Catalytically-relevant electron transfer between two hemes $b_L$ in the hybrid cytochrome $bc_1$-like complex containing a fusion of *Rhodobacter sphaeroides* and *capsulatus* cytochromes $b$

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**A B S T R A C T**

To address mechanistic questions about the functioning of dimeric cytochrome $bc_1$, new genetic approaches have recently been developed. They were specifically designed to enable construction of asymmetrically-mutated variants suitable for functional studies. One approach exploited a fusion of two cytochromes $b$ that replaced the separate subunits in the dimer. The fusion protein, built from two copies of the same cytochrome $b$ of purple bacterium *Rhodobacter capsulatus*, served as a template to create a series of asymmetrically-mutated cytochrome $bc_1$-like complexes (B–B) which, through kinetic studies, disclosed several important principles of dimer engineering. Here, we report on construction of another fusion protein complex that adds a new tool to investigate dimeric function of the enzyme through the asymmetrically mutated forms of the protein. This complex (B–B) contains a hybrid protein that combines two different cytochromes $b$; one coming from *R. capsulatus* and the other — from a closely related species, *R. sphaeroides*. With this new fusion we addressed a still controversial issue of electron transfer between the two hemes $b_L$ in the core of dimer. Kinetic data obtained with a series of B–B variants provided new evidence confirming the previously reported observations that electron transfer between those two hemes occurs on a millisecond timescale, thus is a catalytically-relevant event. Both types of the fusion complexes (B–B and B–L) consistently implicate that the heme-$b_L$-$b_L$ bridge forms an electronic connection available for inter-monomer electron transfer in cytochrome $bc_1$.

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1. Introduction

Cytochrome $bc_1$ is an integral component of many biological energy conversion systems. Its role is to oxidize quinol and reduce cytochrome $c$ and to couple these reactions with proton translocation across the membrane. This way it contributes to generation of protomotive force used to synthesize ATP. The enzyme is a homodimer in which each monomer consists of three, universally conserved subunits, cytochrome $c_1$, the FeS subunit and cytochrome $b_L$. Each monomer embeds two catalytic quinone oxidation/reduction sites located on two opposite sides of the membrane (named the Q$_L$ and Q$_S$ sites) and two chains of cofactors that connect the sites together and also allow them to communicate with quinol pool in the membrane and cytochrome $c$ pool outside the membrane (for recent reviews see [1–3]).

The distances between the cofactors in different monomers, as revealed by X-ray crystallography, are large enough to exclude possibility of inter-monomer electron transfer except for one point: a bridge formed by two hemes $b_L$ which in the center of the dimer are at 14 Å edge to edge. This distance appears to be just at the limit of distances between the centers that exchange electrons within micro- to millisecond, a timescale generally considered to be catalytically-relevant [4].

The revelation about the close distance between two hemes $b_L$ inspired an intense discussion about possible electron transfer between the monomers and its role in a catalytic cycle. An assumption that such electron transfer exists means that the cofactor chains and catalytic sites of two monomers form an H-shaped electron transfer system that all together connects functionally all four catalytic sites of the dimer (see Fig. 5A). Indeed, the possibility of electron transfer between the hemes $b_L$ was so appealing that it became an integral part of several
models of the operation of cytochrome bc1 (see examples in refs [5–7]) and also was considered as providing potential means to diminish levels of unpaired electrons, thus lower risks of superoxide generation by cytochrome bc1 [6,8,9].

The kinetic evidence that the electron transfer between the hemes \( b \) takes place on a catalytically-relevant timescale came recently from the studies that used bacterial genetic systems to create asymmetrically-mutated variants suitable for functional studies. One approach was based on a fusion protein that replaced two cytochromes \( b \) in the dimer [10]. The fusion, built from two copies of the same cytochrome \( b \) of purple bacterium *Rhodobacter capsulatus*, was used to create a series of asymmetrically-mutated cytochrome bc1-like complexes (B–B). To examine the path that exclusively relies on the heme \( b \)–\( b \) electron transfer, a cross-mutated variant of B–B was used in which the complementary segments of the dimer were cross-inactivated leaving the inter-monomer path as the only way connecting the catalytic sites (see scheme shown in Fig. 5B). Flash-induced electron transfer measurements performed with this mutant revealed that electron transfer between the two hemes \( b \) takes place on a millisecond timescale [10]. In addition, the functional connection between the catalytic Qo and Qi sites in this mutant was confirmed by analysis of its enzymatic activity both in the membranes and in the isolated form [11]. Another genetic approach exploited a two plasmid system with two different tags to generate and analyze the heterodimeric system developed for *Paracoccus denitrificans* [13], are so far the only known systems that allow studying the operation of the dimeric cytochrome bc1 through asymmetric mutagenesis. In all cases the expression of heterodimers relies on two copies of the same gene that serve as a template for mutagenesis. From the technical point of view this makes those systems challenging in that it requires a special experimental care to guard against genetic recombination to maintain the desired constructs at genetic level [see 14 for discussion on this issue]. Here, we present a new system that overcomes this difficulty. The system follows our original strategy of replacing two separate cytochrome \( b \) subunits in the dimer with a fusion protein, however now instead of fusing two identical cytochromes \( b \) of *R. capsulatus* we created a hybrid protein combining two different cytochromes \( b \) : one coming from *R. capsulatus* and the other — from closely related *R. sphaeroides*. With this new fusion we provide further kinetic evidence for the existence of heme \( b \)–\( b \) electron transfer on a catalytically-relevant timescale.

2. Materials and methods

2.1. Bacterial strains, growth conditions and plasmids

*E. coli* (HB101 and DH5α) were grown in liquid or solid Luria-Bertani (LB) medium supplemented with appropriate antibiotics (ampicillin or kanamycin), at 37 °C. *R. capsulatus* cells were cultivated on liquid or solid mineral-peptone-yeast extract (MPYE) medium supplemented with kanamycin when needed. They were grown at 30 °C in the dark under semiaerobic conditions or in light under anaerobic conditions. Photosynthetic growth abilities were tested on MPYE plates using anaerobic jars (GasPakTM Anaerobe Container System, BD). MT-RBC1, a strain in which the chromosomal copy of petABC operon has been deleted, was used as a host for expression of cytochrome bc1, and its derivatives from expression vectors introduced into MT-RBC1 via triparental crosses [15].

The plasmids pPET1–BL [14], pUC–BLST [14], and pBC9 [16] were used as templates for genetic manipulations. The plasmid pMTS1 [15] (carries a petABC operon coding for three subunits of *R. capsulatus* cytochrome bc1) and its mutagenized derivatives were used as expression vectors.

2.2. Construction of expression plasmids

The plasmid pMTS1–BS used for expression of Bb complex (cytochrome bc1 containing *R. capsulatus* Fe5 and cytochrome c1 subunits and *R. sphaeroides* cytochrome b) was constructed from two plasmids: pPET1–BL and pBC9. The steps of pMTS1–BS construction are described in details in Supplementary data Fig. S1.

