BBA research letter

On the inter-monomer electron transfer in cytochrome bc_1

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ABSTRACT

Cytochrome bc_1 is a structural and functional homodimer. The catalytically-relevant inter-monomer electron transfer has been implicated by a number of experiments, including those based on analyses of the cross-dimer mutated derivatives. As some of the original data on these derivatives have recently been questioned, we extend kinetic analysis of these mutants to confirm the enzymatic origin of the observed activities and their relevance in exploration of conditions that expose electron transfer between the monomers. While obtained data consistently implicate rapid inter-monomer electron equilibration in cytochrome bc_1, the mechanistic and physiological meaning of this equilibration is yet to be established.

The catalytic Q cycle of cytochrome bc_1 (mitochondrial complex III) is based on the joint operation of two catalytic quinone binding sites, named the Q_o and the Q_i site, located at two opposite sides of the membrane (see [1] for a recent review). The Q_o site located at the p-side oxidizes hydroquinone (QH_2) while the Q_i site located at the n-side reduces quinone (Q). Electronic connection between these sites is secured by a chain of two hemes b (termed b_L and b_H) which allow for the cross-membrane electron transfer from the Q_o to the Q_i site. Apart from this chain, a connection between the Q_o site and cytochrome c outside of the membrane is secured by a chain composed of Rieske cluster and heme c_1.

Cytochrome bc_1 is structurally a homodimer in which each monomer consists of three catalytic subunits: cytochrome b harboring two hemes b (b_L and b_H), the FeS subunit (harboring Rieske cluster) and cytochrome c_1 (harboring heme c_1) (Fig. 1A, WT). In principle, each monomer is equipped with all the cofactors and catalytic sites necessary for operation of the Q cycle. Intriguingly, the architecture of cofactors revealed by the structures of the dimers of cytochrome bc_1 implicated possibility for electron transfer between monomers. The two hemes b_L (each from one monomer) are separated by ~14 Å distance, which falls within the limit for distances securing productive electron transfer between redox cofactors in proteins [2]. The occurrence of such electron transfer was indeed demonstrated by a number of independent experiments which analyzed asymmetrically-mutated derivatives of the protein directing electrons into the specific paths [3–6]. It became apparent that the two hemes b_L form a bridge in the central part of the dimer connecting electronically cofactor chains in both monomers.

The experimental approaches to test this bridge were challenged by the overall symmetry of the system which included symmetrical

distribution of the cofactors in a dimer and the undifferentiation between the monomers at the genetic level. To overcome these difficulties and obtain the cytochrome bc_1 derivatives with mutations placed asymmetrically in the dimer, two genetic systems were introduced and applied in the photosynthetic bacterium *Rhodobacter capsulatus*. One system resulted in a fusion of two cytochromes b which replaced two separate subunits in the dimer (denoted B—B in Fig. 1A) [3,7]. The other system used two plasmids with two tags coexpressed in the cells [5,8]. In both cases the structural outcome was similar: the experimental accessibility to the cross-inactivated derivatives of cytochrome bc_1 designed specifically to isolate the inter-monomer electron transfer as the only route connecting the Q_o and Q_i sites ($_wB-B^N$ in Fig. 1A). The subsequent kinetic experiments demonstrated that this electron transfer takes place on a millisecond timescale, thus should be considered as a catalytically-relevant event [3–6].

The recent perspective on the Q-cycle critically discussed the results and interpretation of these experiments to conclude that no sufficient proofs in support of inter-monomer electron transfer exist [9]. In particular, this review analyzed a Michaelis-Menten dependence of the enzymatic activities of asymmetrically-mutated derivatives of B—B originally reported in [4]. This analysis led to the suggestion that the profile of dependence of $_wB-B^N$ showed "some anomalous process independent of substrate" thereby cannot be taken as evidence for occurrence of intermonomer electron transfer [9]. Below we explain why this conclusion should be considered premature and not consistent with other experimental data.

The suggested anomaly referred to an apparent lack of the measured activities of $_wB-B^N$ on concentration of substrate (cytochrome c)

deduced from the observation that $_wB-B^N$ approached V_{max} relatively fast (much faster than the other forms). However, one should bear in mind the specific conditions of this experiment: the activities were measured under very low ionic strength (0 mM NaCl) to enhance the interactions between cytochrome bc_1 and cytochrome c which secure the highest turnover rates with lowering the Michaelis-Menten constant (K_m). This seemed justified, given that the experiments aimed at reaching enzymatic activities close to V_{max} for all forms of B—B in the tested range of cytochrome c concentration, rather than determining K_m. Under these conditions $_wB-B^N$ displayed generally lower activities compared to the native cytochrome bc_1 (WT) but its V_{max} was still significant. Thus, a plausible interpretation of the fast approaching V_{max} in this form is that the interaction of cytochrome c with the enzyme may not be rate-limiting at low ionic strength, even at low concentrations of cytochrome c. Fig. 1B & C provides experimental support for this interpretation.

