedding a mutant enzyme in the membrane lipid bilayer appears to compensate for the loss of heme b_H by preventing its disassembly, progressive loss of enzyme activity and partial dissociation of the monomers occurs upon detergent dispersion and renders purifications difficult.

Our earlier works on subunit maturation and assembly of *R. capsulatus* cyt bc_1 (14, 43) indicated that the Fe/S protein, cyt *b* and cyt c_1 subunits follow independent pathways for prosthetic group acquisition and maturation to yield a stable cyt *b*- c_1 sub-complex to which the Fe/S subunit could be added even *in vitro* (15). How the monomers dimerize to form the active enzyme is unknown. Our finding that co-expression of two inactive cyts bc_1 yields an active hetero-dimeric variant with distinct features suggests that cyt bc_1 monomers, or their subunits, associate independently to yield homo- and hetero-dimeric cyt bc_1 variants. A likely possibility is the association of cyt *b* and cyt c_1 into a cyt *b*- c_1 dimeric sub-complex followed by the addition of two Fe-S protein subunits to form the active dimeric cyt bc_1 (14). Whether first the complete monomers are formed, and then are dimerized, might be tested using the genetic system described in this work to further elucidate the biogenesis process of the cyt bc_1 .

An important finding of this work is that, under appropriate conditions, not only does inter-monomer electron transfer occur between the hemes b_L of cyt bc_1 monomers (Fig. 7), but also the rate and efficiency of this event is high enough to sustain physiological *Ps* growth of *R. capsulatus*. The cyt *b* reduction kinetics in the presence of antimycin A at an $E_h = 100$ mV

observed with the hetero-dimeric cyts bc_1 were slower than the similar kinetics seen with a wild type enzyme. Note that the measured cyt b reduction rates in the presence of antimycin A encompass multiple, indirectly coupled steps, and in some cases (*e.g.*, A and D constructs) the signal to noise ratio are poor rendering them less reliable. None the less, semi-quantitative estimations of these rates (by curve fitting to a single exponential equation) (Fig. 6) suggested that while the wild type membranes exhibited rates of $\sim 325 \text{ sec}^{-1}$, those containing hetero-dimeric cyts bc_1 rates ranged from $55 - 60 \text{ sec}^{-1}$ (A and D constructs) to $150 - 180 \text{ sec}^{-1}$ (B and C constructs). In any event, in hetero-dimeric cyts bc_1 , inter-monomer hemes $b_L-b_L-b_H$ electron transfer rates are comparatively slower than intra-monomer hemes b_L-b_H rates (Fig. 7). Moreover, depending on the amounts of hetero-dimeric variants, more cyts c (cyts c_2 and c_y) dependent cyt bc_1 - RC cyclic electron transfer was observed upon light activation of a given amount of RC. Strains producing larger amounts of hetero-dimeric enzymes exhibited more pronounced cyt b and cyt c reduction kinetics, possibly improving RC-cyt c_y/c_2 -cyt bc_1 coupling to better support Ps growth. Future studies producing different hetero-dimeric cyts bc_1 variants will further address these issues in details.

Studies similar to those described in this work have been reported recently (29, 30). The work with *Paracoccus denitrificans* used two plasmids to co-express a native and a Q_o site-defective cyt bc_1 variant that were tagged by two different epitope tags in order to purify and analyze the homo- and hetero-dimeric enzymes produced. It was reported that a purified hetero-dimeric cyt bc_1 with only one active Q_o site was as active as a native enzyme with two active Q_o sites in the time scales used, and considered to reflect inter-monomer electron transfer (30). A second related work used a modified *petABC*, where *petB* encoding cyt b was duplicated and the two copies were linked in frame using a small exogenous sequence encoding a dodecapeptide, and a carboxyl terminal epitope tag was added to facilitate purifications (29). This construct yielded a non-native cyt bc_1 -like enzyme akin to a cyt bc_1 dimer with its two cyt b subunits substituted by a covalently linked “cyt b -cyt b ” hybrid. Inter-monomer electron transfer between the hemes $b_L-b_L-b_H$ of this artificial cyt bc_1 -like enzyme was shown to occur using appropriately mutated cyt b -cyt b fusion subunit, and light activated kinetic spectroscopy (29). The Ps growth ability and reversion rate of these mutants as well as the subunit stoichiometry, dimeric state or 3D structures of these enzymes have not been reported (29). Comparisons of the properties of the artificial cyt bc_1 -like variants with two fused cyt b with the more native-like hetero-dimeric enzymes as reported in this work would be informative.

