



Hybrid fusions show that inter-monomer electron transfer robustly supports cytochrome bc_1 function in vivo



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ABSTRACT

Electronic connection between Q_o and Q_i quinone catalytic sites of dimeric cytochrome bc_1 is a central feature of the energy-conserving Q cycle. While both the intra- and inter-monomer electron transfers were shown to connect the sites in the enzyme, mechanistic and physiological significance of the latter remains unclear. Here, using a series of mutated hybrid cytochrome bc_1 -like complexes, we show that inter-monomer electron transfer robustly sustains the function of the enzyme in vivo, even when the two subunits in a dimer come from different species. This indicates that minimal requirement for bioenergetic efficiency is to provide a chain of cofactors for uncompromised electron flux between the catalytic sites, while the details of protein scaffold are secondary.

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1. Introduction

Cytochrome bc_1 (ubihydroquinone:cytochrome c oxidoreductase or mitochondrial complex III) is a multi-subunit enzyme which transfers electrons between quinone molecules and cytochrome c and couples this electron transfer to proton translocation across the membrane. This way cytochrome bc_1 contributes to generation of the protonmotive force used for cellular ATP production [1]. The enzyme operates according to the Q cycle, in which the net translocation of protons is a result of the joint action of two quinone oxidation/reduction sites (named the Q_o and Q_i sites) located at two opposite sides of the membrane [2]: the Q_o site oxidizes hydroquinone (quinol) and releases protons, the Q_i site reduces quinone and uptakes protons.

The Q_o and Q_i sites and cofactor chains are embedded within three subunits: cytochrome b (harbors Q_o , Q_i , heme b_H and heme b_L), cytochrome c_1 (harbors heme c_1) and the FeS subunit (harbors 2Fe–2S iron–sulfur cluster (FeS)) [1,3]. The Q_o and Q_i sites are connected by a chain composed of two hemes (heme b_L and heme b_H), while a chain composed of FeS and heme c_1 connects the Q_o site with cytochrome c pool. In Fig. 1A, these two chains are depicted as parts of the upper and lower branches, respectively. The scheme also shows that cytochrome bc_1 is a homodimer.

Each monomer contains all structural elements necessary to perform the catalytic Q cycle: the Q_o and Q_i sites and cofactors of the upper and lower branches. Intriguingly, crystal structures of the dimer revealed short distance between two hemes b_L , each coming from a different monomer [4,5]. This creates a possibility for inter-monomer electron transfer [6]. The idea of electron exchange between the monomers was adopted in mechanistic concepts describing operation of the dimer [7–11]. Recent experiments have indicated that this reaction is an integral part of ensemble of electron transfer reactions that take place within the dimer [12–16]. The existence of this connection converts the cofactor chains of a dimer into an H-shaped electron transfer system that connects all four catalytic sites [12].

At present, the inter-monomer electron transfer in cytochrome bc_1 is a matter of intense debate [11,14,17,18]. In particular, its physiological significance is not clear. This is because in the case of the fully operational dimer, the inter-monomer electron transfer can in principle be considered as an alternative to the intramonomer electron transfer. In this context an important question that needs addressing is whether the inter-monomer electron transfer can secure energetic efficiency of the enzyme at level that allows supporting the cytochrome bc_1 -dependent growth of cells. The results shown here not only confirm the physiologic competence of inter-monomer electron transfer, but also demonstrate that electronic connections in this system are generally robust over structural alterations and can sustain in vivo the catalysis in the non-native heterogeneous assemblies, when two cytochrome b parts of the dimer core come from different species.