Fig. 1 compares the enzymatic activities of WT and the three fusion-based derivatives: B—B, $_w^NB-B$ – a form with only one monomer active, and $_wB-B^N$, measured at two different ionic strengths. As the low ionic strength conditions correspond to conditions of our earlier measurements [4], $_wB-B^N$ shows a relatively fast approach of V_{max} (Fig. 1B), consistent with the original report. However, at higher ionic strength (100 mM NaCl), when interaction between cytochrome c and cytochrome bc_1 is weaker, the turnover rate approaches the V_{max} at much higher concentrations of cytochrome c (Fig. 1C). Such a curvature stretched over larger cytochrome c concentrations is clearly recognizable for all tested forms, including $_wB-B^N$. This is expected for conditions that deliberately make interaction of cytochrome c with cytochrome bc_1 rate-limiting. It is evident that all tested forms exhibit a Michaelis-Menten dependence on substrate, therefore the measured activities are clearly of enzymatic origin. This dismisses the major argument based on which the evidence for inter-monomer electron transfer was questioned in the review [9].

It is clear that various kinetic experiments with membranes and isolated cytochrome bc_1 complexes and its asymmetrically-mutated derivatives provided an indication that inter-monomer electron transfer can connect functionally the Q_o and Q_i sites [3–6,10]. The proteins that were examined in these experiments derived from aerobically grown cultures of photosynthetic bacteria. Such conditions are routinely used in preparation of samples for the *in vitro* kinetic assays. With these bacteria, one can in principle also test the functionality of cytochrome bc_1 derivatives *in vivo* by checking the ability of the cells to grow under photosynthetic conditions (where the growth is cytochrome bc_1-dependent). Such tests, if performed, are clearly indicated. However, in the
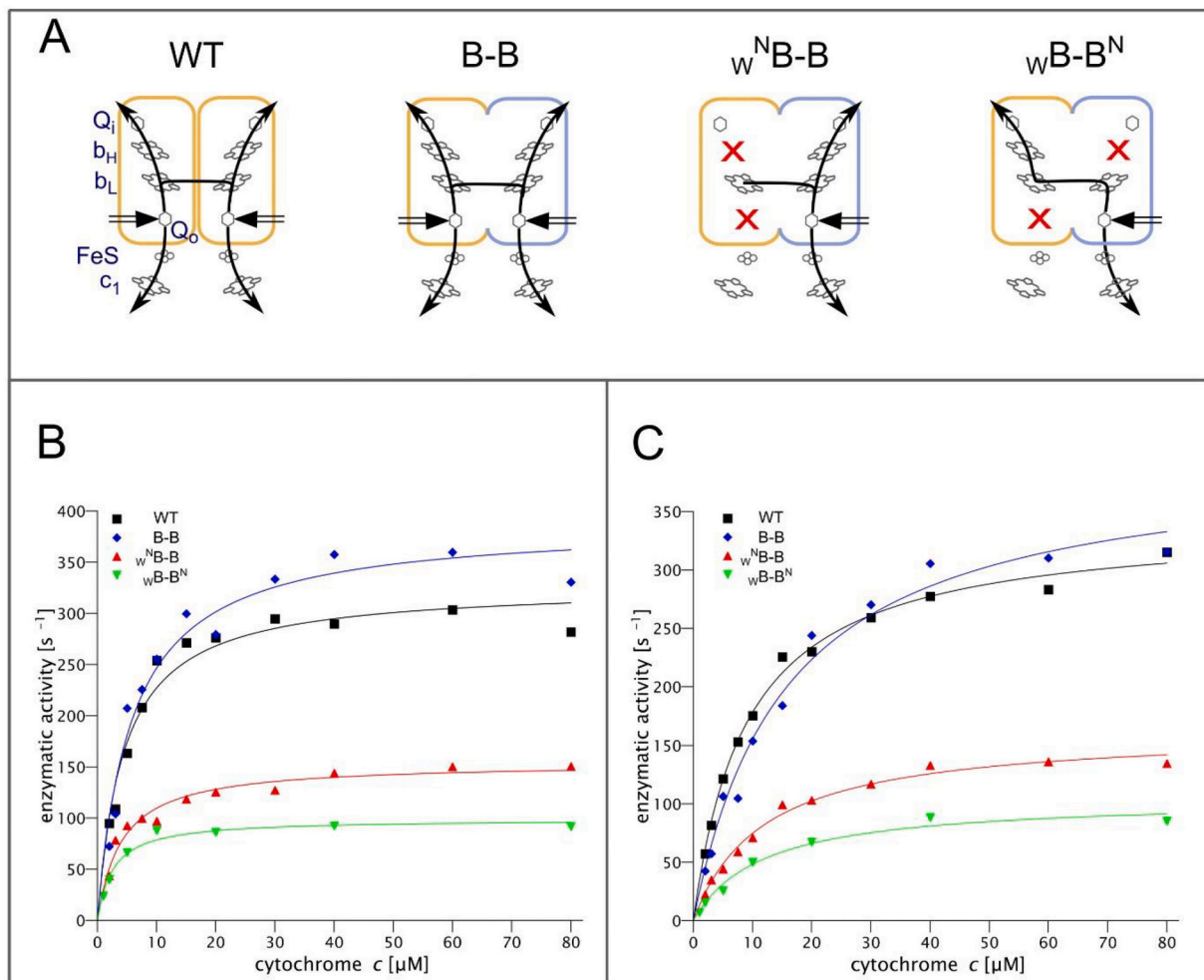
Fig. 1. A. Schematic representation of possible electron paths (black arrows) in native cytochrome bc_1 (WT) and its mutated derivatives. B—B is a derivative in which the two b subunits are fused into one protein. $_w^NB-B$ is a form of B—B in which catalytic sites (Q_o and Q_i) in one monomer are inactivated. $_wB-B^N$ is a cross-inactivated mutant of B—B (contains inactive Q_o site in one monomer and inactive Q_i site in the other monomer). "W" denotes G158W mutation inactivating the Q_o site, "N" denotes H212N mutation inactivating the Q_i site. B, C. The dependence of enzymatic activity on cytochrome c concentration for proteins indicated in panel A measured in 50 mM Tris, pH 8.0, 1 mM EDTA, 0.01 % DDM without addition of NaCl (B) or containing 100 mM NaCl (C). Steady-state enzymatic activities were determined spectroscopically by 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzohydroquinone (DBH_2)-dependent reduction of cytochrome c, as described in [16]. Protein samples were described previously [3,4,7].