Experimental observation of inter-monomer electron transfer (Fig. 7) is highly significant with respect to the “hetero-dimeric Q cycle” model, which postulates dismutation of the two SQ molecules accumulated at the Q_i sites to Q and QH_2 to initiate the next turnover of cyt bc_1 (26). Recently, Cooley has discussed structural elements of cyt b that might govern Q_o - Q_i sites interactions across the membrane, such as the formation of SQ at a Q_i site allowing the cognate Q_o site *ef* loop to act as a temporal “gate” for the initiation of next QH_2 oxidation (42). If such a gate exists, then how long this gate remains “closed” might depend on how stable the SQ is at the Q_i site. Q_i site mutants with increased SQ K_{stab} at the Q_i site, like the cyt b :H217R substitutions produce functional but less active homo-dimeric cyts bc_1 variants (38), suggesting that extreme stability of SQ may be required to inactivate cyt bc_1 completely. In agreement with this suggestion, a fused cyt b -cyt b containing cyt bc_1 -like enzyme formed of one fully active and one both Q_o and Q_i sites inactive monomers apparently remains active (29). Undoubtedly, ongoing use of different genetic systems that produce hetero-dimeric cyt bc_1 variants would be invaluable to assess the validity of current catalytic models invoking dimeric cyt bc_1 .

Acknowledgments

The authors thanks N. Selamoglu for critical reading of the manuscript, and acknowledge the Johnson Research Foundation for use of EPR and kinetic spectroscopy facilities, without which this work would have been impossible, and thank Drs. P. L. Dutton and C. Moser for their help.

This work was supported by grants from NIH (R01 GM 38237) and DOE (91ER 20052) to F. D.

Abbreviations

Cyt <i>bc</i>₁	ubihydroquinone: cytochrome <i>c</i> oxidoreductase
EPR	electron paramagnetic resonance
Fe/S	iron sulfur
Q_o	hydroquinone oxidation
Q_i	quinone reduction
SQ	semiquinone
Ant	antimycin A
Myx	myxothiazol
Stig	stigmatellin
MPYE	mineral-peptone-yeast-extract
QH₂	hydroquinone
Q	quinone
MOPS	4-morpholinepropanesulfonic acid
DBH₂	decylbenzohydroquinone
Flag	FLAG epitope
Strep	Strep-tactin epitope

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Figure 1. The three dimensional structure of cytb₁ dimeric and its cofactors

The three dimensional structure (PDB # 1ZRT for *R. capsulatus*) (left) of cytb₁ depicts it as a dimeric enzyme with two identical monomers, M1 and M2, each formed of the Fe/S protein (light blue), cyt b (dark blue in M1 and yellow in M2) and cyt c₁ (beige). The figure is generated using Pymol (DeLano Scientific, San Carlos, CA), and the cofactors of each monomer ([2Fe2S] cluster, hemes b_L and b_H and c₁) are shown in different colors (blue in M1 and red in M2). Black and red arrows indicate the electron transfer pathways between the cofactors and the Q_O/Q_I active sites of cytb₁, respectively. Inter-monomer hemes b_L – b_L distance is indicated with a dotted line, but for the sake of clarity the distances (11) separating the cofactors are not shown. The right panel shows a schematic depiction of a homo-dimeric cytb₁, with its cofactors and Q_O/Q_I active sites, as described for the left panel.

Figure 2. A genetic system to study intra- and inter-monomer interactions in cyt *bc*₁ dimer: cyt *b*-S:Y147A + cyt *b*-F:H212N hetero-dimeric cyt *bc*₁ variant in *R. capsulatus*