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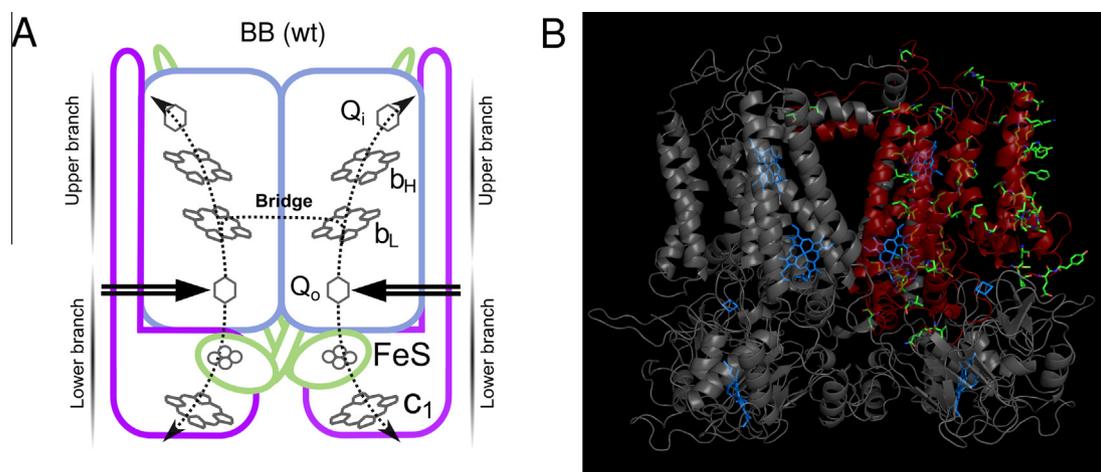


Fig. 1. Catalytic core of cytochrome bc_1 . (A) H-shaped electron transfer system formed by cofactor chains of: cytochrome b (blue), cytochrome c_1 (purple) and FeS subunits (green). Electron entry sites (double arrow), electron paths (dotted lines). (B) Crystal structure of *R. capsulatus* cytochrome bc_1 dimer (PDB: 1ZRT). Heme and FeS cofactors are blue. Cytochrome b subunit of one monomer is shown in red and depicts those amino acids that are different in *R. sphaeroides* (green sticks) to illustrate the heterogeneity of hybrid fusion protein used in this study.

2. Methods

2.1. Mutant strains

A genetic system for expression of hybrid B_S complex and hybrid fusion B_S - B complex in *Rhodobacter capsulatus* was described in [15]. Details of construction of expression vectors for mutant derivatives of $B_S B_S$ and B_S - B is described in the [Supplementary material](#).

2.2. Bacterial growth, genes and proteins

R. capsulatus strains were grown under semiaerobic/dark or photoheterotrophic conditions as described in [19,20]. When describing the type of growth, the term “aerobic growth” referred to aerobic growth under semiaerobic conditions (at relatively low oxygen concentration), while “photoheterotrophic growth” referred to growth under anaerobic conditions in light as a source of energy. For isolation of proteins from photoheterotrophically grown cultures, 100 μ l of overnight 2 ml liquid aerobic culture was spread on each of 14 MPYE plates which then were incubated in anaerobic jars (GasPak, BD) for 48 h.

2.3. Membrane proteins isolation and purification

Preparations of protein complexes from semiaerobic liquid cultures were as described in [21] with modifications reported in [19]. Complexes were isolated by affinity chromatography, Strep-tag purification was performed as described in [19]. SDS-PAGE of purified complexes was performed as described in [22]. To prepare the membranes from the photoheterotrophically grown cultures, cells from 14 MPYE plates were scraped to the MPYE medium and centrifuged (6000g, 20 min). The obtained pellet was subjected to the same purification steps as above.

2.4. Genetic analyses

The size and DNA sequence of the fusion genes present in cells grown photoheterotrophically were verified by restriction analyzes and DNA sequencing of plasmids isolated from *R. capsulatus* and amplified in *Escherichia coli* HB101. The double restriction digestion was performed with *Bst*XI (New England Biolabs) and *Sfu*I (Roche Diagnostics) enzymes, yielding shorter DNA fragment

(1950 bp) in case of cytochrome b gene or longer DNA fragment (3325 bp) for cytochrome $b_S b$ gene.