case of the original B—B protein and its derivatives, the functional tests *in vivo* were not possible to perform, given that the photosynthetic conditions had to be avoided [7]. Only the subsequent construction of the hybrid fusion of two cytochrome *b* genes coming from two different bacterial species [10] provided a system suitable for performing photosynthetic tests. With these tests, complementary evidence was obtained that the intermonomer electron transfer is able to sustain the function of the enzyme *in vivo* [11]. Independently, similar conclusions were reached from the results of the experiments based on the two plasmid system [6].

On a final note, it is worth to emphasize that electron transfer between the monomers takes place through virtually identical redox centers separated by no more than 1.4 nm, thus fast electron tunneling between these centers is expected [2,12]. Given the ensemble of information on this cofactor pair, any assumption that this electron transfer is somehow prevented or slowed down seems to have no physical justification. On the other hand, indications for inter-monomer electron transfer in cytochrome bc_1 come not only from experiments directly aiming to address this issue (as described above), but also from spectroscopic explorations primarily focusing on other mechanistic issues. For example, the presence of the fast-relaxing semiquinone in the Q_i site (SQ_i) detected in the native enzyme with inhibited Q_o site was explained as resulting from electron transfer through the hemes *b* across the dimer [13]. This allows for electron from the Q_i site in one monomer to move towards the Q_i site in the second monomer, yielding SQ_i and oxidized heme b_H in both monomers (explained in detail in [13]). Also, the inter-monomer interactions were evoked to explain the most recent kinetic data on electron transfer in cytochrome bc_1 activated by photoinduced ruthenium complexes [14]. Clearly, the evidence for occurrence of electron transfer between monomers is growing. What remains to be understood are its physiologic and mechanistic implications. Some proposals, discussed in various reviews (see examples in [12,15]), make this issue a fascinating subject for further studies.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Artur Osyczka reports financial support was provided by National Science Centre Poland.

Data availability

Data will be made available on request.

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