The plasmid pMTS1–BSBST used for expression of Bb–B complex (cytochrome bc1–like complex in which two separate cytochrome bc1 subunits in dimer are replaced with a hybrid cytochrome bBS built of *R. sphaeroides* and *R. capsulatus* cytochromes b fused together) and its mutagenized derivatives containing various combinations of point mutations of cytochrome b: G158W, H198N and H212N, were constructed according to description in Supplementary data Fig. S2.

2.3. Isolation of membranes and proteins, electrophoresis and Western blot

The chromatophore membranes were prepared from semiaerobically grown cultures of *R. capsulatus* as described in [17]. Membranes solubilization with n-dodecyl-β-D-maltoside (DDM) and protein purifications were performed as described in [14]. The Bbc complex was purified using DEAE-Biogel column (BioRad), while the BBS–B complexes were purified using Strep-tag affinity chromatography (IBA Biotagno
gy). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described in [18]. The Western blot against Strep-tag was performed according to the protocol supplied by IBA with HRP-streptactin.

2.4. Optical and electron paramagnetic resonance spectroscopy and flash-induced electron transfer measurements

Optical spectra for b– and c-type cytochromes were recorded at room temperature using Shimadzu UV-2450 spectrophotometer. The difference spectra were obtained with samples that were first oxidized by an addition of potassium ferricyanide and then reduced by using either sodium ascorbate or a minimal amount of solid sodium dithionite. Continuous wave electron paramagnetic resonance (EPR) spectra of 2Fe–2S cluster in chromatophores were measured according to the protocol described in [19]. Flash-induced electron transfer of Bb and BBS–B complexes were performed as described in [20]. For the measurements, chromatophore membranes were suspended in 50 mM MOPS buffer pH 7, containing 100 mM KCl, 1 mM EDTA, 3.5 μM valinomycin, and appropriate redox mediators (7 μM 2,3,5,6-tetramethyl-1,4-phenylenediamine, 1 μM phenazine methosulfate, 1 μM phenazine ethosulfate, 5.5 μM 1,2-naphthoquinone, 5.5 μM 2-hydroxy-1,4-naphthoquinone). The samples were poised at an ambient potential of 100 mV. Transient cytochrome c and b reduction kinetics were followed at 550–540 nm and 560–570 nm, respectively. Inhibitors antimycin A and myxothiazol were used at a final concentration of 7 μM.

3. Results

3.1. Cytochrome b of *R. sphaeroides* can replace native cytochrome b in *R. capsulatus* cytochrome bc1

As prerequisite for experiments of fusing two different cytochromes b described in the following sections, we have tested the effect of replacing native cytochrome b of *R. capsulatus* cytochrome bc1, with that coming from the closely related strain, *R. sphaeroides*. To this end we constructed an expression vector pMTS1–BS, which in the place of native *R. capsulatus* petB gene in petABC operon contained the gene *fcbB* coding for *R. sphaeroides* cytochrome b (Fig. 1). The major steps of pMTS1–BS construction are summarized in Supplementary data Fig. S1.
We introduced this plasmid to *R. capsulatus* MT-RBC1 strain and found that an appropriately assembled and functional cytochrome *bc*$_1$ (named BS) was expressed in the cells. The absorption redox difference spectra of chromatophore membranes confirmed presence of hemes *b* and *c* (peaks at 560 and 550 nm in Fig. 2A, respectively). The isolated from membranes BS complexes showed similar optical spectra and contained all three catalytic subunits, cytochrome *b*, *c*$_1$, and FeS, as seen on SDS-PAGE gels (not shown). Light-induced electron transfer measurements in chromatophore membranes confirmed that BS showed typical phases of electron transfer related to the action of the catalytic sites and responded in native-like manner to an addition of specific inhibitors of cytochrome *bc*$_1$ (myxothiazol for the *Q*$_o$ site and antimycin for the *Q*$_i$ site) (Fig. 2B). From kinetic transients of cytochrome *c* reduction and cytochrome *b* re-oxidation in the absence of inhibitor it is clear that the enzyme is fully operational (both the reduction of cytochrome *c* and the re-oxidation of heme *b* proceed to completion). However the rates of the light-induced reactions were lower, comparing to the respective rates of native cytochrome *bc*$_1$ and also the amplitude of heme *b* reduction in the presence of antimycin was smaller (Fig. 2B, BS vs WT). This indicates that in the presence of this inhibitor the distribution of electrons within the cofactor chains is altered in BS (see ref. [20]).

The reason of these effects is currently unknown but may reflect some structural distortions associated with a necessity to accommodate *R. sphaeroides* cytochrome *b*. For example, the lack of subunit *IV*, which in native cytochrome *bc*$_1$ of *R. sphaeroides* naturally interacts with cytochrome *b* subunit [21] but is missing in native cytochrome *bc*$_1$ of *R. capsulatus*, and thus is not present in BS either, may contribute to these effects. (Detailed analysis of BS is under way and will be a subject of separate studies.) Consistent with the kinetic results, the strain expressing BS showed Ps$^+$ phenotype indicating that this enzyme is functional in vivo.

Based on these results we concluded that cytochrome *b* of *R. sphaeroides* is capable of replacing native cytochrome *b* in *R. capsulatus* cytochrome *bc*$_1$, and the engineered BS retains functional and structural properties of cytochrome *bc*$_1$.

### 3.2. Hybrid cytochrome bSb obtained by fusion of cytochromes b from *R. sphaeroides* and *R. capsulatus* assembles with other core subunits to form B$_c$–B$_s$ complex

The fusion of cytochromes *b* from *R. capsulatus* and *R. sphaeroides* was achieved adopting similar strategy that was previously used to fuse two cytochromes *b* of *R. capsulatus* [10]. At genetic level, this strategy requires modification of the expression vector so that the operon coding for cytochrome *bc*$_1$ contains cytochrome *b* gene (*petB*) extended in frame with an additional copy of cytochrome *b* gene. While in our previous work, the two halves of the fusion gene were alike, each containing a sequence of the same *petB* gene originated from *R. capsulatus* [10], in this work we constructed a fusion gene assembled from two different genes: *fbcB* and *petB* (Fig. 3).