3. Results

Photoheterotrophic growth of *R. capsulatus* requires obligatory presence of the functional cytochrome bc_1 , thus the most straightforward way to verify in vivo functionality of specific mutant derivatives of this complex is to test the capability of the mutant cells to grow under the photoheterotrophic conditions [20]. In this work, we used this approach in combination with the asymmetric mutagenesis to assess physiological relevance of the inter-monomer electron transfer in cytochrome bc_1 . The asymmetric mutagenesis was accomplished using the system based on expression, in *R. capsulatus* cells, of cytochrome bc_1 -like complexes in which two separate cytochrome b subunits of the dimer core were replaced with a hybrid fusion of *Rhodobacter sphaeroides* and *R. capsulatus* cytochromes b (this protein complex is named B_S - B) [15]. The structures of these two cytochromes b are similar, but not identical [3,23]. In fact, the 10% difference in amino acid composition makes the fusion protein heterogeneous, offering a unique opportunity to test electron transfers in vivo in an enzyme in which the asymmetry does not concern just the point mutations knocking out parts of individual branches of the system (see below), but also several other amino acid positions randomly spread throughout the protein (Fig. 1B).

Fig. 2 depicts the cofactor knockout patterns that have been tested using B_S - B as the protein template for mutagenesis. Without any additional mutations, all the cofactors and the catalytic sites of B_S - B form an H-shaped electron transfer system. This system, characteristic also for the native cytochrome bc_1 dimer, consists of two upper and two lower branches connected together by the two-heme bridge (Fig. 2A). All the remaining patterns shown in Fig. 2 refer to the derivatives of B_S - B containing specific mutations that knock out individual branches in various combinations (Fig. 2B–F).

${}^w B_S$ - B^w (Fig. 2B) contains mutations knocking out the Q_o site in both halves of the fusion protein, thus inactivating the two lower branches of the system (the two superscripts “ w ” refer to equivalents of mutation G158W in *R. capsulatus* cytochrome b [24]). ${}^w B_S$ - B^w (Fig. 2C) contains mutations knocking out the Q_i site in both halves of the fusion protein, thus inactivating the two upper branches (the two superscripts “ w ” refer to equivalents of H217W mutation in *R.*

