Proton translocation by the cytochrome bc_1 complexes of phototrophic bacteria: introducing the activated Q-cycle†

Armen Y. Mulkidjanian*[a,b]

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The cytochrome bc_1 complexes are proton-translocating, dimeric membrane ubiquinol:cytochrome *c* oxidoreductases that serve as "hubs" in the vast majority of electron transfer chains. After each ubiquinol molecule is oxidized in the catalytic center *P* at the positively charged membrane side, the two liberated electrons head out, according to the Mitchell's Q-cycle mechanism, to different acceptors. One is taken by the [2Fe-2S] iron–sulfur Rieske protein to be passed further to cytochrome c_1. The other electron goes across the membrane, via the low- and high-potential hemes of cytochrome *b*, to another ubiquinone-binding site *N* at the opposite membrane side. It has been assumed that two ubiquinol molecules have to be oxidized by center *P* to yield first a semiquinone in center *N* and then to reduce this semiquinone to ubiquinol. This review is focused on the operation of cytochrome bc_1 complexes in phototrophic purple bacteria. Their membranes provide a unique system where the generation of membrane voltage by light-driven, energy-converting enzymes can be traced via spectral shifts of native carotenoids and correlated with the electron and proton transfer reactions. An "activated Q-cycle" is proposed as a novel mechanism that is consistent with the available experimental data on the electron/proton coupling. Under physiological conditions, the dimeric cytochrome bc_1 complex is suggested to be continually primed by prompt oxidation of membrane ubiquinol via center *N* yielding a bound semiquinone in this center and a reduced, high-potential heme *b* in the other monomer of the enzyme. Then the oxidation of each ubiquinol molecule in center *P* is followed by ubiquinol formation in center *N*, proton translocation and generation of membrane voltage.

1. Introduction

Cytochrome bc_1-complexes of animals and bacteria (hereafter bc_1) are oligomeric membrane enzymes that function as quinol:cytochrome *c* oxidoreductases.[1–7] They utilize the free energy of redox reaction to translocate protons across the energy-transducing membrane, from its *n*egatively charged *n*-side to the *p*ositively charged *p*-side. Proton transfer leads to the formation of difference in electrochemical activity of protons across the membrane ($\Delta\tilde{\mu}_{H^+}$). The latter is contributed by chemical (ΔpH) and electrical ($\Delta\psi$) components and reaches approx. 200–250 mV under physiological conditions.[8] Thus, at steady state, bc_1 translocates protons against remarkable back pressure.

Recently bc_1 has drawn attention as one of the major sources of reactive oxygen species (ROS), which are formed when oxygen molecules interact with semiquinone radicals that serve as intermediates in the catalytic cycle of bc_1. The accumulation of the ROS-damaged cellular compounds is believed to cause aging; it has been reported that the worms *Caenorhabditis elegans* that carried mutations decreasing the activity of bc_1 lived longer.[9] Hence, the importance of clarifying the semiquinone chemistry inside the bc_1 can be hardly overestimated.

The cytochrome bc_1 complex (see Fig. 1) is an intertwined dimer both in crystals[5–7,10–15] and in solution.[16] The catalytic core of each bc_1 monomer is formed by three subunits: the membrane-embedded cytochrome *b* and the membrane-anchored iron–sulfur Rieske protein and cytochrome c_1. Each cytochrome *b* carries one low- and one high-potential heme (b_l and b_h, respectively). The number of subunits in cytochrome bc_1-complexes varies from only 3 catalytic ones in some bacteria up to 11 in the mitochondrial bc_1.

To explain the ability of the bc_1-containing proteoliposomes to translocate two protons across the membrane per each oxidized quinol,[17,18] Mitchell has suggested the Q-cycle mechanism.[19,20] The mechanism invoked a bifurcation of electrons at the site of quinol oxidation, as originally suggested by Wikström and Berden,[21] and, in addition, implied that electrons, after passing through cytochrome *b*, reduce a quinone molecule from the other side of the membrane, as shown in Fig. 1. According to the current, structure-based Q-cycle models,[5,10–15,22–26] quinol molecules are oxidized at the interface between cytochrome *b* and the mobile [2Fe–2S] cluster-carrying domain of the Rieske protein (hereafter the FeS domain, see Fig. 1). This interface forms the catalytic center *P* of the enzyme (which corresponds to center *o* in the original Mitchell's notation). One electron is accepted by the FeS domain to be passed further, *via* cytochrome c_1, to external, mobile *c*-type cytochromes.[27] The other electron goes to heme b_l and then

[a]*A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119899, Moscow, Russia*
[b]*School of Physics, University of Osnabrück, D-49069, Osnabrück, Germany. E-mail: amulkid@uos.de; Fax: +49-(541)-9692656; Tel: +49-(541)-9692680*

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moves across the membrane, *via* heme b_h, to the further quinone-binding center *N* (center *i* in the Mitchell's notation). In center *N*, a ubiquinone molecule can be reduced first to a semiquinone anion $Q_N^{\bullet -}$ and then, after the oxidation of the next ubiquinol in center *P*, to a Q_NH_2 ubiquinol. This ubiquinol molecule can be oxidized by bc_1 as well, so that two charges are ultimately translocated across the membrane per each ubiquinol processed by bc_1.

Depending on the presence of inhibitors and on crystallization conditions, the FeS domain was found in different positions, reflecting its rotation by approx. 60°, upon shuttling an electron from center *P* to cytochrome c_1, from a position where the FeS cluster is docked to the heme b_l (FeS_b) into the position, where the cluster interacts with the cytochrome c_1 heme (FeS_c).[5,7,10–14,28–31]

The X-ray structures show that antimycin A (hereafter antimycin), which blocks the oxidation of heme b_h *via* center *N*,[32,33] binds next to this heme.[13,28,34] Confirming the earlier insightful suggestions,[35,36] the crystal structures revealed two distinct, although partly overlapping inhibitor-binding sites in center *P*.[5–7,11–15,28–30,37–39] Myxothiazol, methoxy-acrylate (MOA) stylbene-type inhibitors, and a non-oxidisable ubiquinol analogue 2,3,4-trimethoxy-5-decyl-6-methyl-phenol (TMDMP) occupy positions that are *proximal* to heme b_l. Some other inhibitors bind *distally*, on the interface of cytochrome *b* and the docked FeS domain. These are, in particular, stigmatellin and 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT)-type inhibitors. Stigmatellin, which seems to imitate either ubiquinol or anionic ubiquinone species,[5,29,40] binds between His-161 of the Rieske protein and Glu-272 of cytochrome *b* (to conform with literature in the field, hereafter the avian numeration of amino acid residues is routinely used). Correspondingly, it has been argued that a ubiquinol molecule binds in the same way, and that His-161 and Glu-272 serve as acceptors of the first and second protons, respectively, upon ubiquinol oxidation.[5,14,26,29,31,37,41]

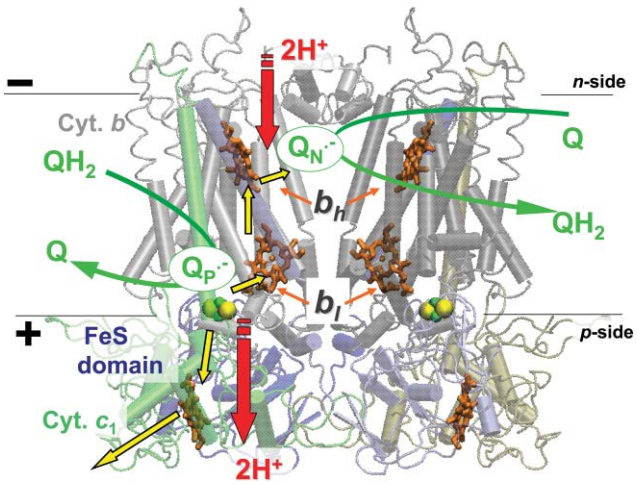

Fig. 1 Overview of structure and function of the cytochrome bc_1 complex. The scheme shows the Q-cycle scheme[20] as plotted over the X-ray structure of a dimeric bc_1 of *Rb. capsulatus* (PDB entry 1ZRT[7]). Colour code: grey, cytochrome *b* subunits; blue, the Rieske protein subunits; green, cytochromes c_1; orange, hemes; yellow-green, the FeS clusters. Yellow arrows, ET steps; red arrows, proton transfer steps. The figure was produced with the help of the VMD software package.[152]

The Mitchell's idea of doubling the enzyme efficiency by internal electron cycling is generally accepted. The further details on enzyme operation remain, however, controversial, as discussed in several recent reviews.[24–26,31,42] Under discussion are (i) the exact mechanism of ubiquinol oxidation and the role of the transiently formed $Q_P^{\bullet -}$ semiquinone in this reaction,[24–26,31,42,43] (ii) the conformational cross-talk between the quinone-binding centers,[22,26,44,45] and (iii) the possibility of electron exchange between the monomers *via* two b_l hemes.[10,22,46–49] One more debatable question is the mechanism of electron/proton coupling in bc_1. This mechanism is routinely studied with chromatophores—the energosomes of purple phototrophic bacteria. Chromatophores can be obtained, as sealed inside-out vesicles of inner cellular membrane with diameter of 300–600 Å, by disruption of the cells of *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*[50–52] (see Fig. 2). With these vesicles, the $\Delta\psi$ generation by photosynthetic enzymes can be synchronously triggered by flashes of light, traced *via* electrochromic spectral changes of native carotenoids[50] and correlated with the redox and pH changes measured either under the compatible conditions or even in the same samples.[51–53] The flash-induced changes in the redox state of cytochromes and in pH can be monitored optically,[50–56] whereas the generation of $\Delta\psi$ can be followed not only optically,[50–58] but also by capacitive electrometry.[58,59]

Besides providing the possibility to study bc_1 in a pulsed mode and to follow the $\Delta\psi$ changes, chromatophores have several other advantages: (i) the cytochrome bc_1 complexes of purple bacteria are simple and are made of only 3 or 4 subunits;[7,60,61] (ii) mutants with site-specific amino acid substitutions in the subunits of the bc_1 are available;[62] (iii) from an evolutionary perspective, purple bacteria are close to the bacterial ancestors of mitochondria;[63] not surprisingly, the X-ray structure of a simple three-subunit bc_1

Armen Y. Mulkidjanian received his academic degrees from the Moscow State University, Russia (PhD in 1984 and Dr. Sc. in 2006) and the University of Osnabrück, Germany (Dr. rer. nat. habil. in Biophysics, 2002). He is currently a Senior Research Scientist at the A. N. Belozerky Institute of Physico-Chemical Biology of the Moscow State University, an Adjunct Professor at the School of Bioengineering and Bioinformatics of the Moscow State University and a Visiting Lecturer at the University of Osnabrück. His research is focused on the molecular mechanisms of proton transfer by the membrane energy-converting enzymes.