The first half of the fusion gene contained the sequence of *fbcB* from *R. sphaeroides* while the second half contained the sequence of *petB* from *R. capsulatus* with the sequence encoding Strep-tag at its 3’ end (this fusion gene was named *fbcB/petB*). The other two genes of the operon *petABC*, encoding the FeS subunit and cytochrome *c*$_1$, were left unchanged. All three genes were expressed using a vector...
pMTS1–BSBST, which was a derivative of pMTS1 containing fbcB/petB in the place of petB gene. The major steps of construction of pMTS1–BSBST are described in Supplementary data Fig. S2. Fig. 4 summarizes the results of expression of pMTS1–BSBST in MT–RBC1 cells. First, spectroscopic measurements of membranous fractions revealed the presence of redox cofactors characteristic for cytochrome bc1-type complexes: absorption redox difference spectra showed presence of hemes b and c (peaks at 560 and 550 nm in Fig. 4A, respectively) while EPR showed presence of Rieske protein appropriately interacting with occupants of the Qo site [22] (characteristic g0 value of the spectrum of Fig. 4B). Second, Western blots revealed the presence of the fusion protein of correct size (two times larger than cytochrome b) in the membranes (Fig. 4C). The fusion protein was also clearly visible on SDS-PAGE of complexes isolated from the membranes using affinity chromatography (Fig. 4D). The electrophoretic profile of isolated complexes showed that the fusion protein was accompanied by the two remaining subunits of cytochrome bc1: cytochrome c1 and the FeS subunit, consistent with spectroscopic features just described. These results provided first indication that the membranes contained a cytochrome bc1-like complex built of the hybrid fusion protein (named cytochrome bSB) assembled together with cytochrome c1 and the FeS subunit (the entire complex was named Bs–B). Further kinetic experiments confirmed that Bs–B did assemble in the membranes (see below). In the remaining text the system of expression of Bs–B in R. capsulatus cells will be referred as the sphaer–caps system. For consistency, the system of expression of B–B described earlier in [10,14] will be named as the caps–caps system.

We note that Western blot and Coomassie blue-stained gels revealed also traces of a protein in size corresponding to native cytochrome b (Fig. 4C, D). The amount of this cytochrome b in relation to the fusion protein was always significantly smaller, as exemplified on gel in Fig. 4D. These results indicate that in addition to the dominant fraction of Bs–B, the membranes contain a small fraction of cytochrome b either alone or assembled with cytochrome c1 and FeS subunits. At present, the origin of this phenomenon is not clear. Given that the fusion constructs are commonly reported to encounter problems with proteolysis upon expression and/or isolation of proteins [23–27], we favor an explanation that in our case it is also a result of partial degradation of protein, especially of a foreign R. sphaeroides portion of the fusion protein with retention of the R. capsulatus part (this part contains Strep-tag used for Western blot.

### Fig. 3
Operon organization and subunit composition of the fusion hybrid Bs–B complex. In the petABC operon, the petB gene was replaced by a hybrid fusion gene fbcB/petB composed of fbcB of R. sphaeroides and petB of R. capsulatus (orange and blue). The remaining genes in the operon are: petA coding for the FeS subunit (green), and petC coding for cytochrome c1 (violet). The expression of this operon resulted in a formation of the Bs–B complex in which the two cytochrome b subunits in the dimer are replaced with hybrid cytochrome bSB (a fusion of cytochromes b of R. sphaeroides and R. capsulatus). DNA sequence and amino acid composition of the linker is shown in black. Orange and blue letters indicate the last and the first codons/amino acid residues that were left unchanged in R. sphaeroides and R. capsulatus gene/protein, respectively. ST – sequence coding for the Strep-tag (gray).

### Fig. 4
Spectroscopic properties and Western blot/SDS-PAGE analyses of Bs–B. (A) Optical redox difference spectra of b- and c-type hemes in chromatophore membranes isolated from the Bs–B strain. Dithionite minus ferricyanide spectra – solid lines, ascorbate minus ferricyanide spectra – dashed lines. (B) X-band continuous wave EPR spectra of the 2Fe–2S cluster of WT and Bs–B complexes in chromatophore membranes. Dotted line shows the position of g0 transition. (C) Western blot against Strep-tag II (IBA Biotagnology) of chromatophore membranes isolated from wild type (lane 1), the B–B strain (lane 2) and the Bs–B strain (lane 3). M, Molecular weight marker. Names cyt. bb and cyt. bSB depict the protein shown in line 2 and 3, respectively. (D) Coomassie blue stained SDS-PAGE analysis of complexes isolated using affinity chromatography (Strep-tag) from wild-type (lane 1), the B–B strain (lane 2) and the Bs–B strain (lane 3). M, Molecular weight marker. Names cyt. bb and cyt. bSB are the same as in C.
Fig. 5. Symmetric and asymmetric knockout patterns in the fusion hybrid B–B complex. (A) B–B complex without mutation — four branches for electron transfer are open forming an H-shaped electron transfer system characteristic for the intact wild-type cytochrome b; (B) wB5–B*N and (C) wB5–B w — two branches across removed (cross-inactivation) and heme bL–bL connection maintained; (D) wB5–B w — both lower branches removed; (E) wB5–B w — two branches across removed and heme bL–bL connection disrupted; (F) wB5–B*N — both upper branches removed (note that this form is drawn schematically but was not obtained as a fusion protein complex). W, N (superscript), and N (superscript) refer to G158W, H198N, and H212N point mutations in cytochrome b, respectively. Black arrows indicate functional branches. Black double arrows indicate electron entry point at the Qi site. Red crosses display distribution of G158W, H198N, and H212N point mutations in B–B complexes.

3.3. B–B protein accommodates several point mutations introduced in symmetric and asymmetric patterns

In the next series of experiments, we introduced point mutations to pMTS1–BSBST template repeating the strategy earlier to create symmetrically and asymmetrically mutated B–B complexes [10] (Fig. 5). The point mutations included G158W to inactivate the Qo site and the lower branch of the H-shaped electron transfer system [22] and H212N to inactivate the Qi site and the upper branch of this system [8] (numbering corresponds to the sequence of R. capsulatus cytochrome b). The asymmetric combinations contained an equivalent of G158W in one half of the hybrid gene and an equivalent of H212N in the other half (to obtain wB5–B w or B*N–B w, Fig. 5B, C). The symmetric combinations contained equivalents of G158W or H212N in both halves of the gene (to obtain wB5–B w or B*N–B w, Fig. 5D, F). Table 1 and Fig. 6 summarize the results of expression of pMTS1–BSBST derivatives containing appropriate mutations in MT-RBC1 strain. Table 1 also compares these results with those obtained previously when the same combinations of mutations were tested with the caps–caps system [10,14].

From Table 1 and Fig. 6, it is clear that B–B complex containing the fusion protein is assembled in all cases, except for B*N–B w. Most importantly, both asymmetric combinations wB5–B w and B*N–B w resulted in an assembly of the fusion protein. The sphaer–caps system allowed also for an assembly of the complex containing one of the symmetric mutation patterns (i.e. wB5–B w). The latter pattern was previously unavailable with the caps–caps system, which in general did not tolerate the presence of the same mutation in both halves of the fusion protein [14].

In this work we also tested a new asymmetric pattern wB5–B w or wB5–B w (for sphaer–caps or caps–caps system, respectively) (Fig. 5E). This combination contained G158W in the first half of the fusion protein and an equivalent of H198N introduced in the second half and was specifically designed to perform a series of genetic and kinetic control experiments described in next paragraphs. In cytochrome b, mutation H198N replaces one of the histidine ligand to iron of heme bL with non-competent asparagine and, as previous studies with R. sphaeroides have indicated, results in an assembly of the cytochrome bL complex with an impaired Qi site [28]. This mutation has not been described earlier for R. capsulatus, but our initial experiments confirmed that also in this species H198N mutant assembles as cytochrome bL with impaired Qo site (manuscript in preparation). We thus used H198N and G158W to create a form intended to disable both the Qi, sites, each by a different point mutation. Those two point mutations were separated from each other in DNA sequence of petB, which was important from a genetic point of view for the planned experiments (this separation was one of the reasons for selection of H198N over other mutations in cytochrome bL that are also known to inactivate the Qo site but are closer to G158W in sequence).