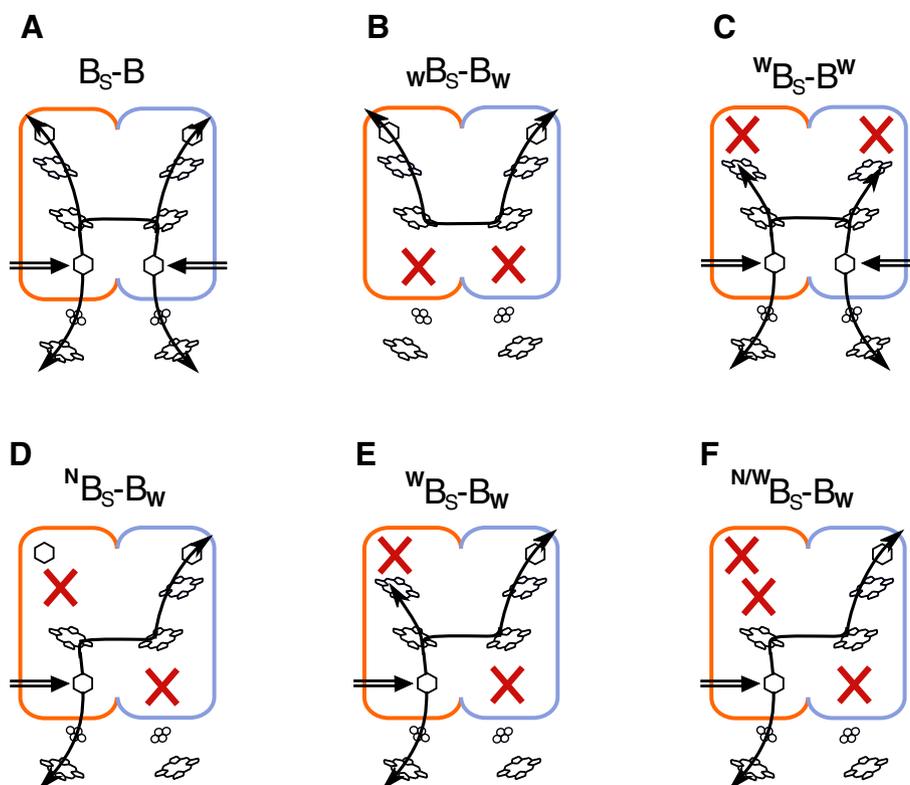


Fig. 2. Electron paths in hybrid fusion complexes with various cofactor knockout patterns. (A) B_S - B complex contains all electronic connections characteristic of native cytochrome bc_1 (see Fig. 1A). (B and C) Symmetric patterns disrupt all connections between the Q_o and Q_i sites. (D–F) Three variants of asymmetric patterns cross-inactivate the complex and leave one possible connection between the Q_o and Q_i sites (involving the inter-monomer electron transfer). Orange and blue show two halves of the fusion protein corresponding to cytochromes b of *R. sphaeroides* and *R. capsulatus*, respectively. Red crosses indicate cofactor knockouts.

capsulatus cytochrome b , which has knockout effects similar to those described earlier for H217L [25]. wB_S - B_W and $^W B_S$ - B^W are the two control forms of cytochrome bc_1 -like complexes containing the fused cytochrome b inactivated in such a way that a complete turnover of the enzyme involving the joint action of the Q_o and the Q_i sites is not possible.

Lower panel of Fig. 2 depicts three variants of the cross-inactivated complex: $^N B_S$ - B_W , $^W B_S$ - B_W and $^{N/W} B_S$ - B_W . The cross-inactivation refers to inactivation of the Q_i site (upper branch) in one half of the fusion protein and the Q_o site (lower branch) in the other half. The annotations “w” and “ W ” refer to the same mutations that were described above (for the forms of Fig. 2B and C), while superscript “N” refers to a mutation that knocks out heme b_H (“N” is an equivalent of H212N in *R. capsulatus* cytochrome b [6]). H212N inactivates the upper branch as H217W, but in a different manner: while H217W blocks just the Q_i site allowing electrons to advance as far as to heme b_H , H212N eliminates the electron transfer from heme b_L to heme b_H . “ $^{N/W}$ ” combines both ways of inactivating the upper branch in one monomer. All three cross-inactivated forms preserve the electronic communication between the active Q_o site in one half and the active Q_i site of the other half. This communication is possible due to the presence of the intact heme b_L - b_L bridge.

Fig. 3A shows aerobic growth in dark of the *R. capsulatus* strains transformed with mutated genes coding for the B_S - B derivatives described above. In all cases the cells under these growing conditions expressed the complexes with the fusion protein. This was confirmed genetically by verifying the size and the sequence of the plasmids isolated from the respective *R. capsulatus* strains and also by analyzing the subunit composition of complexes isolated by Strep-tag affinity chromatography. SDS-PAGE profiles of B_S - B and its four derivatives were compared with the profile of wild-type cytochrome bc_1 (Fig. 4A). The derivatives include all

three variants of cross-inactivated complex and one symmetrically inactivated $^W B_S$ - B^W . The profile of wB_S - B_W is omitted here as it was already shown in [15]. It is clear that in lanes 3–7 the predominant band of ~100 kDa corresponds to the fusion protein (cytochrome b_5b) that replaces the native cytochrome b (lane 2). The SDS profiles (high intensity of the 100 kDa band comparing to the bands of cytochrome c_1 and the FeS subunit) reflect an increased probability of dissociation of subunits not-containing Strep-tag during the purification using the affinity chromatography. In these profiles a band corresponding in size to cytochrome b is also detectable, which possibly comes as a result of partial degradation of a fusion protein upon expression and/or isolation of proteins [15]. We note that this cannot be a result of genetic recombination, as established in [15]. We also note that the background of cytochrome b would not affect the meaning of the results of photoheterotrophic growth tests of the cross-inactivated forms as any complex containing identical halves of the fusion protein would be inactive, thus not able to support the photoheterotrophic growth of the cells.