A. The *petABC* of *R. capsulatus* is composed of *petA*, *petB* and *petC* genes encoding the Fe/S protein, cyt *b* and cyt *c*₁ subunits, respectively. The different plasmids carrying this operon (left), their replicon type and antibiotic resistance markers (right), as well as the location of cyt *b* mutations in *petB* (•, middle) that they carry are shown together with the Strep (S) and Flag (F) epitope tags added at the carboxyl terminal end of cyt *b*. The brackets are to indicate that pED2 and pHY103 carry the same cyt *b* mutation but in two different replicons with the same antibiotic resistance marker, and the same is also the case for pED3 and pHY101. **B.** Co-expression of a Kan^R plasmid carrying *petABC* with cyt *b*-S:Y147A mutation and a Tet^R plasmid carrying *petABC* with cyt *b*-F:H212N mutation yields the homo-dimeric cyt *b*-S:Y147A (left) and cyt *b*-F:H212N (right) as well as the hetero-dimeric cyt *b*-S:Y147A + cyt *b*-F:H212N (middle) cyt *bc*₁ variants. The thin dotted arrows refer to residual electron transfer that occurs in the homo-dimeric cyt *b*-S:Y147A variant, whereas the thick red dashed arrows, and thick blue solid arrows correspond to electron transfer to the high and low potentials cofactors of cyt *bc*₁, respectively. Note that inter-monomer hemes *b*_L to *b*_L electron transfer is indicated by a thick blue solid arrow. **C.** SDS/PAGE-immunoblot analyses of Strep- and Flag-tagged cyt *b* in chromatophore membranes of strains harboring different combinations of Kan^R and Tet^R plasmids (A to D, as indicated) producing hetero-dimeric cyt *bc*₁ variants. Approximately 10 µg of total proteins were used and cyt *b* contents were determined using polyclonal antibodies specific to *R. capsulatus* cyt *b* (*b*) as well as commercially available antibodies against the Strep (S) and Flag (F) epitope tags. Properties of the plasmids used are described in Table 1.

Figure 3. Photosynthetic growth phenotypes and biochemical properties of *R. capsulatus* strains producing hetero-dimeric cyt *bc*₁ variants

A. Photosynthetic (Ps) growth on enriched medium of *R. capsulatus* strains harboring plasmids pMTS1, pMTS1-S and pMTS1-F producing wild type, pED2 and pED3 producing homo-dimeric cyt *b*-S:Y147A and cyt *b*-F:H212N, and pED2+pED3 producing hetero-dimeric cyt *b*-S:Y147A + cyt *b*-F:H212N cyt *bc*₁ variants are shown. Cells harboring pMTS1, pMTS1-S and pMTS1-F are Ps⁺, those harboring pED2 or pED3 are Ps⁻, and those harboring both pED2 and pED3 are Ps^{+/-} (*i. e.*, slow growth). Shown above the plate a small section where rare Ps⁺ revertants in pED2+pED3 cultures (indicated by arrows) are observed. **B.** Chromatophore membranes of appropriate *R. capsulatus* strains described in **A** and grown by respiration in enriched medium were analyzed by SDS-PAGE (15%)/immunoblots using approximately 10 μg of total proteins. The cyt *b* and cyt *c*₁ subunits were determined using polyclonal anti-cyt *b* or anti-cyt *c*₁ (right) or anti-Strep II and anti-Flag M2

(left) antibodies as indicated. **C.** Total *b*-type and *c*-type cyts contents of chromatophore membranes (0.3 mg/mL) obtained from appropriate *R. capsulatus* strains (as described in **A**) grown by respiration in enriched medium were determined by ascorbate (dashed lines) or dithionite (solid lines) reduced *minus* ferricyanide oxidized optical difference spectra. Note the differences seen between the stains harboring different plasmids and producing different cyt *bc*₁ variants. **D.** Low spin heme EPR spectra from redox-poised chromatophore membranes obtained from appropriate *R. capsulatus* strains (as described in **A**) grown by respiration in enriched medium. The ambient redox potential (E_h) values at which the spectra were recorded were 255, 260, 300 and 290 mV for membranes derived from cells harboring pMTS1, pED2, pED3 and pED2+pED3 plasmids, respectively. Experimental conditions used were as follows: temperature, 10K; microwave power, 10 mW at 9.420 GHz; modulation amplitude, 10 G at 100 kHz, number of scan, 4, and note that no baseline correction was used. **E.** [2Fe-2S] EPR spectra of redox-poised chromatophore membranes obtained from appropriate *R. capsulatus* strains (as described in **A**) grown by respiration in enriched medium. The E_h values at which the spectra were recorded were 123, 112, 116 and 126 mV for membranes derived from cells harboring pMTS1, pED2, pED3 and pED2+pED3 plasmids, respectively. Experimental conditions used were as follows: temperature, 20K; microwave power, 2 mW at 9.416 GHz; modulation amplitude, 20 G at 100 kHz; number of scan, 1.