As Table 1 indicates, the form containing equivalents of H198N and G158W assembled as fusion protein only as wB5–B w in the sphaer–caps system. For the caps–caps system, the results were similar to those previously described for all symmetrically mutated forms (B*N–B w, wB5–B w) [14].

In general, from the comparison shown in Table 1 it appears that B–B have more structural flexibility to accommodate larger number of mutational patterns than B–B. Possibility to analyze those combinations of B–B that were previously unavailable with B–B (i.e. wB5–B w, wB5–B w) detection and affinity chromatography). At the same time we are certain that this cannot be due to genetic recombination leading to shortening of the fused gene. The results shown in the next paragraphs have demonstrated that the sphaer–caps system exhibits high genetic stability.

Our initial attempts to eliminate completely this background of cytochrome b have proven that this was not a straightforward task. We did not investigate this issue any further as for the main purpose of this work it was not necessary. We reasoned that as long as B–B complex was assembled in the membranes and there was a possibility to perform all appropriate control experiments, the background of cytochrome b would not compromise the kinetic experiments that were a subject of present studies.

Table 1

| Name of fusion protein (caps–caps system) | Assembly of B–B | Name of fusion protein (sphaer–caps system) | Assembly of B–B |
|------------------------------------------|----------------|---------------------------------------------|----------------|
| B–B                                     |                | B*N–B                                       |                |
| wB5–B w                                 |                | wB5–B w                                    |                |
| H5–B w                                  |                | H5–B w                                      |                |
| wB5–B w                                 |                | wB5–B w                                    |                |
| H5–B w                                  |                | H5–B w                                      |                |
| wB5–B w                                 |                | wB5–B w                                    |                |
| H5–B w                                  |                | H5–B w                                      |                |
| wB5–B w                                 |                | wB5–B w                                    |                |
| H5–B w                                  |                | H5–B w                                      |                |

W, N (subscript), and N (superscript) indicate position of mutation corresponding to G158W, H198N, and H212N in cytochrome b subunit, respectively. “+” indicates assembly of the complex containing the fusion protein. “−−” indicates lack of the fusion protein (for B*N–B w, wB5–B w, B*N–B w, wB5–B w) or the presence of dominant fraction of the complex with fused protein (for B*N–B w) see ref. [14].
The sphera-caps system exhibits high genetic stability

We have previously observed that in the caps-caps system, the cells carrying genes coding for B-B did not grow photosynthetically (exhibited Ps− phenotype). However, the photosynthetic growth conditions allowed for selection of revertants. The cells that regained Ps+ phenotype carried plasmids containing only a short version of the gene (corresponding in size to a single copy of petB) (Fig. 7B). The reversions to Ps+ occurred with a frequency of 10⁻³–10⁻⁴, which was estimated from the number of cells that were able to grow photosynthetically at given concentration of cells. The tests involved serial dilutions experiments where the number of colonies that can grow under photosynthetic conditions was compared to the total number of cells equal to the number of cells growing under aerobic conditions (an example of the result for a given concentration of cells is shown in Fig. 7A, top).

Similar tests were now performed for the cells carrying pMTS1–BSBST (used for expression of B-B) in the sphera-caps system. First, we checked the cells expressing B-B without additional mutations and observed that the number of colonies that grow under photosynthetic and aerobic conditions for given concentrations of the cells was always similar (Fig. 7A, middle). We also found that the cells grown under photosynthetic conditions retained the original plasmid pMTS1–BSBST with intact fused gene fbcB/petB and showed no signs of a short copy of the gene (Fig. 7B). Furthermore, the SDS profile of complexes isolated from the membranes of these cells (Fig. 7C, lane 2) indicated that they contained B-B complex with fusion protein (the SDS profile of the complexes obtained from the photosynthetic cultures was very similar to that obtained from the semiaerobic cultures, see, lane 3 of Fig. 4D). This all was a first indication that the frequency of genetic recombination in the sphera-caps system is low and that this system is genetically more stable than the caps-caps system.

We note that because the cells expressing B-B show some background of unfused cytochrome b subunit (Fig. 4C, D), the Ps+ phenotype in itself cannot be used as an argument in discussions about possible functionality of B-B in vivo. Clearly, other experiments are needed to assess it (such experiments are currently under way).

To further assess genetic stability of the sphera-caps system we analyzed the non-functional WBS–BN variant which had two of its Qo sites disabled by two different mutations positioned in protein sequence 40 amino acids apart (G158W in one half and an equivalent of H198N in the other). In this case, although the mutant cells expressing WBS–BN complex were Ps−, as expected, there was a theoretical possibility of homologous recombination between parts of the fusion gene resulting in a sequence that would remove deleterious mutation and restore the functional Qo site. Those types of recombinant cells...
3.5. Light-induced electron transfer in B<sub>fl</sub>–B<sub>W</sub> derivatives confirm occurrence of fast electron transfer between two hemes

Fig. 8 compares kinetic traces of light-induced electron transfer in chromatophore membranes containing various forms of B<sub>fl</sub>–B<sub>W</sub>. In the chromatophores containing B<sub>fl</sub>–B<sub>W</sub> complex without any additional mutations in the absence of any inhibitors, hemes c (c<sub>1</sub> and c<sub>2</sub>) of cytochromes c were rapidly photo-oxidized then reduced, while heme b<sub>H</sub> of cytochrome b was rapidly reduced and re-oxidized (Fig. 8A). Antimycin, inhibitor of the Q<sub>H</sub> site, greatly diminished heme c reduction phase and fully abolished heme b re-oxidation phase leaving only its reduction phase. Myxothiazol, inhibitor of the Q<sub>L</sub> site, abolished heme c reduction phase and also fully abolished heme b reduction and re-oxidation phases. From this data it is clear that B<sub>fl</sub>–B<sub>W</sub> exhibits all phases of electron transfer reminiscent of the functional catalytic Q<sub>H</sub> and Q<sub>L</sub> sites connected together, as known for the wild-type enzyme and described earlier for B–B<sub>W</sub> [10].

Most significantly, asymmetric N<sub>BW</sub>–B<sub>W</sub> exhibited kinetic behavior consistent with the same mode of operation (Fig. 8B). Again, there was a clear and large phase of antimycin-sensitive reduction of hemes c. Heme b reduction and re-oxidation phases were antimycin-sensitive: in the absence of any inhibitors heme b<sub>H</sub> was rapidly reduced and re-oxidized, while in the presence of antimycin re-oxidation phase was eliminated and only reduction phase was observed. Similar kinetic results were obtained for the mirror asymmetric form wB<sub>BW</sub> (not shown).