Armen Y. Mulkidjanian

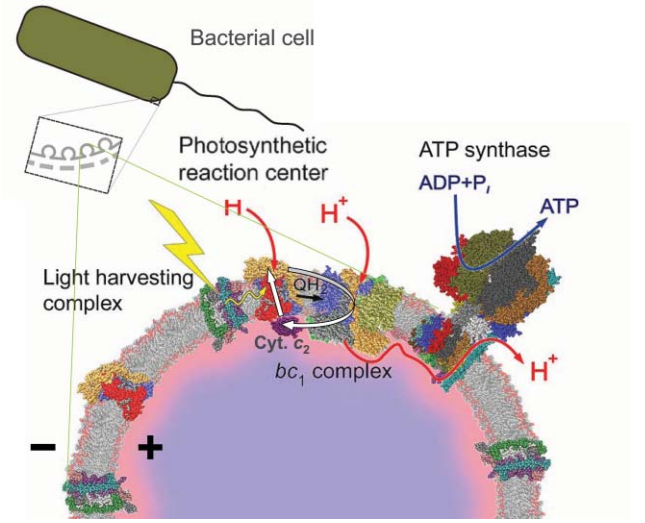

Fig. 2 Schematic presentation of electron cycling in a chromatophore membrane (the figure is reproduced from ref. 26 with permission). White arrows depict electron cycling between the RC and the bc_1. The red color of the interfacial water layer indicates its higher acidity at steady state, which can increase the protonic backpressure over the cytochrome bc_1 complex.[153]

from *Rb. capsulatus* overlaps with the three core subunits of the mitochondrial bc_1[7] and the same specific inhibitors are efficient in both systems pointing to common catalytic mechanisms. The characteristic properties of the redox cofactors of *Rhodobacter* bc_1 are summarized in Table 1.

In this review, only the flash-induced reactions in bc_1 are surveyed. The data on generation of $\Delta\psi$ and ΔpH in response either to continuous illumination or to substrate addition are difficult to interpret in relation to molecular mechanisms of bc_1. The reader has to consult earlier works[52,64] for surveys on the steady operation of bc_1 in photosynthetic membranes.

2. Electron cycling in chromatophore vesicles from purple phototrophic bacteria

As depicted in Fig. 2, an absorption of a light quantum leads to a transmembrane charge separation between the water-soluble cytochrome c_2 and the secondary quinone Q_B of the photosynthetic reaction center (RC).[51,52,55,56] The charge separation yields potential reductants and oxidants for the bc_1, *i.e.* molecules of ubiquinol and of oxidized cytochrome c_2, respectively. The bc_1, in essence, catalyses the ET from ubiquinol back to cytochrome c_2. The availability of specific inhibitors of center *P*, such as myxothiazol and stigmatellin, helps to discriminate the reactions in bc_1 from other flash-induced events. In some cases, inhibitors of center *N* (*e.g.* antimycin) might be useful, in their presence electrons cannot pass heme b_h, so that only one "half-turnover" of bc_1 takes place.

When the membrane ubiquinone pool is completely oxidized, the flash-induced turnover of bc_1 is triggered by the arrival of a ubiquinol molecule that is formed in the RC. At neutral pH, the oxidation of this ubiquinol leads to partial reduction of heme b_h at approx. 3 ms followed by slower (i) cytochrome c_1 re-reduction by electrons coming from ubiquinol, (ii) $\Delta\psi$ generation, and (iii) proton release to the *p*-side of the membrane. These three reactions correlate with each other and take approx. 10–20 ms.[22,55,56,58,65–70]

When there is plenty of ubiquinol in the membrane (under reducing conditions that correspond to the physiological situation),[71] the bc_1 turnover is triggered by the migration of an electron vacancy from the flash-oxidized primary donor P_{870}^+, *via* cytochrome c_1, to the FeS domain[27,72] (see Fig. 1 and 2). The oxidation of Q_PH_2 results in the re-reduction of cytochromes *c* at 2–5 ms. The $\Delta\psi$ generation proceeds with the same rate. No redox changes of cytochrome *b* can be resolved: apparently, under these conditions the hemes of cytochrome *b* are oxidized faster than are reduced.[51,52,55,56,65–67]

3. Correlations between $\Delta\psi$ generation, proton translocation, and ET reactions

The views on the mechanism of $\Delta\psi$ generation by bc_1 have changed dramatically with time.‡ As the last summarising articles on this topic were published more than 15 years ago,[58,73] a historical consideration of the subject might be appropriate.

3.1 Electrogenic reactions in bc_1: the pre-structural age

3.1.1 $\Delta\psi$ generation. Jackson and Crofts were the first to trace the light-induced $\Delta\psi$ generation in bc_1 of *Rb. sphaeroides* by measuring the red electrochromic shift of carotenoid pigments.[74] Later they showed that the extent of carotenoid bandshift dropped in the presence of antimycin.[57] Under controlled redox conditions, the extent and rate of the antimycin-sensitive component of $\Delta\psi$ generation rose upon decrease in redox potential of the medium (E_h) with an apparent midpoint redox potential at pH 7.0 (E_m^7)

‡ It took some time until the bc_1 of *Rhodobacter* was isolated and the relation of the cytochrome *b* component of chromatophores to the ubiquinol:cytochrome *c* oxidoreductase (complex III) of mitochondria became unambiguous.[4,155,156] For simplicity, the notion bc_1 is used hereafter even in relation to earlier studies where the cytochrome bc_1 complex has not been identified as such yet.

Table 1 Cytochrome bc_1 complex of *Rb. capsulatus*: electrochemical and spectral properties of the redox cofactors

Redox cofactor	E_m^7 (apparent midpoint redox potential at pH 7.0 in mV)	λ_{max}/nm	g_x (EPR)
Heme b_l	−90 ÷ −115 (heme b_h reduced)[51,60] Approx. 0 (heme b_h oxidized)[128,149]	558 and 565[51,60]	
Heme b_h	+50 ÷ +60[51,115]	560.5[51,115]	
Cytochrome c_1	+290 ÷ +340[60,72]	552[60,72]	
[2Fe–2S] cluster	+270 (FeS_c)[60,145] ÷ +460 (FeS_b)[144]	—	1.800 (ubiquinone present)[150,151] 1.774 (ubiquinone absent)[150,151]

of ~150 mV.[55,56,75–79] These findings prompted diverse hypotheses on governing the cytochrome *b* turnover by a component with E_m^7 of ~150 mV. The component was putatively attributed either to a bound quinone "Q_Z" or to cytochrome b_{155}[55,56,77–79] (*vide infra* section 3.2.2.1). The oxidation of the flash-reduced cytochrome *b* and the re-reduction of the cytochrome *c* component accelerated in the presence of uncouplers, so that the energy storing step in bc_1 was attributed to ET from cytochrome b_{50} (heme b_h in current terms) to cytochrome *c*. At this stage, it was not clear yet whether the Q-cycle operates in phototrophic bacteria or not.[55,56,77–79]

The major breakthrough was coupled with the introduction of center *P* inhibitors, UHDBT and myxothiazol in the first line.[35,65,80,81] These inhibitors, when added over antimycin, helped to discriminate the reactions that accompanied the ubiquinol oxidation in center *P*. In the presence of antimycin, one ubiquinol molecule could be still oxidized in response to a flash and the concomitant reduction of heme b_h, re-reduction of cytochrome c_1 and $\Delta\psi$ generation proceeded with approx. same rate.[82] For the bc_1 of *Rhodobacter*, Crofts[4,82] has suggested the modified Q-cycle scheme that incorporated some earlier ideas of Garland.[83] According to this scheme, the center *P* turns over twice to produce a Q_NH_2 ubiquinol. The oxidation of the first ubiquinol molecule in center *P* leads to the formation of a $Q_N^{\bullet -}$ semiquinone[84] (in fact, the electron is shared between $Q_N^{\bullet -}$ and the reduced b_h heme (b_h^{red})). The redox equilibration in the low-potential branch of bc_1 (after oxidation of Q_PH_2 to $Q_P^{\bullet -}$ by the FeS cluster) can be written as:

$$Q_P^{\bullet -} + b_h^{ox} Q_N \rightarrow Q_P + b_h^{red} Q_N \leftrightarrow Q_P + b_h^{ox} Q_N^{\bullet -} \text{ (1st turnover)} \quad (1)$$