We report a success in obtaining $^W B_S$ - B^W as complex containing the fused cytochrome b_5b . In view of the results from our earlier experiments, introducing mutations symmetrically to the fusion protein (i.e., the same mutation present in both halves of the protein) does not always guarantee the proper expression of the fusion protein [15,19]. Indeed, $^W B_S$ - B^W appears as the first example of the fusion protein with successful inactivation of the two upper branches (so far the symmetric inactivation was only possible with the two lower branches in wB_S - B_W [15]).

Fig. 3B shows aerobic growth of other *R. capsulatus* strains that were used as additional controls in further photoheterotrophic growth tests. MT-RBC1 is devoid of genes coding for cytochrome bc_1 . All other strains expressed the homodimeric cytochrome bc_1 (containing two cytochrome b subunits). BB refers to native

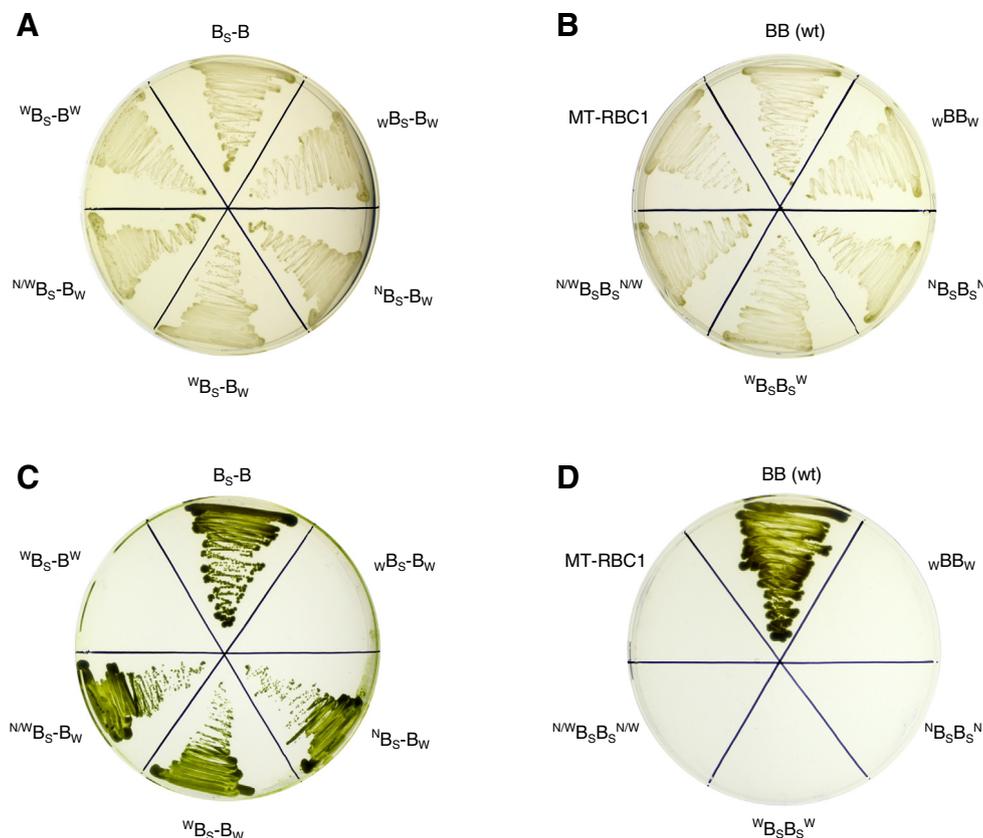


Fig. 3. Testing in vivo functionality of inter-monomer connection between the catalytic sites of cytochrome bc_1 . Aerobic growth of *R. capsulatus* expressing (A) hybrid fusion B_S - B and its derivatives or (B) homodimeric cytochrome bc_1 : BB or B_S B and their derivatives. (C and D) Photoheterotrophic growth of the strains depicted in (A and B), respectively. MT-RBC1 is a strain devoid of cytochrome bc_1 [20].

cytochrome bc_1 of *R. capsulatus*, wBB_W is its derivative – a mutant with G158W in cytochrome b . The remaining three mutants are derivatives of *R. capsulatus* cytochrome bc_1 having a native cytochrome b replaced with cytochrome b of *R. sphaeroides* ($B_S B_S$). $N^N B_S B_S^N$ and $W B_S B_S^W$ are mutants with H212N and H217W in cytochrome b , respectively, while $N^N W B_S B_S^{N/W}$ is a double mutant H212N/H217W.