In the asymmetric form N<sub>BW</sub>–B<sub>W</sub> the functional connection between the Q<sub>H</sub> and Q<sub>L</sub> sites can only be accomplished if electrons are transferred between the hemes b<sub>H</sub>. This is because in this mutant electrons enter the b chain (reflected as flash-induced reduction of heme b<sub>H</sub>) only through one active Q<sub>H</sub> site and leave this chain (reflected as flash-induced oxidation of heme b<sub>L</sub>) only through one active Q<sub>L</sub> site, but each of these two sites is located on a separate half of the fusion protein (Fig. 5C). Thus, to reach the active Q<sub>H</sub> site, electrons that entered the enzyme through the active Q<sub>L</sub> site must use the path: heme b<sub>L</sub>–heme b<sub>H</sub>–heme b<sub>W</sub>. This also means that when the Q<sub>H</sub> site is inactive (in the presence of antimycin), heme b<sub>H</sub> cannot be reduced in flash experiments unless the quinol-derived electron is transferred from one heme b<sub>L</sub> (that associated with active Q<sub>L</sub> site) to another heme b<sub>W</sub> (that associated with inactive Q<sub>H</sub> site).

A profound antimycin-sensitive phase of cytochrome c reduction and the Q<sub>H</sub>-site-mediated re-oxidation of heme b<sub>H</sub> seen in N<sub>BW</sub>–B<sub>W</sub> indicate that the functional connection between the Q<sub>H</sub> and Q<sub>L</sub> sites is preserved in this mutant. At the same time, the reduction of heme b<sub>H</sub> in the presence of antimycin confirms that this heme is reducible by electrons coming from the active Q<sub>L</sub> site. We note that consistent with hemes c and b reduction/oxidation kinetics, N<sub>BW</sub>–B<sub>W</sub> displayed all cytochrome bc<sub>1</sub>-related phases of carotenoid band shifts typical for native cytochrome bc<sub>1</sub> confirming full turnover of the cross-inactivated enzyme in the absence of any inhibitors (not shown). Thus, in light of the above considerations, kinetic traces of the asymmetric N<sub>BW</sub>–B<sub>W</sub> clearly indicate that electron transfer between two hemes b<sub>H</sub> must take place on catalytically-relevant timescale. This result is fully consistent with our earlier demonstration of existence of heme b<sub>H</sub>–b<sub>L</sub> electron transfer reported for cross-inactivated wB<sub>W</sub>–B<sub>W</sub> constructed using the caps–caps fusion system [10].

We note that re-reduction of cytochromes c in the absence of inhibitors reaches similar level in both N<sub>BW</sub>–B<sub>W</sub> and B<sub>fl</sub>–B<sub>W</sub>, but at the same time the amplitude of heme b<sub>H</sub> reduction in N<sub>BW</sub>–B<sub>W</sub> in the presence of antimycin is smaller comparing to the respective amplitude of B<sub>fl</sub>–B<sub>W</sub> (Fig. 8B and A). This indicates that, in the post-flash redistribution, electrons equilibrate on cofactors chains to the same final levels in N<sub>BW</sub>–B<sub>W</sub> and B<sub>fl</sub>–B<sub>W</sub> as long as the Q<sub>H</sub> and Q<sub>L</sub> sites communicate with the quinone and cytochrome c pools. On the other hand, when the outflow of electrons through the Q<sub>H</sub> site is blocked by antimycin the final distribution of electrons is different and reduction of heme b<sub>H</sub> in N<sub>BW</sub>–B<sub>W</sub> is less complete. At this stage deciding what causes this shift in the final equilibrium is difficult, however this result should not be considered unexpected, especially in light of similar changes in electron distribution observed in antimycin-inhibited cytochrome bc<sub>1</sub> when a barrier for a particular electron transfer reaction was specifically modified [20].

Fig. 8C, D show the results of the controls that involved two fusion forms designed to have heme b<sub>H</sub>–b<sub>L</sub> electron transfer eliminated: wB<sub>W</sub>–B<sub>W</sub> and wB<sub>W</sub>–B<sub>N</sub> (Fig. 5D and E, respectively). These controls deserve particular attention as, for reasons discussed earlier in [14], they were not previously available with the caps–caps fusion system.
In \( \text{wB}_5\text{B}_4\text{N}, \) no kinetic phases of heme \( c \) reduction or heme \( b \) reduction and re-oxidations were observed (Fig. 8C). Furthermore, addition of either antimycin or myxothiazol had no effect on the kinetic traces. These results are similar to the effects of G158W [10,22] and report that both the Q₉ sites in \( \text{wB}_5\text{B}_4\text{N} \) are inactive. \( \text{wB}_5\text{B}_4\text{N} \) demonstrates that the complex containing the fusion protein can be fully activated when the Q₉-site-inactivating mutation is present in both of its halves.

Kinetic traces recorded for the second control, \( \text{wB}_5\text{B}_4\text{B}_N \) are shown in Fig. 8D. The heme \( c \) reduction phase was almost fully suppressed (we note that the residual cytochrome \( c \) reduction kinetics seen in the absence of any inhibitors must have come from the half of \( \text{wB}_5\text{B}_4\text{N} \) containing the equivalent of H198N, as we also observed such residual activity in the H198N mutant) and there were no signs of any phases of heme \( b \) reduction or re-oxidation. Furthermore, the traces recorded for heme \( b_H \) were not sensitive to antimycin or myxothiazol. Clearly, \( \text{wB}_5\text{B}_4\text{B}_N \) shows no signs of functional connection between the \( Q \) and \( Q \) sites nor electron transfer between two hemes \( b_H \). We emphasize that the kinetic traces of \( \text{wB}_5\text{B}_4\text{B}_N \) are clearly different from those of \( \text{wB}_5\text{B}_4\text{B}_w \) (Fig. 8D vs B).

The form \( \text{wB}_5\text{B}_4\text{B}_N \) adds to \( \text{wB}_5\text{B}_4\text{B}_w \) as another version of control with both of the \( Q \) sites inactivated (Fig. 5D, E). But in the case of \( \text{wB}_5\text{B}_4\text{B}_N \), unlike in \( \text{wB}_5\text{B}_4\text{B}_w \), there exists a possibility of genetic recombination between parts of the fusion gene to obtain pseudo-native form of the enzyme. Occurrence of such reversions at significant level would manifest itself as a background of native-like kinetic traces visible in flash-induced electron transfer measurements. The results obtained with \( \text{wB}_5\text{B}_4\text{B}_N \) clearly demonstrate that this is not the case. The lack of any background of native-like kinetics in chromatophores containing \( \text{wB}_5\text{B}_4\text{B}_N \) is consistent with the observation that the spher–caps system exhibits high genetic stability, thus recombinations between parts of the fused gene that would obscure the kinetic results do not occur.