The second turnover ends with ubiquinol formation in center *N* after binding of two protons from the *n*-side:

$$Q_P^{\bullet -} + b_h^{ox} Q_N^{\bullet -} \rightarrow Q_P + b_h^{red} Q_N^{\bullet -} \xrightarrow{+2H^+} Q_P + b_h^{ox} Q_N H_2 \text{ (2nd turnover)} \quad (2)$$

Myxothiazol, when added over antimycin, decreased the flash-induced $\Delta\psi$ generation in chromatophores. Hence, the reduction of heme b_h was suggested to be electrogenic.[85] This suggestion was consistent with the transmembrane location of the two cytochrome *b* hemes, as deduced from molecular modelling.[86,87] The extent of the respective electrogenic component was estimated as 30–60% of the whole membrane dielectric.[58,73,85,88,89]

It is noteworthy that the E_m value of heme b_h in chromatophores of *Rhodobacter*, unlike that of the Q_N/Q_NH_2 couple, is pH-independent at pH > 7.5,[51] so that the E_m of the ubiquinol/ubiquinone couple becomes lower than that of heme *b* under alkaline conditions. Therefore the heme b_h could be reduced *via* center *N* by the RC-generated ubiquinol in the presence of myxothiazol at pH > 8.0.[90,91] The reaction was accompanied by a blue carotenoid bandshift indicating a discharge of $\Delta\psi$; this shift was not observed in the presence of antimycin.[73,88] This *negative* electrogenesis was more pronounced in the presence of $\Delta\psi$.[73,88] It was suggested that the voltage generation by bc_1 is a two-step ET reaction, where an electron goes first from heme b_l, at the *p*-side of the membrane, to heme b_h in the middle of the membrane, and then from heme b_h to Q_N at the membrane *n*-side.[73,88] The reversion of the latter reaction upon heme b_h reduction *via* center *N* was suggested to account for the blue carotenoid bandshift. To explain why $\Delta\psi$ prevented the oxidation of heme b_h but not its reduction, the transmembrane ET from heme b_h to Q_N was considered to be the least favourable step of the cycle. The dielectric distance between heme b_h and Q_N was estimated either as 40%[73] or as 60%[88] of the membrane dielectric. Accordingly, the proton transfer reactions were assumed to proceed at the membrane/water interfaces and to be electrically silent.[73,88]

Another considered possibility has been the involvement of transmembrane proton displacements in $\Delta\psi$ generation by bc_1.[20,58,59,68,69,92–96] In particular, the binding of the RC-generated ubiquinol to center *N*, as measured in the presence of myxothiazol by an electrometric technique, induced an antimycin-sensitive "negative" component of $\Delta\psi$ even at neutral pH when no reduction of heme b_h *via* center *N* could be expected. It was speculated that the binding of ubiquinol to center *N* could lead to the formation of an anion QH^- and electrogenic proton release.[58,59] A small myxothiazol-sensitive component of $\Delta\psi$ generation was resolved by capacitive electrometry in the mutants of *Rb. sphaeroides* lacking heme b_h. It was suggested that proton release from center *P* might be electrogenic.[96] In the resulting tentative picture of bc_1, the centers *P* and *N* were protein-buried and connected with external aqueous phases by proton conducting channels.[58] Proton transfer along these channels was assumed to be electrogenic.[58,59]

Furthermore, the above noted kinetic disparity between the faster reduction of heme b_h, on one hand, and the slower $\Delta\psi$ generation and cytochrome c_1 re-reduction, on the other hand, as seen under oxidizing conditions in the absence of antimycin,[55,56,58,65,66,68,69,95] could indicate poor coupling between the transmembrane ET towards heme b_h and the generation of $\Delta\psi$. It was suggested that protons, which are released in center *P* upon ubiquinol oxidation, are not ejected out of bc_1 but stay inside it for electrostatic compensation of the injected electron(s).[68,69,95,97] It was hypothesized (i) that these protons are released after the negative charge at reduced cytochrome *b* is neutralized by proton binding in center *N* upon ubiquinol formation and (ii) that the coupled events of proton binding and release account for the major electrogenic reaction in bc_1.[68,69,95] It is worth mentioning that at the same time it was realised that the electrostatic neutralization of injected electrons by the trapped protons is an inherent feature of the cytochrome *c* oxidase.[98]

3.1.2 Proton binding and release. Proton transfer reactions in chromatophores were measured by applying pH dyes.[50,54–56,68,69,81,99–107] Because the inner chromatophore volume is 4–5 orders of magnitude smaller than the external one, hydrophilic pH dyes, when added to chromatophore solution, report the pH-changes in the external phase. Using this approach, it was shown that the flash-induced proton binding by chromatophores, as measured in the absence of pH-buffers, was partly sensitive to antimycin.[50,56,75,81,103–107] Correspondingly, this antimycin-sensitive component of pH-transients was attributed to bc_1. In current terms, the reaction could be ascribed to proton binding in center *N* upon ubiquinone reduction and protonation. The relative extent of antimycin-sensitive proton binding increased in the presence of a K^+ ionophore valinomycin.[50,56,81,105–107] In the chronologically last work on this issue,[107] the odd behaviour of *Rb. sphaeroides* chromatophores, as studied under oxidizing conditions (E_h ~ 300 mV), was described. Under these conditions no antimycin-sensitive proton binding was observed although the $\Delta\psi$ generation by bc_1 was pronounced. The antimycin-sensitive proton binding

became observable only in the presence of valinomycin. The authors wrote that "proton binding occurs only when the carotenoid band shift is collapsed in milliseconds, whereas, conversely, the carotenoid band shift is stably generated when proton binding is not observed". A measurement error was unlikely because a significant antimycin-insensitive proton binding by the RC was always observed and could serve as a reliable reference.

To measure the pH changes in the tiny internal space of chromatophores, they were soaked by dense solution of a hydrophilic pH dye phenol red that was then rinsed off. The proton release into the chromatophore "lumen" took about 20–30 ms at pH about 6.0.[104] Saphon and Gräber measured the proton release into the interior of *Rb. sphaeroides* chromatophores *via* the fluorescence quenching of 9-aminoacridine.[108] This technique enabled to estimate the relative extent of the flash-induced acidification as 0.1–0.3 pH units but was not fast enough to determine the time constant. Jackson and co-workers have measured the same proton release at the *p*-side with whole cells, spheroplasts and right-side-out vesicles of *Rb. capsulatus*; in all these cases, the *p*-side was facing the external phase.[100–102] In whole cells and spheroplasts the proton release was distinctly slower than $\Delta\psi$ generation.[100–102] In smaller right-side-out vesicles the proton release had the same rate as the voltage generation.[102]§ The relation between proton release from bc_1, $\Delta\psi$ generation, and redox-reactions of cytochrome *b* was studied within chromatophores of *Rb. capsulatus* by using neutral red (NR) as an amphiphilic pH-dye.[68] In the presence of NR, an additional flash-induced absorption rise with a time constant of approx. 10 ms was seen under oxidizing conditions. The transient could be abolished by the addition either of a penetrating pH buffer glycyl–glycin, or a K^+/H^+ exchanger nigericin, or myxothiazol.[68] These absorption changes of NR were attributed to the acidification of chromatophore lumen by bc_1. The onset of acidification correlated with $\Delta\psi$ generation by bc_1 and was slower than the flash-induced reduction of heme b_h at ~3 ms.[68,69]

3.2 Electrogenic reactions in bc_1: the post-structural era

3.2.1 $\Delta\psi$ generation under oxidizing conditions. The crystal structures (see *e.g.* Fig. 1) have revealed that centers *P* and *N* are embedded in the protein matrix and connected by water channels with the external aqueous phases[5,7,14,28] confirming thus the hypothetical topology of bc_1 that was suggested earlier by Drachev and co-workers.[58,59] The edge-to-edge distance between two b_l hemes in the bc_1 dimer (see Fig. 1) was found to be <11 Å.[7,10–15] The proximity of two hemes added credit to the earlier suggestions on electron exchange between the two bc_1 monomers.[46,47] In fact, electrons, according to the rate–distance dependence for biological ET,[109] are likely to equilibrate between the two bc_1 monomers on the time scale of turnover. Gopta and co-workers have exploited this feature to rationalise their data on the operation of a substrate-limited bc_1. When less than one ubiquinol molecule was available per dimeric bc_1 under oxidizing conditions, the flash-induced $\Delta\psi$ generation and cytochrome c_1 re-reduction proceeded at 30–40 ms although the myxothiazol-sensitive reduction of heme b_h took about 3 ms.[22] Similar discrepancy was observed earlier when bc_1 turned over only once because of weak intensity of the actinic flash.[69] A scheme of a dimeric Q-cycle was put forward where (i) the electron exchange between the monomers is possible *via* two hemes b_l, (ii) the ubiquinol binding in two centers *P* is alternating, so that the two consequent ubiquinol oxidation reactions take place in different bc_1 monomers, (iii) the $Q_N^{\bullet-}$ semiquinone formation is not electrogenic because protons stay inside bc_1 to compensate the negative charge of injected electrons, (iv) the major electrogenic reaction is coupled with the formation of Q_NH_2 and is due to electrogenic proton transfer towards $Q_N^{\bullet-}$ and to the release, from the *p*-side, of those protons that compensated the surplus negative charges in bc_1 during the previous steps of the catalytic cycle.[22]

The electron transfer between the bc_1 monomers has been recently demonstrated.[48,49] The support for the alternating sites mechanism has been provided by Trumpower and co-workers.[42,44,45] The evidence in favour of the suggested, still controversial, mechanism of $\Delta\psi$ generation is surveyed in detail below.