Panels C and D in Fig. 3 show the results of photoheterotrophic growth tests performed with the same mutants as in Fig. 3A and B, respectively. As expected, the strains expressing BB or B_S - B grow photoheterotrophically, while all the strains expressing the symmetrically-inactivated forms of the complexes – do not. This includes two complexes with the fusion protein, i.e., derivatives of B_S - B (wB_S - B_W and $W B_S$ - B^W), the mutant of cytochrome bc_1 (wBB_W), as well as derivatives of $B_S B_S$ ($N^N B_S B_S^N$, $W B_S B_S^W$, and $N^N W B_S B_S^{N/W}$). Photosynthetic incompetence of these strains is correlated with a lack of functional cytochrome bc_1 ; the symmetric inactivation effectively disrupts the electronic connections between all the Q_o and the Q_i sites (Fig. 2B and C).

Remarkably, all three strains expressing the cross-inactivated forms of B_S - B ($N^N B_S$ - B_W , $W B_S$ - B^W , $N^N W B_S$ - B_W) can grow photoheterotrophically similarly to the cells expressing native cytochrome bc_1 (Fig. 3C). To confirm that expression of the B_S - B complexes containing the appropriately mutated fusion protein did take place under photoheterotrophic conditions in these strains, we performed analyzes of both the DNA and the protein complexes isolated from the cells grown photoheterotrophically. At the DNA level, restriction analysis of isolated expression plasmids amplified in *E. coli* confirmed the presence of the fused gene of the expected length (Fig. 4B). Moreover, the DNA sequencing confirmed that the

mutations originally introduced to the gene (N , W , $N^N W$ and w) were retained at original positions during the photoheterotrophic growth.

At the protein level, the SDS-PAGE profiles of the complexes isolated from the membranes showed a prominent band of ~ 100 kDa corresponding to the fusion protein (Fig. 4C). This band was accompanied by the bands corresponding to the two other subunits: cytochrome c_1 and FeS. It is of note, that the results of Fig. 4B and C provide first verification of expression of asymmetrically mutated complexes isolated from cultures grown under photoheterotrophic conditions.

4. Discussion

To efficiently translocate protons across the bioenergetic membrane, cytochrome bc_1 must have functional Q_o and Q_i sites and those two sites must be electronically connected together. In the H-shaped electron transfer system of the native cytochrome bc_1 dimer, the Q_o and the Q_i sites are connected not only by the cofactor chains of the same monomer (upper and lower branches making connections with the Q_o and Q_i sites), but also by the chain built of the lower branch of one monomer and the upper branch of the other [12]. The latter connection requires electron transfer between the two monomers which occurs through the bridge formed by two hemes b_L . This additional link increases a number of available electron paths and allows the quinone catalytic sites to communicate across the dimer. This engineering principle provides one testable prediction: the disruption of the catalytic Q -cycle and consequent loss of bioenergetic function of the enzyme will not occur unless all possible connections linking the Q_o with