Fig. 5F presents schematically the cofactor pattern in the mutant allowing to test the conditions when the active \( Q \) site mediates reduction of heme \( b_H \) but further electron transfer to heme \( b_H \) is prevented. Although such a mutant was not obtained as fusion protein complex (see Fig. 6, lane 6), the characteristic light-induced kinetic transients of the corresponding \( b_H \) knockout (cytochrome \( bC \), mutant lacking both hemes \( b_H \)) [8] are available for comparison with the transients of \( \text{wB}_5\text{B}_4\text{B}_N \). As described previously [10], and also shown in Fig. S3A, the \( b_H \) knockout does not exhibit profound antimycin-sensitive phase of cytochrome \( c \) reduction present in wild-type cytochrome \( bC \) (Fig. 2B) and in \( \text{wB}_5\text{B}_4\text{B}_N \) (Fig. 8B). The \( b_H \) knockout neither shows heme \( b_H \) reduction/re-oxidation phases in the absence of inhibitors, nor heme \( b_H \) reduction in the presence of antimycin (Fig. 5B), as observed at 560–570 nm in wild-type cytochrome \( bC \) (Fig. 2B) and in \( \text{wB}_5\text{B}_4\text{B}_N \) (Fig. 8B). The involvement of heme \( b_H \) in the \( b_H \) knockout can be seen as antimycin-insensitive reduction phase at 566–573 nm (Fig. S3C). The clear differences between the kinetic traces of the \( b_H \) knockout with that of \( \text{wB}_5\text{B}_4\text{B}_N \) rule out the possibility that the latter ones result from electron transfer reactions involving just heme \( b_H \) without participation of heme \( b_H \) (and the \( Q \) site in the absence of inhibitors).

To sum up the results of control experiments, the kinetic traces recorded for two control fusion forms \( \text{wB}_5\text{B}_4\text{B}_N \) and \( \text{wB}_5\text{B}_4\text{B}_N \) and for the \( b_H \) knockout show no signs of functional connection between the catalytic \( Q \) and \( Q \) sites, nor the heme \( b_H \) reduction/reoxidation reminiscent of heme \( b_H \) electron transfer (Fig. 8C, D). This further substantiates the conclusion that the kinetic traces of the cross-inactivated asymmetric form \( \text{wB}_5\text{B}_4\text{B}_N \) (Fig. 8B) do reveal heme \( b_H \) electron transfer and functional connection between the catalytic sites.

4. Discussion

Our earlier work has shown that a genetic approach of fusing two cytochrome \( b \) subunits in cytochrome \( bC \) offers an attractive opportunity to address crucial bioenergetic questions related to the mechanisms of operation of this enzyme. This in particular concerned controversial issues of possible allostery within the dimeric complex and possibility of communication between the monomers. Experimental results addressing these points have demonstrated that monomers operate independently, but at the same time — can exchange electrons using the electron-transfer bridge formed by two hemes \( b_H \) in the core of the dimer [10,11,14].

Mechanistic conclusions were drawn from kinetic analysis of the mutants containing a fusion protein (cytochrome \( \text{bb} \)) assembled with other core subunits to form cytochrome \( bC \)-like complex named \( B-B \). The point mutations introduced to cytochrome \( \text{bb} \) enabled inactivation of individual segments of cofactor chains in various symmetric and asymmetric combinations, exposing various electron transfer paths within \( B-B \) for kinetic testing. The path that specifically exposed the electron transfer between two hemes \( b_H \) was identified in the asymmetric form \( \text{wB}-B^N \), in which the complementary parts of the fusion protein were cross-inactivated [10].

From the protein engineering point of view this fusion system (referred in this paper as the \( \text{caps}–\text{caps} \) system) came as a remarkable example of flexibility within the whole protein expression and assembly system, which clearly was able to adopt itself to accommodate \( B-B \) and several of its mutant derivatives. Because, however, the fusion was based on the two copies of the same gene, the risk of genetic recombination (to remove one copy of a gene or exchange complementary fragments of a gene) imposed a necessity of experimental care to implement protocols that ensured that samples used for kinetic analysis were devoid of unwanted background of recombined proteins. This present work provides an attractive alternative template for asymmetric mutagenesis: a cytochrome \( bC \)-like complex with a new fusion protein expressed from a gene of improved genetic stability. This new system allowed us to obtain a whole family of mutants that included the cross-inactivated variants together with an extended set of control forms to further analyze electronic communication between two hemes \( b_H \).

The system was based on a fusion of two cytochromes \( b \) one coming from \( R. \) sphaeroides and the other from \( R. \) capsulatus (the \( \text{sphaer–caps} \) system). Because the new fusion comprised two different genes, the \( \text{sphaer–caps} \) system turned out to be genetically more stable than the \( \text{caps–caps} \) system. Indeed, 17.6% of difference between the two genes in the \( \text{sphaer–caps} \) system appeared sufficient to lower the frequency of recombination between the genes [29,30] orders of magnitude in comparison to the \( \text{caps–caps} \) system. At the same time, the structure of those two closely related cytochromes is very similar [31,32] (90.4% of similarity based on primary sequence) and, as we have shown here, not only cytochrome \( b \) of \( R. \) sphaeroides can replace native cytochrome \( b \) in \( R. \) capsulatus cytochrome \( bC \), but also hybrid cytochrome \( b^B \) (a fusion of \( R. \) sphaeroides and \( R. \) capsulatus cytochromes \( b \)) assembled with other subunits in membranes of \( R. \) capsulatus cells to form a hybrid cytochrome \( bC \)-like complex. This latter complex, named \( B-B \), corresponds to the previously described \( B-B \) [10].

Using \( B-B \) as a template we prepared the cross-inactivated variants \( \text{wB}_5\text{B}_4\text{B}_N \) and \( \text{wB}_5\text{B}_4\text{B}_N \) which repeated the asymmetric combination of mutations in \( \text{wB}-B^N \) originally used to test the electron transfer between the two hemes \( b_H \). In addition, we prepared the control forms \( \text{wB}_5\text{B}_4\text{B}_N \) and \( \text{wB}_5\text{B}_4\text{B}_N \) which had both of the \( Q \) sites of the complex inactivated by mutations and thus allowed us to test the conditions when electron transfer between two hemes \( b_H \) was not possible within the fusion protein.

The flash-induced experiments performed with \( \text{wB}_5\text{B}_4\text{B}_N \) and \( \text{wB}_5\text{B}_4\text{B}_N \) showed the presence of kinetic phases reminiscent of the functional connection between the \( Q \) and \( Q \) sites. In addition, these experiments revealed reduction of heme \( b_H \) in the presence of antimycin. As all these reactions in those mutants can only be accomplished if the heme \( b_H \) electron transfer takes place, it is clear that the kinetic traces proved that these two hemes exchange electrons on a catalytically-relevant
timescale. $^{9}$B$_{5}$–B$_{W}$ and $^{10}$B$_{5}$–B$_{N}$ demonstrated this reaction in the same manner as the previously described $^{5}$B–B$^{*}$.[10]

On the other hand, the traces of $^{9}$B$_{5}$–B$_{W}$ and $^{10}$B$_{5}$–B$_{N}$ showed neither signs of functional connection between the $Q_{0}$ and $Q_{1}$ sites nor signs of reduction of heme $b_{1}$ in the presence of antimycin, confirming that the heme $b_{1}$–$b_{1}$ electron transfer does not occur in those two mutants. The observation that the kinetic traces of those two controls clearly differ from the traces of cross-inactivated $^{9}$B$_{5}$–B$_{W}$ and $^{10}$B$_{5}$–B$_{N}$ further substantiated the conclusion that the latter ones did reveal heme $b_{1}$–$b_{1}$ electron transfer.