3.2.2 Resolution of discrete catalytic steps in a Zn^{2+}-treated bc_1 of *Rb. capsulatus*. The above cited data on the mismatch between the faster heme b_h reduction, on the one hand, and the slower $\Delta\psi$ generation and re-reduction of cytochrome c_1, on the other hand,[22,55,56,58,65,66,68,69,70,95] were obtained with a pre-oxidised ubiquinone pool. Only under these conditions a (partial) flash-induced reduction of heme b_h could be seen and correlated with other reactions in bc_1. Under conditions close to the physiological ones, which imply a half-reduced ubiquinone pool[71], the oxidation of cytochrome *b* is faster than its reduction, so that the flash-induced redox-changes of cytochrome *b* are "invisible".[51,55,65,66] Recently it has been shown that implementation of Zn^{2+}, a well-established inhibitor of mitochondrial bc_1,[110,111] could help to resolve the partial reactions in bc_1 even under reducing conditions. The Zn^{2+} ions retarded the oxidation of heme b_h and made the kinetics of its reduction visible even when the ubiquinone pool was half-reduced.[53,112–114] When added at <100 μM, Zn^{2+} ions slowed down the bc_1-due voltage generation without affecting its extent. Hence, the bc_1 remained functional. In the presence of Zn^{2+}, the flash-induced oxidation of ubiquinol led to the reduction of heme b_h at 1–2 ms. Thereby only the half of heme b_h content was reduced. The rate of heme b_h reduction was independent of Zn^{2+} concentration at <100 μM of the latter. The re-reduction of cytochrome c_1 by ubiquinol, $\Delta\psi$ generation, and proton release into the chromatophore lumen proceeded slower; the rates of these reactions were sensitive to the Zn^{2+} concentration. At 50 μM of Zn^{2+}, the slower steps took about 10–20 ms. The kinetic mismatch was seen even in response to weak flashes that triggered only one turnover in some bc_1 monomers—under these conditions the heme b_h was still reduced at 1–2 ms, while cytochrome c_1 was re-reduced at ~7 ms and the $\Delta\psi$ generation was even slower.[53,112–114] These time constants neatly correspond to those reported by Zhu and co-workers, who, by using an ultra-fast microfluidic mixer and a freeze-quenching device, coupled with EPR, have determined the pre-steady state kinetics of ubiquinol oxidation by mitochondrial bc_1.[115] They found that the FeS cluster was reduced, after a lag of 100 μs, with half time of 250 μs. A compatible reduction kinetic was also observed for cytochrome b_l. The time constants of ~2.5 ms and ~6 ms were observed for the reduction of heme b_h and re-reduction cytochrome c_1, respectively.

§ The slower acidification in larger cells and spheroplasts, as compared to the vesicles, was caused, most likely, by the dependence of the surface protonic equilibration rate on the size of cells/vesicles, as elucidated elsewhere.[157]

Based on studies of Zn^{2+}-treated preparations, the turnover of bc_1 was suggested to proceed in two steps, at least[26,53,113], as depicted in Fig. 3. During the first, apparently Zn^{2+}-insensitive step (see Fig. 3A), ubiquinol is oxidized in center *P*, the FeS domain takes the first electron and first proton from ubiquinol, while the other electron is transferred *via* heme b_l to heme b_h across the membrane. The second proton remains in center *P*. During the second, Zn^{2+}-sensitive step (see Fig. 3B), the FeS domain re-reduces cytochrome c_1, protons are released into the chromatophore interior, heme b_h is oxidized *via* center *N* and $\Delta\psi$ is generated.[26,53,113]

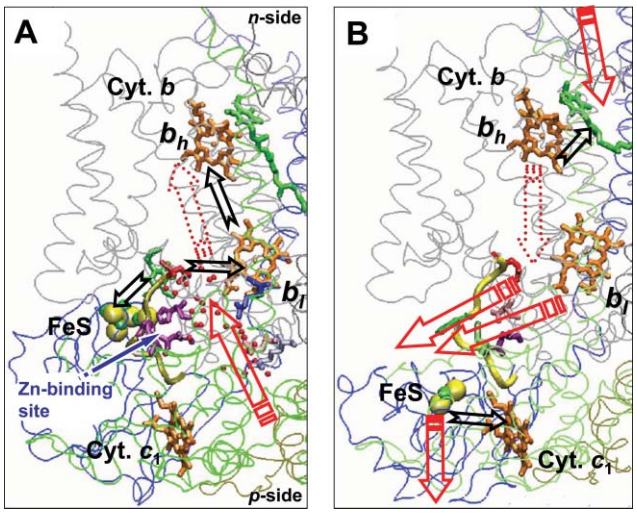

Fig. 3 Tentative scheme of electron and proton transfer during the initial steps of bc_1 turnover (the figure is reproduced from ref. 26 with permission). Black arrows, ET events; red arrows, proton transfer events; dark-red dotted arrows, dielectric relaxation of protein–water. The redox centers are colored as in Fig. 1. Below the yeast numbering of amino acid residues is given by straight letters, while that of *Rb. capsulatus* is given in italic letters. The Glu-272 (*Glu-295*) of cytochrome *b* is colored red. The segment of the *ef* loop that interferes with the movement of the FeS domain is shown as a thick yellow tube (cytochrome *b* residues from 260 to 270 (from *283* to *293* in *Rb. capsulatus*). The figure was produced with the help of the VMD software package.[152] (A) Fast step of ubiquinol oxidation in center *P*. The picture is a compilation of two crystal structures of the yeast bc_1 with the FeS domain docked to cytochrome *b* in the presence of stigmatellin: the water chains from the high resolution structure (PDB entry 1EZV[14]) are superimposed over the structure of a dimeric yeast bc_1 co-crystallized with cytochrome *c* (PDB entry 1KYO[15]). The bound stigmatellin in center *P* was replaced by ubiquinol. Water molecules, which are found in the vicinity of center *P* are shown as red balls. The four amino acid residues, which correspond to the Zn^{2+}-binding ligands of the chicken bc_1, are depicted in violet. Thereby Ser-268 of yeast was replaced by histidine, as in *Rb. capsulatus* (*His-291*) and chicken; other residues are His-253 (*His-276*), Asp-255 (*Asp-278*) of cytochrome *b* and His-185 of cytochrome c_1 (no evident counterpart in *Rb. capsulatus*). (B) Slower step of ubiquinone reduction in center *N*. The picture is based on the structure of the chicken bc_1 with the FeS domain in the "cytochrome c_1" position (PDB entry 1BCC[11]). Ubiquinol in center *N* is shown in the same position as it is found in the yeast bc_1 (PDB entry 1EZV[14]). The four amino acid residues, which bind Zn^{2+} in the chicken bc_1 are colored as follows: cytochrome *b*, Asp-253 (*His-276*), pink, Glu-255 (*Asp-278*), violet, His-268 (*His-291*), green, cytochrome c_1, His-121 (no evident counterpart in *Rb. capsulatus*), light green.

These studies of bc_1 revealed several features that deserved explanation, namely:

(1) Only a half of the total heme b_h content was reduced in response to a flash, even when the experimental conditions (pH, temperature, H_2O/D_2O) were varied.[53,112–114] The reduction of only half of heme b_h content was surprising. During the studies of Zn^{2+}-treated samples, the ubiquinone pool was kept half-reduced by the succinate/fumarate redox couple; the redox poise of the sample corresponded to 80–100 mV at pH 7.5 of the measurements.[53,112–114] The heme b_h with $E_m^{7.5}$ of ~20 mV[51] was expected to be oxidized in the dark and to be completely reduced in response to a flash. Such a complete reduction of the heme b_h content could be indeed observed, but only after antimycin was added to the sample.[53,112–114]

(2) When weak exciting flash was used, so that ubiquinol was oxidized only by some bc_1 monomers, the rate and relative extent of $\Delta\psi$ generation was comparable with that observed after a saturating flash.[53,113,114] This observation was in variance with the behaviour of bc_1 under oxidizing conditions when the $\Delta\psi$ generation slowed down and diminished dramatically under single-turnover settings.[22,69,70]

(3) The transmembrane ET from center *P* to heme b_h took 1–2 ms and was not accompanied by notable $\Delta\psi$ generation.[53,112–114] One would expect (i) that the transmembrane movement of a negative charge should contribute to $\Delta\psi$ generation and (ii) that the onsets of heme b_h reduction and of voltage generation should match each other.

(4) Zn^{2+} ions concomitantly slowed down the $\Delta\psi$ generation, proton release into the lumen, re-reduction of cytochrome c_1 and oxidation of heme b_h.[53,112–114] Such a kinetic match, as observed even under single-turnover conditions, deserves explanation as long as the oxidation of heme b_h and the re-reduction of cytochrome c_1 proceed at different sides of the membrane.

Below I attempt to justify these observations by considering the catalytic cycle of bc_1 in some detail.