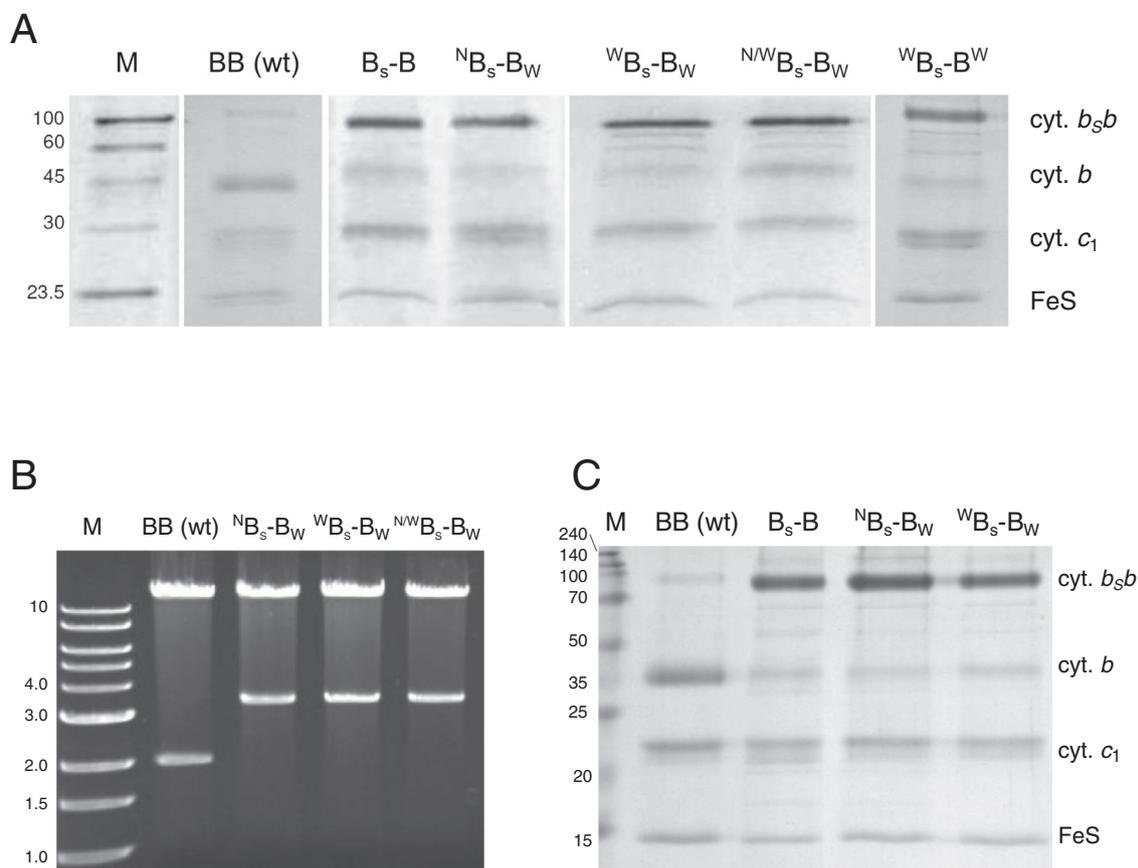


Fig. 4. Protein and gene analysis for strains expressing the fusion complexes. (A) SDS-PAGE profiles of the complexes purified from cells grown under semiaerobic conditions. (B) Restriction analysis of plasmid DNA isolated from cells grown under photoheterotrophic conditions. (C) SDS-PAGE profiles of the complexes purified from cells grown under photoheterotrophic conditions. M – mass marker (kDa or kb), cyt. *b_Sb* – hybrid fusion of cytochromes *b* in B_S-B complex, cyt. *b* – monomeric cytochrome *b*, remaining lanes descriptions correspond to the names of the mutants shown in Figs. 2 and 3.

the Q_i sites are blocked. Our work confirmed this principle at in vivo level with a series of mutants that exposed various electron paths for functional testing.

The loss of the cytochrome *bc*₁-dependent photoheterotrophic growth of the cells was observed in all symmetrically-mutated derivatives of cytochrome *bc*₁ in which either both upper or both lower branches were inactivated (Fig. 3C and D). Such inactivations cut all possible connections linking the Q_o with the Q_i sites and thus the enzyme was not functional in vivo under the conditions used.