Figure 4. Biochemical properties of partially purified *R. capsulatus* cyt *b*-S:Y147A + cyt *b*-F:H212N hetero-dimeric cyt *bc*₁ variants

A. Homo-dimeric (FF and SS) and hetero-dimeric (FS) cyts *bc*₁ variants purified from cells harboring pED2+pED3 by DEAE-Biogel chromatography were analyzed by SDS-PAGE (12.5%)/immunoblots using approximately 5 µg proteins and anti-Strep II (α-Strep) or anti-Flag M2 (α-Flag) monoclonal antibodies. Fe/S protein, cyt *b* and cyt *c*₁ subunits of cyt *bc*₁ are indicated. Note that Flag tagged (pMTS1-F) and Strep tagged (pMTS1-F) wild type, as well as homodimeric cyt *b*-S:Y147A (pED2) and cyt *b*-F:H212N (pED3) cyt *bc*₁ variants were also purified, but not shown for the sake of clarity. **B.** Total *b*-type and *c*-type cyts contents of purified materials obtained from appropriate *R. capsulatus* strains (as described in Fig. 2A) were used to determine their ascorbate (dashed lines) or dithionite (solid lines) reduced *minus* ferricyanide oxidized optical difference spectra. Note that these spectra are similar to those obtained using appropriate chromatophore membranes shown in Fig 3C. **C.** Low spin heme EPR spectra of purified materials obtained from appropriate *R. capsulatus* strains (as described in Fig. 2A). All samples were oxidized directly in the EPR tube by adding 5 µL 10mM ferricyanide. The experimental conditions were as in Fig. 3D, and a “non signal” portion of a baseline spectrum simulated by a third-order polynomial fit subtracted as baseline correction.

Figure 5. Light-induced, time-resolved cytochrome *b* reduction and cytochrome *c* re-reduction kinetics of various *R. capsulatus* strains

R. capsulatus strains harboring plasmids pMTS1 producing wild type, pED2 and pED3 producing homo-dimeric cytochrome *b*-S:Y147A and cytochrome *b*-F:H212N, respectively, and pED2+pED3 producing hetero-dimeric cytochrome *b*-S:Y147A + cytochrome *b*-F:H212N cytochrome *bc*₁ variants were analyzed. In each case, chromatophore membranes corresponding to an amount of RC equal to 0.30 μM were resuspended in 50 mM MOPS buffer (pH 7.0) containing 100 mM KCl at an E_h of 100 mV. The amount of RC in each case was determined from the extent of its photo-oxidation after a train of 10 flashes, separated by 50 ms at an E_h of 380 mV (upper panel) and an extinction coefficient of $29 \text{ mM}^{-1} \text{ cm}^{-1}$ at 605 minus 540 nm, as described in Material and Methods. In all samples, cytochrome *c* re-reduction kinetics (middle panel) were monitored at 550 minus 540 nm, in the absence of inhibitor (no), or in the presence of 10 μM myxothiazol (Myx) which abolishes QH_2 oxidation at the Q_0 site, or 10 μM stigmatellin (Stig), which abolishes electron transfer from the [2Fe-2S] cluster to heme *c*₁ by immobilizing the head domain of the Fe/S protein subunit at the Q_0 site. Similarly, cytochrome *b* reduction kinetics (lower panel) were monitored at 560 minus 570 nm, in the absence of inhibitor (no), or in the presence of 10 μM of the Q_i site inhibitor antimycin (Ant). Note that the samples of pED2 were analyzed using slightly different equipment, which yielded a higher signal to noise ratio than the other samples.

Figure 6. Light-induced, time-resolved cyt *b* reduction kinetics exhibited by *R. capsulatus* strains harboring different plasmid combinations yielding hetero-dimeric cyt *bc*₁ variants

The cyt *b* reduction kinetics were monitored using chromatophore membranes prepared from appropriate cells (pMTS1, pED2+pED3, pED3+pHY103, pHY101+pHY103 and pED2+pED3) grown in enriched medium either under respiratory growth conditions. All plasmid combinations (A to D) are described in Fig. 2 and Table 1. Experimental conditions were as described in Fig. 5, using chromatophore membranes containing 0.30 μ M of RC, appropriate mediators (Materials and Methods), 3 μ M valinomycin, and 10 μ M antimycin A. Samples were poised at 100 mV. Note that the extent and rate of cyt *b* reduction changes depending on the strains, with cells harboring identical replicons exhibiting more pronounced kinetics, possibly reflecting better electronic couplings in membranes harboring heterogeneous cyt *bc*₁ subpopulations.