3.2.2.1. EPR-silent $Q_N^{\bullet-}$ semiquinone in a dimeric cytochrome bc_1 *complex.* The observations (1) and (2) can be accounted for by invoking the data on potentiometric redox titrations of bc_1. On one hand, these titrations have revealed a particular high-potential form of heme b_h in the bc_1 of *Rhodobacter*[5,55] and of mitochondria.[116–118] In chromatophores of *Rhodobacter*, the E_m^7 of this "b_{155}" was estimated as ~150 mV.[5,55] The "very high potential" cytochrome *b* was not detectable after the addition of antimycin; therefore it was suggested that b_{155} represented a special state of heme b_h (hereafter denoted as $b_{h,150}$) induced by its interaction with a quinone species in center *N*.[5,90,118] On the other hand, Palmer and co-workers, while performing a parallel potentiometric titration of isolated yeast bc_1 by optical and EPR spectroscopy,[116,119] found that ~50% of the oxidized heme b_h was EPR silenced by its antiferromagnetic interaction with a semiubiquinone when the ubiquinone present was reduced by half. It was concluded that bc_1 contains two populations of ubisemiquinone, namely a minor EPR-visible one (~0.1 per bc_1 monomer) and a major EPR-silent one (~0.5 per bc_1 monomer). Rich and co-workers came to the same conclusion upon analysing their data on potentiometric titration of bc_1 in the beef hearth submitochondrial particles (SMP).[118] By invoking the pioneering EPR spectroscopy data of De Vries and co-workers,[46,47] Crofts argued that the $b_{h,150}$ state resulted from "reversal of the second electron transfer of the normal forward reaction".[5,98] It was suggested that at E_h < 150

mV the oxidation of an ubiquinol molecule from the membrane pool *via* center *N* might yield a semiquinone and a reduced heme b_h.[5,90] The E_m^7 value of the Q_N/Q_NH_2 couple, as revealed from the potentiometric titration of the EPR-visible $Q_N^{\bullet-}$ signal, was indeed found to be ~150 mV in the case of *Rb. sphaeroides* chromatophores.[84]

As long as a monomeric bc_1 is considered, this explanation of the $b_{h,150}$ phenomenon is rather paradoxical. It implies that an interaction of heme b_h with a negatively charged $Q_N^{\bullet-}$ causes an *increase* in the E_m value of the heme, conversely to expectations based on electrostatics. The paradox, however, can be solved by invoking the above noted electron exchange inside a bc_1 dimer. Although such electron exchange, as explicitly suggested by some authors,[10,22,24,46,47,120] has been recently experimentally demonstrated,[48,49] it is not proved yet whether the exchange can proceed on the timescale of the bc_1 turnover. However, in relation to the pulsed experiments, there is no thinkable way to prevent an electron equilibration between the bc_1 monomers *before* the measurements, on a timescale of seconds. Such an equilibration is expected to proceed in the following way.

(1) Ubiquinol binding in center *N* would result in formation of a $Q_N^{\bullet-}b_h^{red}$ pair at redox poise corresponding to $E_h < 150$ mV, as suggested by Crofts and co-workers.[5,90]

(2) Provided that electron can migrate inside the bc_1 dimer, the electrostatic repulsion between electrons at $Q_N^{\bullet-}$ and at b_h^{red} would favour the spillover of the electron from the b_h heme next to $Q_N^{\bullet-}$ to the heme b_h of the other bc_1 monomer:

$$QH_2 + b_h^{ox} \mid b_h^{ox} \rightarrow Q_N^{\bullet-}b_h^{red} \mid b_h^{ox} \rightarrow Q_N^{\bullet-}b_h^{ox} \mid b_h^{red} \quad (3)$$

Then, because the heme iron is paramagnetic in the b_h^{ox} state:[116]

(i) accounting for the data of Palmer and co-workers,[116,119] one EPR silent semiquinone $Q_N^{\bullet-}b_h^{ox} \mid b_h^{red}$ would be present in the majority of bc_1 dimers (its estimated amount of 0.5 per one bc_1 monomer[116,118,119] corresponds to one semiquinone per one bc_1 dimer).

(ii) in a minor fraction of enzymes, the $Q_N^{\bullet-}$ semiquinone could be EPR visible either because the electron has not got to the other monomer ($Q_N^{\bullet-}b_h^{red} \mid b_h^{ox}$), or because (one) heme b_h was already pre-reduced at equilibrium ($Q_N^{\bullet-}b_h^{red} \mid b_h^{red}$, note that the iron atom of b_h^{red} is diamagnetic[116]). At neutral pH the relative extent of EPR visible $Q_N^{\bullet-}$ seems to be ~0.1 per bc_1 monomer, at most.[84,116,119,121]

The EPR silence of the $Q_N^{\bullet-}b_h^{ox} \mid b_h^{red}$ state is in agreement with the X-ray data that show Q_N only 5 Å away from heme b_h[14], *i.e.* close enough for antiferromagnetic quenching.

As already noted, the E_m of heme b_h in chromatophores of *Rhodobacter*, unlike that of the Q_N/Q_NH_2 couple, is pH-independent at pH > 7.5.[51] Then one should expect an increase in the relative amount of the EPR-visible $Q_N^{\bullet-}b_h^{red} \mid b_h^{red}$ state at alkaline pH owing to the increase in the amount of reduced heme b_h next to $Q_N^{\bullet-}$. This expectation is fulfilled: at alkaline pH, the relative extent of the EPR-visible $Q_N^{\bullet-}$ semiquinone increased up to 0.4 per bc_1 monomer in chromatophores of *Rb. sphaeroides.*[84] Supposedly because of the same reason, the relative amount of the EPR-visible $Q_N^{\bullet-}$ semiquinone increased upon alkalisation also in the cytochrome bc_1 complexes from other sources: up to 0.26–0.5 in the SMP,[84,122], and up to ~0.5 in the bc_1 of *Paracoccus denitrificans.*[121] The p*K* value of 7.5, which is well-established for b_h of *Rhodobacter*, is sufficient to explain the increase in the amount of the EPR-visible $Q_N^{\bullet-}$ at high pH values; there is no need to invoke additional functional p*K* of $Q_N^{\bullet-}$ in the neutral range.

Hence, when the ubiquinone pool is half-reduced, two electrons seem to be distributed over a bc_1 dimer; they reside at heme b_h and at $Q_N^{\bullet-}$ semiquinone of *different monomers*, as described by scheme (1). Under physiological conditions of a half-reduced ubiquinone pool,[71] the state $Q_N^{\bullet-}b_h^{ox} \mid b_h^{red}$ is the stable "ground" state of bc_1; it is depicted as state A in Fig. 4 where a tentative catalytic cycle is schematically shown for a bc_1 dimer.

Then, in response to a flash of light:

(i) accounting for our observations[53,112–114], not more than a half of the total b_h content could be reduced—the other half is already pre-reduced in the dark as $Q_N^{\bullet-}b_h^{ox} \mid b_h^{red}$;

(ii) ubiquinol formation in center *N* would occur already in response to the first turnover of center *P*. Already the oxidation of the first Q_PH_2 molecule would lead to the reduction of heme b_h next to the $Q_N^{\bullet-}$ semiquinone followed by the oxidation of this heme and ubiquinol formation in center *N* (see Fig. 4):

$$Q_P^{\bullet-} + Q_N^{\bullet-}b_h^{ox} \mid b_h^{red} \rightarrow Q_P + Q_N^{\bullet-}b_h^{red} \mid b_h^{red} \rightarrow Q_P + Q_NH_2b_h^{ox} \mid b_h^{red} \quad (4)$$

Since the major electrogenic reaction in bc_1 seems to be coupled with the formation of the Q_NH_2 ubiquinol[22] (see also section 3.2.2.3 below), the suggested rational explains why the flash-induced $\Delta\psi$ generation by bc_1, as measured under reducing conditions in a single-turnover setup, was similar to that after a saturating flash.[53,112–114]

One could ask why the $Q_N^{\bullet-}b_h^{ox} \mid b_h^{red}$ state is not oxidized by the membrane ubiquinone further to yield a $Q_N^{\bullet-}b_h^{ox} \mid b_h^{ox}Q_N^{\bullet-}$ state. The reason might be the inability of the bc_1 dimer to stabilize two $Q_N^{\bullet-}$ semiquinones at once. The X-ray structure of crystals formed from bc_1 dimers as crystallographic units has revealed one Q_N bound per bc_1 dimer.[15]

According to the suggested scheme, already the first turnover of center *P* can yield Q_NH_2 under reducing, physiological conditions. This feature is related to the long-lasting dilemma on the apparent incompleteness of a single Q-cycle turnover. Indeed, the oxidation of one Q_PH_2 ubiquinol provides only one electron for center *N*, whereas two electrons are needed to yield a product—a Q_NH_2 ubiquinol. Mitchell has been concerned by this problem; he has considered a possibility that while one electron comes to the quinone in center *i* (center *N*) from heme b_h, another one is supplied by some other source. He hypothesized that this second electron is provided by the succinate dehydrogenase in mitochondria, by the RC in chromatophores of phototrophic bacteria, and by the photosystem II in plants.[20] However, the bc_1 operates with a H^+/e^- stoichiometry of 2 even alone, when incorporated into liposomes.[17,18] Therefore, the common believe has been that a catalytic cycle of bc_1 requires two turnovers of center *P*,[4,82,83,123] as described by eqn (1) and (2) in section 3.1.1. The scheme (4) offers another solution: here an oxidation of only one ubiquinol molecule in center *P* by the "pre-activated" bc_1 is sufficient to yield a Q_NH_2 ubiquinol. In other words, the Q-cycle is complete in a single turnover of center *P*.