The lack of electron transfer between two hemes $b_{1}$ in kinetic traces of $^{9}$B$_{5}$–B$_{N}$ deserves particular emphasis, as this is a variant in which the recombination between the parts of fusion gene to restore the functional $Q_{0}$ site was theoretically possible. Such recombination was described recently by Hong et al. ([33] who constructed similar types of mutants $^{9}$B–B$^{*}$ or $^{10}$B–B$_{N}$ in R. sphaeroides cells (in those forms the complex was fully inactivated by mutations H198N and H212N) and observed that they grew photosynthetically. As the photosynthetic growth in those cases must have come from the reverted forms that were effectively selected during photosynthetic cultivation, the authors assumed that similar reversions occurred in the cross-inactivated forms used to test heme $b_{1}$–$b_{1}$ electron transfer. Based on this assumption they raised concern that our kinetic traces of cross-inactivated $^{9}$B–B$^{*}$ ([10] did not reveal heme $b_{1}$–$b_{1}$ electron transfer but rather originated from the pseudo-native contaminants. The results presented here do not support this view. The cells expressing $^{9}$B–B$_{N}$ did not grow photosynthetically and the estimated frequency of reversion was very low. Consistent with this behavior, the kinetic traces of $^{9}$B–B$_{N}$ showed no signs of native-like activity. Yet the kinetic traces of cross-inactivated $^{9}$B$_{5}$–B$_{W}$ matched the traces recorded earlier for the $^{9}$B–B$^{*}$. Clearly, the traces of all our cross-inactivated forms revealed electron transfer between the hemes $b_{1}$. It follows that our original kinetic experiments with $^{9}$B–B$^{*}$ that were prepared using the caps–caps system ([10] were free of any pseudo-native contaminants despite the fact that recombination frequency was larger in this system comparing to the sphaer–caps system.

It should be emphasized that the effect of selection of cells that expressed unfused cytochrome $b$ in R. sphaeroides under photosynthetic conditions described in ref. [33] is consistent with our observations made for the caps–caps system ([14]. However, we do not share a view that the photosynthetic selection in these cases reflects in vivo enforcement for monomeric electron transfer, as suggested [33]. While various reasons can be envisaged for this selection (see discussion in ref. [14]), it is clear that it took place irrespective of whether the original mutations within the fusion gene enforced the inter-monomer electron transfer within B–B or not.

It should also be emphasized that the reported by Hong et al. difficulties with interpretation of kinetic experiments [33] originate from the fact that they used photosynthetic growth conditions selecting recombined proteins to prepare the samples for kinetic analysis. We, on the other hand, specifically avoided this type of selection in our preparations.

The successful expression of hybrid cytochrome $b_{1}$–$b_{1}$ and its assembly with other core subunits to form B$_{5}$–B$_{W}$ adds to B–B as another remarkable example of the overall structural plasticity of cytochrome $b_{1}$ design and a flexibility within the whole protein expression and assembly system (see [34] for discussion of this issue). However, like in the case of B–B and its derivatives, some distortions from the native structure cannot be ruled out. In this context, one may wonder how much these proteins, which all should undoubtedly be treated as model proteins, resemble native cytochrome $b_{1}$. The biochemical, spectroscopic and kinetic properties of these proteins (in particular the occurrence of all of the characteristic kinetic phases of electron transfer) allow us to be confident that the overall mode of operation follows the catalytic cycle of native cytochrome $b_{1}$. The fact that heme $b_{1}$–$b_{1}$ electron transfer was confirmed independently by the asymmetric forms of the cytochrome $b_{1}$–$b_{1}$ fusion complexes coming from two different genetic systems (caps–caps and sphaer–caps) provides strong evidence in support of the notion that the heme–b–b$_{1}$ bridge forms an electronic connection available for inter-monomer electron transfer in cytochrome $b_{1}$.

The experimental evidence for the electron transfer between hemes $b_{1}$ emerging from our studies and supported by other independent investigations ([12] opens doors to discussions about the physiological significance of the intermonomer electron transfer for cytochrome $b_{1}$ operating in living cells. In this respect, one of the crucial aspects that needs detailed investigation concerns the ratio of intra-monomer vs inter-monomer electron transfer ([1,9,35]. This ratio is likely to change in response to the changes in redox conditions and/or changes in the membrane potential. In addition, the ratio might be affected by certain mutations that inactivate or impair parts of the protein and are associated with the process of accumulation of mitochondrial mutations occurring in mitochondrially-coded cytochrome $b$ subunit. Future studies with asymmetrically mutated forms of cytochrome $b_{1}$ should provide information on those and other related issues to advance our general understanding on the operation of this complex enzyme.

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Appendix A. Supplementary data

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References

[1] W.A. Cramer, S.S. Hasan, E. Yamashita, The Q cycle of cytochrome bc complexes: a structure perspective, Biochim. Biophys. Acta 1807 (2011) 788–882.

[2] D.M. Kramer, W. Nitschke, J.W. Cooley, The cytochrome bc complexes, and related bc complexes: the Rieske/cytochrome$\hat{b}$ complex as the functional core of a central electron/proton transfer complex, in: N. Hunter, F. Daldal, M.C. Thurnauer, J.T. Beatty (Eds.), The purple photosynthetic bacteria, Springer, The Netherlands, (2009) 451–473.

[3] E.A. Berry, D.-W. Lee, L.-S. Huang, F. Daldal, Structural and functional mutations of the cytochrome$\hat{bc}$ complex, in: N. Hunter, F. Daldal, M.C. Thurnauer, J.T. Beatty (Eds.), The purple photosynthetic bacteria, Springer, The Netherlands, 2009, pp. 425–450.

[4] C.C. Page, C.C. Moser, X. Chen, P.L. Dutton, Natural engineering principles of electron tunneling in biological oxidation–reduction, Nature 402 (1999) 47–52.

[5] J.W. Cooley, D.-W. Lee, F. Daldal, Across membrane communication between the $Q_{0}$ and $Q_{1}$ active sites of cytochrome$\hat{bc}$, Biochemistry 48 (2009) 1888–1899.

[6] R. Covian, B.L. Trumpower, Regulatory interactions in the dimeric cytochrome$\hat{bc}$ complex: the advantages of being a twin, Biochim. Biophys. Acta 1777 (2008) 1079–1091.

[7] A.Y. Multikidjanian, Activated Q cycle as a common mechanism for cytochrome$\hat{bc}$, and cytochrome$\hat{b}f$ complexes, Biochim. Biophys. Acta 1797 (2010) 1858–1868.

[8] A. Osyczka, C.C. Moser, F. Daldal, P.L. Dutton, Reversible redox energy coupling in electron transfer chains, Nature 427 (2004) 607–612.