All three cross-inactivated forms of B_S-B (^NB_S-B_W, ^WB_S-B_W and ^{N/W}B_S-B_W) effectively supported photoheterotrophic growth of the cells, in spite of the fact that the connection between the Q_o and Q_i sites at the level of the same monomer was blocked. In those forms, the intact bridge allowed the inter-monomer electron transfer to connect the lower branch and the Q_o site of one monomer with the upper branch and the Q_i site of the other. In fact, the cross-inactivations forced the enzyme to use the path involving the inter-monomer electron transfer if all reactions of the catalytic Q-cycle were to be completed. Our results clearly demonstrate that enzymes relying just on this path perform their bioenergetic function in vivo.

These results are fully consistent with the results of our previous kinetic experiments performed in vitro with asymmetrically-mutated variants of the fusion complexes. The measurements of flash-induced electron transfer in membranes demonstrated that inter-monomer electron transfer occurs in milliseconds or less and thus is a catalytically-relevant event [12,15]. Furthermore,

the measured activities of isolated complexes confirmed the competence of inter-monomer electron transfer in supporting the multiple enzymatic turnovers [14].

Our results are also fully consistent with the in vitro and in vivo results obtained independently with the two-plasmid system developed for the *R. capsulatus* to probe the inter-monomer electron transfer through the asymmetrically-mutated heterodimers of cytochrome *bc*₁ [13,16]. In this case the cross-inactivation was achieved by compiling F144R or Y147A mutation (inactivating the Q_o site) present in one copy of specifically-tagged cytochrome *b* gene with H217L or H212N mutation (inactivating the Q_i site) present in another copy of differently-tagged cytochrome *b* gene. The expression of the mutated heterodimers in the cells grown under semiaerobic conditions was confirmed biochemically and the occurrence of inter-monomer electron transfer was supported by kinetic measurements. Furthermore, the mutated strains grew photoheterotrophically indicating that the inter-monomer electron transfer can support the function of cytochrome *bc*₁ in vivo.

It is clear that both systems (i.e., the system based on fusion protein used in this work and the two-plasmid system) consistently indicate that cytochrome *bc*₁ complexes modified specifically so that the only route connecting the Q_o site with the Q_i site involves electron transfer between the two hemes *b_L*, are enzymatically active and efficient to sustain the cytochrome *bc*₁-dependent photoheterotrophic growth of the cells. This further rules out the arguments against the existence of inter-monomer electron transfer [17] (detailed discussion dealing with these arguments can be found in [15,26]).

The *in vivo* demonstration of the enzymatic competence of electron path involving the cross-communication of Q_o and Q_i sites can be considered as a step forward towards understanding the physiological significance of the inter-monomer electron transfer in cytochrome bc_1 . This still remains an open issue. Clearly, the tests conducted so far concerned specific conditions where the inter-monomer electron transfer was imposed to the system by eliminating other possible connections present in the native dimer. Considering living cells, this could correspond to the situation when parts of the enzyme are damaged by mutations (a case specifically relevant to mitochondria, where mutations, involving those in cytochrome b subunit of complex III, appear to accumulate with age [27]). In such cases, the advantage of the apparent redundancy of the components of the H-shaped electron transfer system is to allow the function of the partly damaged enzyme. In cases when all parts of the enzyme are operational, the inter-monomer electron transfer, constituting an integral part of the entire electron transfer system, is expected to compete kinetically with the intramonomer electron transfer. How this translates into the physiological operation of the enzyme, in particular in the context of its possible regulatory function and the role in reactive oxygen species generation, is currently unknown and requires further study.

All known cytochrome bc_1 complexes are homodimers characterized by high structural symmetry between the monomers [3,28]. While the symmetry concerns both the architecture of cofactor chains and the amino-acid composition/structure of proteins, its mechanistic and structural roles are not clear. Our results show that functional *in vivo* complex assembles not only when the symmetry in the cofactor chains is broken, but also when the protein core is heterogeneous. This implies a key principle and minimal requirement to secure bioenergetic efficiency: the chains of cofactors must support catalytically-competent electron transfer between the quinone binding sites located on the opposite sides of the membrane. Once this is achieved, the enzyme appears to be able to use *in vivo* any of the paths that are available (intra- or inter-monomer electron transfer) in a manner robustly tolerant to structural alterations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2014.07.117>.

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