If reaction (3) is exothermic, then the $Q_N^{\bullet-}b_h^{ox} \mid b_h^{red}$ state would be trapped once formed. Because of electrostatic attraction to the heme iron, the $Q_N^{\bullet-}$ semiquinone would be tightly bound (as it happens in the RC[124]). The bound $Q_N^{\bullet-}$ would be destined to wait for the second electron coming from center *P via* cytochrome *b*

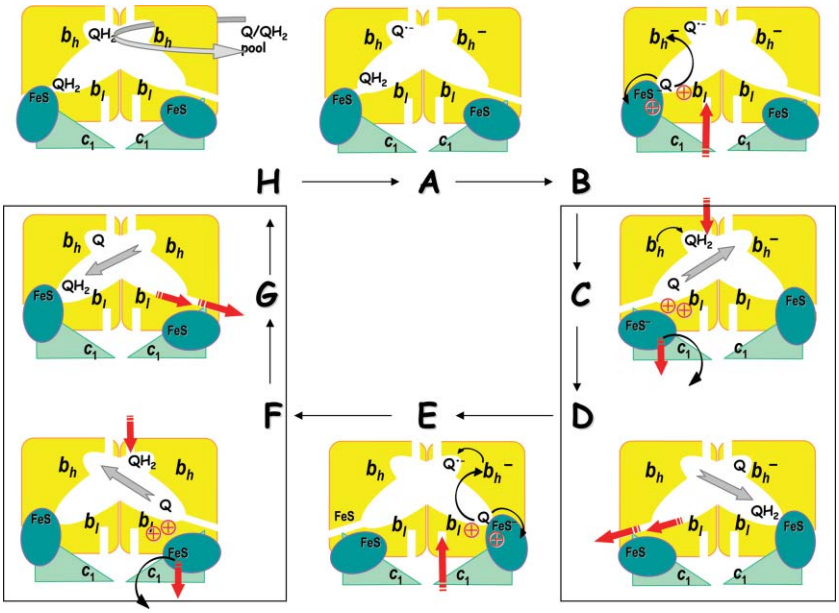

Fig. 4 Activated Q-cycle in a dimeric cytochrome bc_1 complex (the scheme has been first presented during the 13th EBEC Meeting).[154] Thin black arrows, electron transfer steps; thick red arrows, proton transfer steps; thick gray arrows, quinone/quinol exchange reactions. Protons are depicted as red, circled crosses. The tentative mechanism of the first ubiquinol oxidation, as presented on panels from A to D, is based on the single-turnover data[53,112–114] and on the comparative analysis of different bc_1 structures.[26,113,136] The following steps (panels from E to H), which describe the second turnover of center *P*, are hypothetical; they are based on symmetry considerations and on the need to complete the catalytic cycle. For the other explanations, see the text. (1) Panel A, initial activated state. When ubiquinol is present, the bc_1 dimer is in an activated $Q_N^{\bullet-}b_h^{ox} \mid b_h^{red}$ state. The docking of the oxidized FeS domain to cytochrome *b* leads to the formation of a quinol-oxidizing catalytic site in center *P*. (2) Panel B, the faster oxidation of Q_PH_2 (see also Fig. 3A). The ubiquinol oxidation leads to the coupled electron and proton transfer to the FeS cluster yielding a $Q_P^{\bullet-}$ semiquinone. The reduced and protonated FeS domain remains docked to cytochrome *b* for the time being. The electron goes from $Q_P^{\bullet-}$ to heme b_l; the released proton resides in center *P*. The electron then crosses the membrane going from heme b_l to the only oxidized heme b_h of the bc_1 dimer, next to the $Q_N^{\bullet-}$ semiquinone. This transmembrane ET seems to be electrostatically compensated. (3) Panels C and D, the slower formation of Q_NH_2 (see also Fig. 3B): The FeS domain undocks and moves towards cytochrome c_1. This reaction seems to be coupled to the oxidation of heme b_h by the $Q_N^{\bullet-}$ semiquinone, electrogenic binding of two protons from the *n*-side of the membrane, and formation of a Q_NH_2 ubiquinol. The oxidation of the reduced and protonated FeS domain by cytochrome c_1 is accompanied by the proton release into the water phase. In the same time, the protons, which have compensated the negative charge at cytochrome *b* hemes, get out *via* the now open proton exit. The transfer of all these protons across the membrane dielectric, as well as the re-orientation of the intra-membrane dipoles and charges, account for the observable electrogenic reaction. Only for illustrative purposes, the reactions accompanying this slower step of the catalytic cycle are depicted on two panels C and D; the panels are framed to emphasize that all these reactions are likely to be coupled to each other (this consideration relates also to the panels F and G).

hemes. From the apparent E_m of 150 mV both of $b_{h,150}$[5,55,115] and of the $Q_N^{\bullet-}$ semiquinone in *Rb. sphaeroides*,[84] the ΔG value of reaction (3) can be estimated as ~ -60 meV under conditions where the ubiquinone pool ($E_m^7 \sim 90$ mV) is half-reduced. This free energy is temporally borrowed from the ubiquinol pool to guarantee the steady presence of one $Q_N^{\bullet-}$ per one bc_1 dimer; the loan is returned back when a new ubiquinol molecule is formed in center *N*. A continual priming of bc_1 is secured by the promptness of ubiquinol binding in center *N* (<100 μs).[59]

The suggested ability of the $Q_N^{\bullet-}b_h^{ox} \mid b_h^{red}$ state to exert control over the rate of bc_1 turnover might solve an old controversy concerning the nature of the bound quinone in bc_1. The earlier studies have revealed that the flash-induced electrogenesis and cytochrome *c* reduction in bc_1 of *Rhodobacter* accelerated dramatically with an apparent E_m^7 of ~150 mV.[55,65,67,77–79] Based on these observations, Dutton and co-workers have suggested a tightly bound ubiquinol (Q_Z) in center *P* with E_m^7 of about 150 mV.[55,77–79] This suggestion was supported by the observation that the prompt turnover of bc_1 was unaffected by ubiquinone extraction until only 1–2 ubiquinones per bc_1 remained.[78] The X-ray structures of bc_1 revealed neither ubiquinol nor ubiquinone in center *P*, but, instead, exposed ubiquinone molecule bound in center *N*.[14,15,28] It looks like that the only binding site in bc_1 with a notable affinity to ubiquinol/ubiquinone is the center *N* proper. Taken together, these data might indicate that Dutton and co-workers were correct when they suggested that the acceleration of the bc_1 turnover is related to a bound quinone species with an apparent E_m^7 of ~150 mV. This quinone species, however, seems to be not the ubiquinol in center *P*, but a bound $Q_N^{\bullet-}$ semiquinone in center *N*. In fact, Dutton and co-workers have considered the possibility that the semiquinone in center *N* might control the kinetics of bc_1.[84] They, however, declined this possibility because the amount of the EPR-visible semiquinone was sub-stoichiometric at neutral pH.[84] As argued above, one EPR-silent $Q_N^{\bullet-}$ semiquinone seems to be present per each bc_1 dimer at $E_h < 150$ mV.[116,118,119] Apparently, the formation of the EPR-silent $Q_N^{\bullet-}b_h^{ox} \mid b_h^{red}$ state switches the cytochrome bc_1 complex from the slower double-turnover mode of operation to the faster single-turnover one.

3.2.2.2. Electrostatic compensation of the transmembrane ET towards heme b_h. As noted above, the faster reduction of heme

b_h, as compared to the $\Delta\psi$ generation, might indicate electrostatic compensation of the transmembrane electron transfer to heme b_h.[22,26,68,69,95] More widespread explanation is, however, that the genuine, slower kinetics of heme b_h reduction is masked, in the absence of antimycin, by the oxidation of the heme, so that the observable prompt reduction of heme b_h only *seems* to be faster than the voltage generation.[125] To assess these two possibilities, it is useful to consider a simple kinetic scheme:

$$Q_P^{\bullet-} \xrightarrow{k} b_h \xrightarrow{K_{eq}=k_+/k_-} Q_N \qquad (5)$$

A partial flash-induced reduction of heme b_h is expected if (i) k_+ has the same order of magnitude as k (*kinetic* limitation) or (ii) if K_{eq} is low (even when $k_+ \gg k$, *thermodynamic* limitation). Numerical simulations/analysis of the scheme (5) show that the oxidation of heme b_h can cause apparent acceleration of its reduction rate only in case of kinetic limitation. In the case of thermodynamic limitation, the *observable* rate of heme b_h reduction should be equal to the *genuine* rate of electron delivery to b_h and independent of the observable extent of heme reduction. The extent of flash-induced heme b_h reduction, as seen under oxidising conditions in the absence of antimycin, decreased in the presence of uncoupler[55,126] and increased when the membrane potential was elevated by blocking the proton escape *via* ATP-ase.[69] Thereby the time constant of heme b_h reduction (~3 ms) remained independent of the fraction of the heme staying reduced after the flash.[69] As well, the distance of 5 Å between heme b_h and Q_N[14] is hardly compatible with a kinetically limited oxidation of heme b_h. This evidence strongly supports the thermodynamic control over the oxidation of heme b_h and, accordingly, suggests that the rate of the partial heme b_h reduction in the absence of inhibitors reflects the genuine rate of electron delivery to the heme.

Still, when the membrane ubiquinol pool is oxidized and the substrate ubiquinol comes from the RC, it is difficult to exclude completely a prompt, non-electrogenic reduction of heme b_h *via* center *N* (A. R. Crofts, personal notion). Under reducing conditions, as maintained upon the studies of the Zn^{2+}-treated bc_1, this possibility could be ruled out. The heme b_h was reduced at 1–2 ms by an electron that crossed the membrane coming from center *P*, and still no compatibly fast $\Delta\psi$ onset was observed.[53,112–114]

As argued in more detail elsewhere[26,127], an electrostatic compensation is, in fact, a precondition of ET from heme b_l ($E_m^7 \sim 0$ mV[128]) to heme b_h ($E_m^7 \sim 50$ mV[51,61]) against the backpressure of $\Delta\psi$ (about 100 mV in chromatophores of *Rb. capsulatus* after a single saturating flash[129]). Such an electric silencing could be achieved, in particular, by proton transfer in the direction of ET,[22,26,105] *e.g.* along a water chain that connects the propionates of heme b_l with the external *p*-phase[14,130] While proton *release via* this path, as suggested by Crofts and co-workers[41], would go against the transmembrane electric field, the same field would favour proton re-distribution in the opposite direction, towards heme b_l. Additionally, electrostatic compensation might be achieved *via* re-orientation of water dipoles and of charged protein groups/domains, as discussed in more detail elsewhere[26,53,113]

3.2.2.3. Kinetic correlations in the cytochrome bc_1 *complex and the mechanism of* Zn^{2+} *binding.* The mechanism of kinetic correlation between the re-reduction of cytochrome c_1, $\Delta\psi$ generation, proton release into the chromatophore interior and the oxidation of heme b_h[53,112–114] can be tentatively elucidated if we consider the mode of Zn^{2+} binding by bc_1.