Figure 7. A model depicting inter-monomer electron transfer between the hemes b_L of monomers in a hetero-dimeric cyt bc_1

The three dimensional structure (1ZRT) of *R. capsulatus* cyt bc_1 shown in Fig. 1 is modified to show (front view, left and top view, right) the location and nature of the mutations used in this work, revealing inter-monomer electron transfer between the hemes b_L cofactors of the enzyme. All structural features are as in Fig. 1, except that the cyt b :Y147A and cyt b :H212N substitutions are shown as space filling models in blue on M1 and red on M2 monomers. The absence of heme b_H in one monomer, due to the latter mutation, is indicated by a black ellipsoid, and the residual electron transfer that is unable to support Ps growth but still occurs in the presence of the former mutation is indicated by dotted arrows. The thick black arrows correspond to the productive inter-monomer electron transfer that occurs across the functional Q_0 and Q_i sites of the hetero-dimeric cyt bc_1 . Note that this electron transfer is efficient enough to support Ps growth of *R. capsulatus* but apparently is rate limiting because it can only confer a slow $Ps^{+/-}$ growth phenotype.

Table 1

Characteristics of the different plasmids used in this work

Name	Properties				Ref
	Mutation	<i>a</i> Tag	<i>b</i> Replicon	<i>a</i> Resistance	
pMTS1	No	No	IncQ/P4	Kan ^R	(38)
pMTS1-S	No	S	IncQ/P4	Kan ^R	This work
pMTS1-F	No	F	IncQ/P4	Kan ^R	This work
pED2	<i>petB</i> :Y147A (Q _o site)	S	IncQ/P4	Kan ^R	This work
pED3	<i>petB</i> :H212N (Q _i site)	F	fused incQ/P4+colE1	Tet ^R	This work
pHY101	<i>petB</i> :H212N (Q _i site)	F	IncQ/P4	Tet ^R	This work
pHY103	<i>petB</i> :Y147A (Q _o site)	S	fused incQ/P4+colE1	Kan ^R	This work

^a *petB* is carboxyl terminally epitope-tagged with S (Streptactin) or F (FLAG) tags as indicated, and Kan^R and Tet^R refer to kanamycin and tetracycline resistance, respectively.

^b IncQ/P4 and fused incQ/P4+colE1 refer to pRK-type and composite-type plasmids, respectively.

Table 2

Characteristic of various homo- and hetero-dimeric cyt *bc*₁ variants

Strains	<i>a</i> P _s growth	<i>b</i> _{l/c} ratio	<i>c</i> DBH ₂ Activity %	<i>d</i> E _{m,7} (mV)			<i>e</i> Dimer assembly		
				<i>b</i> _L	<i>b</i> _H	<i>c</i> ₁	Homo	Hetero	
pMTS1	+	1.6	100	-133	67	310	-		
pMTS1-S	+	1.6	106	<i>g</i> nd	nd	nd	SS		
bpMTS1-F	+	1.7	80	nd	nd	nd	FF		
pED2	-	1.7	<i>f</i> _{13.2}	-137	70	305	SS		
pHY103	-	nd	nd	nd	nd	nd	SS		
pED3	-	0.5	<i>f</i> _{9.2}	-145	<i>g</i> na	300	FF		
pHY101	-	nd	nd	nd	nd	nd	FF		
pED2+pED3 (A)	±	0.9	22	-147	73	300	FF+SS+FS		
pED2+pH101 (B)	±	1	19	nd	nd	nd	FF+SS+FS		
pED3+pHY103 (C)	±	0.75	17.5	nd	nd	nd	FF+SS+FS		
pH101+pHY103 (D)	±	0.65	15	nd	nd	nd	FF+SS+FS		

^a P_s refers to photosynthetic growth, and +, -, and ± indicate vigorous, no and slow growth ability, respectively.

^b *b*_{l/c} ratio refers to cyt *b* to cyt *c* ratio determined by optical difference spectroscopy using membranes as described in Materials and Methods.

^c DBH₂ activity indicate the amount of DBH₂; cyt *c* reductase activity determined as described in Materials and Methods; 100 % refers to the cyt *bc*₁ activity (1.74 micromoles of cyt *c* reduced/min/mg of total membrane proteins) exhibited by cells harboring pMTS1. Note the variability of the values obtained possibly reflecting the lability of heterodimeric enzymes.

^d E_{m,7} refers to the redox midpoint potentials of the hemes *b*_L, *b*_H and *c*₁ were approximated as described in Materials and Methods.

^e SS, FF and FS indicate homo- and hetero-dimeric cyt *bc*₁ variants assembled, and for (A), (B), (C) and (D) refer to Fig. 2.

^f Note that 13.2% would correspond to 12.4% by taking as 100% the activity observed with cells harboring pMTS1-S. Similarly, 9.2% would correspond to 11.5% by taking as 100% the activity observed with cells harboring pMTS1-F.

^g nd, not done; na, not applicable.