[9] W.A. Rutherford, A. Osyczka, F. Rappaport, Back-reactions, short-circuits, leaks and other energy wasteful reactions in biological electron transfer: redox tuning to survive life in $O_{2}$, FEBS Lett. 586 (2012) 603–616.

[10] M. Świerczek, E. Gelach, M. Sarewicz, A. Borek, C.C. Moser, P.L. Dutton, A. Osyczka, An electronic bus bar lies in the core of cytochrome$\hat{bc}$, Science 329 (2010) 451–454.

[11] M. Czapla, A. Borek, M. Sarewicz, A. Osyczka, Enzymatic activities of isolated cytochrome$\hat{bc}$–like complexes containing fused cytochrome$\hat{b}$ subunits with asymmetrically inactivated segments of electron transfer chains, Biochemistry 51 (2012) 829–835.

[12] P. Lanciano, D.-W. Lee, H. Yang, E. Darrouzet, F. Daldal, Intermonomer electron transfer between the low-potential hemes of cytochrome$\hat{bc}$, Biochemistry 50 (2011) 1651–1663.

[13] M. Castellani, R. Covian, T. Kleinschroth, O. Anderka, B. Ludwig, B.L. Trumpower, Direct demonstration of half-of the sites reactivity in the dimeric cytochrome$\hat{bc}$ complex, J. Biol. Chem. 285 (2010) 502–510.

[14] M. Czapla, A. Borek, M. Sarewicz, A. Osyczka, Fusing two cytochromes$\hat{b}$ of Rhodobacter capsulatus cytochrome$\hat{bc}$, using various linkers defines a set of protein templates for asymmetric mutagenesis, Protein Eng. Des. Sel. 25 (2012) 15–25.
[15] E. Atta-Asafo-Adjei, F. Daldal, Size of the amino acid side chain at position 158 of cytochrome b is critical for an active cytochrome bc1 complex and for photosynthetic growth of Rhodobacter capsulatus, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 492–496.

[16] C.-H. Yun, R. Beci, A.R. Crofts, S. Kaplan, R.B. Gennis, Cloning and DNA sequencing of the fbc operon encoding the cytochrome bc1 complex from Rhodobacter sphaeroides. Characterization of fbc deletion mutants and complementation by a site-specific mutational variant, Eur. J. Biochem. 194 (1990) 399–411.

[17] M.B. Valkova-Valchanova, A.S. Saribas, B.R. Gibney, P.L. Dutton, F. Daldal, Isolation and characterization of a two-subunit cytochrome b-c1 subcomplex from Rhodobacter capsulatus and reconstitution of its ubihydroquinone oxidation (Qb) site with purified Fe-S protein subunit, Biochemistry 37 (1998) 16242–16251.

[18] A. Osyczka, P.L. Dutton, C.A. Yu, D. Xia, Inhibitor-complexed structures of the FeS cluster and heme flavin protein subunit, Biochemistry 34 (1995) 15979–15996.

[19] E. Cieluch, K. Pietryga, M. Sarewicz, A. Osyczka, Visualizing changes in electron distribution in coupled chains of cytochrome bc1 by modifying barrier for electron transfer between the Fe5 cluster and heme c1, Biochim. Biophys. Acta 1797 (2010) 296–303.

[20] S.-C. Tso, S.K. Shenoy, B. Quinn, L. Yu, Subunit IV of cytochrome bc1 complex from Rhodobacter sphaeroides, J. Biol. Chem. 275 (2000) 15287–15294.

[21] H. Ding, C.C. Moser, D.E. Robertson, M.K. Tokito, F. Daldal, P.L. Dutton, Ubiquinone pair in the Qb site central to the primary energy conversion reactions of cytochrome bc1 complex, Biochemistry 34 (1995) 15979–15996.

[22] M. Jahic, M. Gustavsson, A.-K. Jansen, M. Mertinelle, S.-O. Enfors, Analysis and control of proteolysis of a fusion protein in Pichia pastoris fed-batch processes, J. Biotechnol. 102 (2003) 45–53.

[23] D.-W. Lee, Y. Oztruk, A. Mamedova, A. Osyczka, J.W. Cooley, F. Daldal, A functional hybrid between the cytochrome bc1 complex and its physiological membrane-anchored electron acceptor cytochrome c, in Rhodobacter capsulatus, Biochim. Biophys. Acta 1757 (2006) 346–352.

[24] D.-W. Lee, Y. Oztruk, A. Osyczka, J.W. Cooley, F. Daldal, Cytochrome bc1–c1 fusion complexes reveal the distance constraints for functional electron transfer between photosynthetic components, J. Biol. Chem. 283 (2008) 13973–13982.

[25] H. Hellebust, A. Vede, S.-O. Enfors, Proteolytic degradation of fused protein A13-galactosidase in Escherichia coli, J. Bacteriol. 7 (1988) 185–197.

[26] F. Raneyx, G. Georgiou, In vivo degradation of secreted fusion proteins by the Escherichia coli outer membrane protease OmpT, J. Bacteriol. 172 (1990) 491–494.

[27] S. Yang, H.-W. Ma, L. Yu, C.A. Yu, On the mechanism of quinol oxidation at the Qb site in the cytochrome bc1 complex, J. Biol. Chem. 283 (2008) 28767–28776.

[28] M. Vulic, F. Dionisio, F. Taddei, M. Radman, Molecular keys to speciation: DNA polymorphism and the control of genetic exchange in enterobacteria, Proc. Natl. Acad. Sci. U. S. A. (1997) 9763–9767.

[29] S. Stambuk, M. Radman, Mechanism and control of interspecies recombination in Escherichia coli. I. Mismatch repair, methylation, recombination and replication functions, Genetics 150 (1998) 533–542.

[30] E.A. Berry, L.-S. Huang, L.K. Saechao, N.G. Pon, M.B. Valkova-Valchanova, F. Daldal, X-ray structure of Rhodobacter capsulatus cytochrome bc1 complex with its mitochondrial and chloroplast counterparts, Photosynth. Res. 81 (2004) 251–275.

[31] S. Esser, M. Elberry, F. Zhou, C.A. Yu, D. Xia, Inhibitor-complexed structures of the cytochrome bc1 from the photosynthetic bacterium Rhodobacter sphaeroides, J. Biol. Chem. 283 (2008) 2846–2857.

[32] S. Hong, V. Doreen, A.R. Crofts, Inter-monomer electron transfer is too slow to compete with monomeric turnover in bc1 complex, Biochim. Biophys. Acta 1817 (2012) 1053–1062.

[33] M. Czapla, M. Sarewicz, A. Osyczka, Fusing proteins as an approach to study bioenergetic enzymes and processes, Biochim. Biophys. Acta 1817 (2012) 1847–1851.

[34] V.P. Shinkarev, C.A. Wrathall, Intermonomer electron transfer in the bc1 complex dimer is controlled by the energized state and by impaired electron transfer between low and high potential hemes, FEBS Lett. 581 (2007) 1535–1541.