Skulachev and co-workers were the first to show that Zn^{2+} ions increased the reduction level of cytochromes *b* and decreased that of cytochromes *c* in the mitochondrial bc_1.[110] Link and Jagow, who studied the inhibition of mitochondrial bc_1 by Zn^{2+} as function of its concentration and pH, suggested that Zn^{2+} ions interfered with histidine residue(s) forming the proton outlet from center *P*.[111] Relevantly to this conclusion, Zn^{2+} and some other divalent ions were found to block the proton inlet to Q_B in the RC of *Rb. sphaeroides* by binding to a cluster formed by His-126, His-128 and Asp-124 of the H subunit of the RC with an apparent p*K* of 6.8.[131] As well, Zn^{2+} ions were found to block the proton paths in the cytochrome *c* oxidase.[132,133] Incidentally or not, the affinity of negatively charged histidine-containing patches to Zn^{2+} ions seems to correlate with the proton trapping/proton conducting ability of these patches.[134] In *Rb. capsulatus*, the Zn^{2+}-due effects in bc_1 have diminished with an apparent p*K* of approx. 7.0.[114] This value is close to the p*K* values of Zn^{2+} binding to the mitochondrial bc_1 (pK = 7.2)[111] and to the *Rb. sphaeroides* RC (pK = 6.8)[131] and points to histidine residue(s) as potential Zn^{2+} ligand(s) in *Rb. capsulatus*. Berry and co-workers have crystallized the avian bc_1 in the presence of Zn^{2+}. They found a binding site for Zn^{2+} close to center *P* that was formed by His-121 of cytochrome c_1 and by Asp-253, Glu-255 and His-268 of cytochrome b[135] (see Fig. 3A). All three Zn^{2+} ligands provided by the avian cytochrome *b* have counterparts in *Rb. capsulatus*; these are His-276, Asp-278 and His-291. The constellation of two histidine residues and a carboxyl strikingly resembles the Zn^{2+}/proton binding cluster of the *Rb. sphaeroides* RC,[131] so that this triad was suggested to bind Zn^{2+} in the bc_1 of *Rb. capsulatus*.[53,113,136] This suggestion has been recently confirmed by Venturoli and co-workers who, by using K-edge X-ray absorption spectroscopy, have found that two nitrogen and two oxygen atoms (at <2.20 Å) plus one oxygen (or nitrogen) atom serve as ligands for a single Zn^{2+} ion bound in the bc_1 of *Rb. capsulatus*.[137] The avian Asp-253 and Glu-255 (His 276 and Asp 278 of *Rb. capsulatus*) are close to Glu-272 (Glu-295 of *Rb. capsulatus*), the putative immediate acceptor of the second proton from ubiquinol.[14,26,41] Thus, the path *via* the Zn^{2+}-binding site seems to be the shortest potential proton exit from center *P*. The comparison of different bc_1 structures shows that this exit is open when the FeS domain is not docked to cytochrome *b* (see Fig. 3B). Hence, it is thinkable that protons get the opportunity to escape from center *P* into the water phase only when the FeS domain relocates towards cytochrome c_1. It is noteworthy that His-268 of cytochrome *b* belongs to its *ef* loop, which controls the motion of the FeS domain and serves as a slide-valve for the passing FeS domain.[130,138,139] The participation of the *ef* loop in the binding of Zn^{2+} (*via* His-268 in avian bc_1 and, most likely, *via* His-291 in the bc_1 of *Rb. capsulatus*) might provide a mechanistic rationale for the slowing of the cytochrome c_1 re-reduction by the FeS domain in the presence of Zn^{2+}, as observed with chromatophores of *Rb. capsulatus*.[53,112–114] (see Fig. 3). The binding of Zn^{2+} could constrain the mobility of the *ef* loop and thereby impede the motion of the FeS domain. This rational can account for the concurrent inhibition of cytochrome c_1 re-reduction and $\Delta\psi$ generation by Zn^{2+}: the binding of Zn^{2+} to the histidine patch can slow down (i) the movement of the FeS domain towards cytochrome c_1 (*via* the above mentioned interaction with a histidine residue of the *ef* loop) and, accordingly, (ii) the electrogenic proton release from center *P*.

As it is discussed in more detail elsewhere,[22,26,53,113,136] and as it is depicted in Fig. 3B and panels C and D of Fig. 4, the $\Delta\psi$ generation by bc_1 seems to represent a chain of coupled electrogenic events. (i) The movement of the reduced and protonated FeS domain towards cytochrome c_1 leads to the oxidation of the FeS cluster followed by proton release into the aqueous phase *p*; (ii) the formation/release of the Q_NH_2 ubiquinol in center *N* is accompanied by electrogenic binding of two protons from the *n*-side of the membrane; (iii) the opening of the proton exit from center *P* lets proton(s), which do not compensate any negative charge now, to get out.¶ The transfer of all these protons across the membrane dielectric, together with the resetting of the intra-membrane charges and dipoles, generates $\Delta\psi$. In agreement with experiment (i) the rate of $\Delta\psi$ generation correlates with the rate of cytochrome c_1 re-reduction[22,55,56,77,79,80] and (ii) both these reactions are sensitive to Zn.[26,53,112–114]

The Zn^{2+} treatment has also exposed a kinetic correlation between the re-reduction of cytochrome c_1 (and $\Delta\psi$ generation), on one hand, and the oxidation of heme b_h, on the other hand.[53,112–114] It is noteworthy that such a kinetic correspondence has been observed not only with the bc_1 of *Rhodobacter*[55,65,66] but also upon pulsed studies of the mitochondrial cytochrome bc_1 complex.[140–142] Diverse earlier data on the steady operation of cytochrome bc_1 complexes from different sources also showed correlation between the oxidation of cytochrome *b* and the re-reduction of cytochrome c_1.[32,55,56,143] Around 1981 Mitchell has mentioned in a conversation a possibility that "the electron might be getting hung up on the Rieske" (as noted recently by Crofts[4]). Independently, it has been hypothesized that the ET from the FeS cluster to cytochrome c_1 might be mechanistically controlled by a redox reaction in center *N*.[69] As argued elsewhere,[26] the inability of the electron to leave the FeS cluster until the reaction in center *N* is completed helps to prevent the short-circuiting in bc_1. The structural data provide some hints on how such a control can be achieved. As already noted, the relocation of the FeS domain between its two binding sites seems to be controlled by the *cd* and *ef* loops of cytochrome *b*.[12,130,138,139,144,145] These loops connect, respectively, the C, D and E, F α-helices of cytochrome *b*. The quinone-binding site in center *N*, on the other hand, is formed with the participation of helices D and E of cytochrome *b*.[14,28] The occupancy of center *N* might affect the mechanistic properties of the *ef* and *cd* loops and *vice versa* resulting in coupling between the relocation of the FeS domain and the oxidation of heme b_h. Recently Cooley and coworkers, by increasing the spectral and spatial resolution obtainable with orientation-dependent EPR spectroscopic analysis of ordered membrane preparations, have indeed found correlations between the occupancy of center *N* and the EPR spectra of both cytochrome *b* hemes and the FeS cluster and suggested a hypothetical mechanism of coupling *via* helices of cytochrome *b*.[146]

The above considered correlation between the occupancy of center *N* and the mobility/position/EPR spectra of the FeS domain[146] might be related to the "double occupancy" model of Dutton and co-workers (see ref. 3 and citations therein). These authors studied the EPR line shapes of the Fe–S cluster as function of (i) the amount of ubiquinone/ubiquinol present (varied by solvent extraction and reconstitution), (ii) the redox state of the ubiquinone pool, (iii) the presence of specific inhibitors and (iv) the site-specific mutations in cytochrome *b*. From data analysis, it has been suggested that the center *P* (Q_O site) can accommodate two ubiquinone molecules, namely the strongly (Q_{OS}) and the weakly (Q_{OW}) bound ones. After the crystal structures failed to reveal any bound quinone in center *P*, the double occupancy model has been apparently abandoned. The underlying experimental data, however, deserve explanation. These data can be accounted for, at least qualitatively, by suggestions that (i) Q_{OS} is the ubiquinone that occupies center *N* in the crystal structures,[14,15,28] (ii) the extraction of this strongly bound ubiquinone affects the EPR spectra of the FeS cluster *via* the conformational coupling between centers *N* and *P*.

The oxidation of heme b_h *via* center *N* can lead either (i) to the $Q_N^{\bullet-}$ formation (if a quinone molecule pre-occupies center *N*, see eqn (1)) or (ii) to the formation of ubiquinol Q_NH_2 (if a $Q_N^{\bullet-}$ semiquinone is present in the site, see eqn (2) and eqn (4)) . Which of these two reactions is coupled with the relocation of the FeS domain? This question can be tentatively answered by considering the single turnover data. When bc_1 of *Rb. capsulatus* turned over only once under oxidizing conditions (case (i)), the kinetic correlation was absent: the re-reduction of cytochrome c_1 took approx. 30–40 ms while the electron re-distribution between heme b_h and the Q_N ubiquinone, according to eqn (1), proceeded at <3 ms.[22,69,70] On the contrary, under reducing conditions (case (ii)) the re-reduction of cytochrome c_1 correlated with the oxidation of heme b_h even after weak flashes.[53,112–114] Hence, it seems that the relocation of the FeS domain towards cytochrome c_1 is coupled with the formation of the Q_NH_2 ubiquinol and/or with its release from center *N*. After this point of "no return", there is no danger of short-circuiting, so that the reduced FeS domain can move towards cytochrome c_1 to be oxidized. Besides, the coupling between centers *N* and *P* could drive the unfavourable proton binding upon Q_NH_2 formation by the thermodynamically gainful release of proton(s) from center *P* into the water phase.[136]

The idea of coupling between the "liberation" of the FeS domain and the formation/release of Q_NH_2 gets further support from the already cited study of Cooley and co-workers who concluded that the bound antimycin, on one hand, imitates either the presence of Q_NH_2 or the absence of quinone, and, on the other hand, increases the mobility of the reduced FeS domain maximally as if it were to facilitate its movement away from center *P*.[146] This observation not only provides hints on how the reduced FeS domain can be "released" after Q_NH_2 formation, but also might explain the difference in the behaviour of bc_1 in the absence and in the presence of antimycin, respectively. In chromatophores, $\Delta\psi$ generation and proton release accelerated after the addition of antimycin, while dramatically diminishing in magnitude. Thereby these reactions proceeded approximately as fast as the reduction of heme b_h.[22,58,69,70,73,82,85,89,95] This kinetic match could not be due just to the oxidation of only one ubiquinol molecule in the presence of antimycin. As noted above, the kinetic discrepancy between the faster heme b_h reduction, from one hand, and the slower cytochrome c_1 re-reduction, $\Delta\psi$ generation and proton release, from the other hand, was still observed under single-turnover conditions, as created (in the absence of antimycin!) by ubiquinol shortage or weak flashes.[22,53,69,70,112,114] Most likely,

¶ The expulsion of non-compensating, redundant proton(s) out of center *P*, even *via* a short dielectric distance, can be highly electrogenic. By polarizing water molecules, a non-compensated charge can decrease, up to ~5, the dielectric permittivity inside a water-filled cavity intruding in a hydrophobic medium.[158]

the turnover of center *P* proceeds differently in the absence of antimycin and in its presence. It is known that antimycin affects the mechanistic properties of bc_1 and, in particular, the interaction of the Rieske protein with the cytochrome *b* (see ref. 32, 147 and citations therein). As argued elsewhere,[26,53,69,70] it is thinkable that antimycin, by disrupting the conformational connection between centers *N* and *P*, enables the fast re-location of the reduced FeS domain towards cytochrome c_1. The observation of Cooley and co-workers on the increased mobility of the reduced FeS domain in the presence of antimycin[146] is compatible with this suggestion.

In this framework, it seems plausible that the electrogenic reaction, as observed in the presence of antimycin, is mostly due to the unconstrained and fast (due to the increased mobility of the FeS domain) proton release from center *P*, while the ET to heme b_h is electrically silenced. This suggestion is supported by the relatively small extent of voltage generation in the presence of antimycin,[58,73,85,88,89] by the ability of bc_1 of *Rb. sphaeroides* to generate $\Delta\psi$ even in the absence of heme b_h,[96] and by the kinetic correspondence between proton release into the lumen of *Rb. capsulatus* and $\Delta\psi$ generation, when measured in the presence of antimycin.[69]

3.2.3 Proton binding and release. With structural data in hand, it seems useful to revisit some open problems related to the $\Delta\psi$ generation by bc_1. The crystal structures show heme b_h next to Q_N,[14,28] so that the ET from heme b_h to Q_N is expected to be insensitive to $\Delta\psi$. Therefore the "reverse" electrogenic reaction, as observed upon heme b_h reduction *via* center *N*,[73,88] cannot be due to the transmembrane ET from Q_NH_2 to heme b_h and has to be explained anew. On one hand, the oxidation of ubiquinol in center *N* has to be accompanied by electrically "reverse" proton release to the *n*-side of the membrane, as suggested earlier.[58,59] On the other hand, it is plausible that the reduction of heme b_h *via* center *N* might cause protein rearrangement accompanied by charge displacements, in particular, along the water chain connecting the heme b_l with the aqueous *p*-phase. In the presence of myxothiazol, a minor luminal alkalisation in response to the flash-induced heme b_h reduction *via* center *N* could be revealed by using NR as a pH-dye.[69]

The anomalous absence of proton binding by bc_1 under oxidizing conditions,[56,105–107] as discussed in section 3.1.2, might be due to the pH-dependence of E_m of heme b_h.[51] Because of this feature, the flash-induced turnover of bc_1 should be accompanied by proton trapping from the *n*-side both in the absence of antimycin and in its presence. In the latter case, protons would be taken by ionizable groups next to heme b_h. Hence, the −/+ antimycin difference might diminish to zero under certain conditions. The increase in proton binding after the addition of valinomycin[56,105–107] might reflect the drop in the $\Delta\tilde{\mu}_{H^+}$ backpressure. When the flash-triggered proton release into the chromatophore lumen was measured by NR, the addition of valinomycin doubled the NR response.[68] Hence, $\Delta\tilde{\mu}_{H^+}$, as generated in response to a single saturating flash (>100 mV[129]), was large enough to block the turnovers of bc_1 beyond the first one. In view of these data, the amount of protons trapped in the absence of antimycin should approx. double after the valinomycin addition, while the proton binding by heme b_h in the presence of antimycin should stay independent of valinomycin. As a result, the extent of −/+ antimycin proton binding should increase in the presence of valinomycin, in agreement with experimental observations.[56,105–107] As well, a gradual elevation of the ubiquinol/ubiquinone ratio should increase the number of bc_1 turnovers by raising the reaction driving force. Accordingly, the extent of the antimycin-sensitive proton binding should increase with lowering of E_h, also in agreement with experimental observations.[56,105–107]

Still, to adequately measure the proton trapping by bc_1, one has to compare the intact enzyme with one that is "doubly killed" by the addition of antimycin and myxothiazol. These experiments have to be carried yet.

4. Activated Q-cycle as a consistent mechanism of the cytochrome bc_1 complex

The different facets of proton translocation by the cytochrome bc_1 complex are brought together in Fig. 4. This scheme can be denoted as activated Q-cycle where the cytochrome bc_1 complex enters the catalytic cycle being in an "activated," pre-reduced $Q_N^{\bullet-} b_h^{ox} \mid b_h^{red}$ state, as shown on panel A. As long as ubiquinol is present in the membrane, the activated state is maintained by steady ubiquinol oxidation *via* center *N*, according to eqn (3), yielding a bound semiquinone in this center and a reduced high-potential heme *b* in the other monomer of the enzyme. The almost isoenergetic pre-activation of bc_1 enables the formation of Q_NH_2 upon each turnover of center *P* as described by eqn (4). Otherwise, if bc_1 is not activated (that could happen under non-physiological, oxidizing conditions), the formation of ubiquinol in center *N* requires not one, but two turnovers of center *P*, according to the bicycle mechanism of Crofts *et al.*[4,82] and Garland *et al.*,[83] see eqn (1) and (2). For oxidizing conditions, a tentative scheme of a catalytic cycle in a dimeric bc_1 has been presented elsewhere.[22]

Upon designing the steps of quinone/quinol transfer inside bc_1, as tentatively depicted in Fig. 4, the aim was to minimize the exchange reactions with the membrane quinone pool. Quite unexpectedly, a scheme was obtained where the external ubiquinol enters the catalytic cycle by binding in center *N* (upon the G → H transition), but not in center *P*, as commonly believed. Ubiquinol binding is followed by its oxidation and formation of the $Q_N^{\bullet-} b_h^{ox} \mid b_h^{red}$ state (H → A transition in Fig. 4). The center *P*, in turn, is steadily supplied by ubiquinol molecules coming from center *N*, as shown in Fig. 4 (this possibility has been earlier considered by Rich[148]); it is not obligatory for external ubiquinol molecules to penetrate towards center *P* to be oxidized. This rather odd view on the Q-cycle mechanism is consistent with the higher accessibility of center *N* in the crystal structure[10] and with otherwise peculiar observations that (i) ubiquinol binds in center *N* of *Rhodobacter* by order of magnitude tighter than ubiquinone[84] although center *N* is believed to be the quinone-reducing one; (ii) external ubiquinol molecules interact with center *N* faster than with center *P* in chromatophores of *Rb. sphaeroides*;[59,90,91] (iii) ubiquinone is seen bound in center *N* but not in center *P* in the crystal structures.[5,14,15,28]

At least in one point the presented model carries more resemblance with the original Q-cycle scheme of Mitchell[20] than with its later modifications. Mitchell has considered the possibility of ubiquinol formation in center *N* already after the first turnover of center *P*.[20] As already noted in section 3.2.2.1, he has hypothesised that one electron comes to Q_N from cytochrome *b* while the other

one is donated by some different enzyme.[20] The activated Q-cycle in Fig. 4 implies, similarly to the original Mitchell's scheme,[20] that the oxidation of each ubiquinol in center *P* can be followed by ubiquinol production in center *N*. The mechanism, however, differs from that suggested by Peter Mitchell: according to the scheme in Fig. 4, the *other* electron(s) wait(s) *inside* the dimeric bc_1 after its redox equilibration with the ubiquinol pool *via* center *N*.

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