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Rapid report

Flash-induced turnover of the cytochrome bc_1 complex in chromatophores of *Rhodobacter capsulatus*: binding of Zn^{2+} decelerates likewise the oxidation of cytochrome *b*, the reduction of cytochrome c_1 and the voltage generation

Sergey S. Klishin [a,b], Wolfgang Junge [a], Armen Y. Mulkidjanian [a,b,*]

[a] *Division of Biophysics, Department of Biology and Chemistry, University of Osnabrück, D-49069 Osnabrück, Germany*
[b] *A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119899 Moscow, Russia*

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Abstract

The effect of Zn^{2+} on the rates of electron transfer and of voltage generation in the cytochrome bc_1 complex (bc_1) was investigated under excitation of *Rhodobacter capsulatus* chromatophores with flashing light. When added, Zn^{2+} retarded the oxidation of cytochrome *b* and allowed to monitor (at 561–570 nm) the reduction of its high potential heme b_h (in the absence of Zn^{2+} this reaction was masked by the fast re-oxidation of the heme). The effect was accompanied by the deceleration of both the cytochrome c_1 reduction (as monitored at 552–570 nm) and the generation of transmembrane voltage (monitored by electrochromism at 522 nm). At $Zn^{2+} < 100$ μM the reduction of heme b_h remained 10 times faster than other reactions. The kinetic discrepancy was observed even after an attenuated flash, when bc_1 turned over only once. These observations (1) raise doubt on the notion that the transmembrane electron transfer towards heme b_h is the main electrogenic reaction in the cytochrome bc_1 complex, (2) imply an allosteric link between the site of heme b_h oxidation and the site of cytochrome c_1 reduction at the opposite side of the membrane, and (3) indicate that the internal redistribution of protons might account for the voltage generation by the cytochrome bc_1 complex. © 2002 Elsevier Science B.V. All rights reserved.

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Cytochrome bc_1 complexes of animals and bacteria and cytochrome *bf* complexes of plants (hereafter bc_1 and *bf*, respectively) are electrogenic quinol:cytochrome *c* oxidoreductases (see [1,2] for comprehensive reviews). The X-ray structures of mitochondrial bc_1 showed it as a dimer [3–6]. The membrane-embedded core of each bc_1 monomer is the cytochrome *b* that carries the low and high potential protohemes (b_l and b_h, respectively). Each cytochrome *b* is flanked by the iron-sulfur Rieske protein and cytochrome c_1. The latter two subunits are composed of water-soluble, redox center-carrying domains (headpieces) located at the positively charged *p*-side of the membrane and connected by single α-helices to cytochrome *b*. The bc_1 and *bf* complexes are believed to operate by Mitchell's *Q*-cycle mechanism [7]. According to its current version for bc_1 [1,8], quinol molecules are oxidized at the interface between the FeS cluster-carrying domain of the Rieske protein (here-

* Corresponding author, at address a. Fax: +49-541-969-2870.
E-mail address: mulkidjanian@biologie.uni-osnabrueck.de (A.Y. Mulkidjanian).

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after FeS) and heme b_l. At this so-called center *P*, the ubiquinol is likely to bind between His-161 of FeS and Glu-272 of cytochrome *b* (beef numbering of amino acids) [6,9]. The first electron from ubiquinol is accepted by FeS and then passed, via cytochrome c_1, to the water-soluble cytochrome c_2. X-ray studies have revealed that the delivery of the electron to cytochrome c_1 implies a rotation of the FeS headpiece by 70°, from a position where the FeS cluster is close to b_l (the FeS_b state) into the 'cytochrome c_1' (FeS_c) position [3]. The second electron moves across the membrane, via hemes b_l and b_h, to the other quinone-binding center, *N*, where an ubiquinone molecule is reduced to a semiquinone anion $Q_N^{\bullet-}$. Oxidation of the next ubiquinol at center *P* leads, correspondingly, to the formation of the Q_NH_2 ubiquinol at center *N*. Myxothiazol and stigmatellin block the quinol binding and oxidation at center *P*, whereas antimycin A binds close to center *N* and prevents the quinone reduction by b_h [4,6].

The turnover of bc_1 has been resolved into partial steps with chromatophores (intracellular vesicles) of phototrophic bacteria (see [1,8,10] and references therein). In these preparations, a flash of light leads to the charge separation in the photochemical reaction centers (RC). This event, in turn, triggers the redox reactions in bc_1 that can be monitored optically. The accompanying generation of the transmembrane electric potential difference ($\Delta\psi$) is usually traced via electrochromic changes of native carotenoids.

After the transmembrane location of two hemes in cytochrome *b* was revealed by molecular modeling [11,12], it was customary to think that the transmembrane electron transfer (ET) from b_l to b_h is the main electrogenic reaction in bc_1. Contrary to this expectation, the onset of the (partial) reduction of b_h in chromatophores of *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* was found to be distinctly faster than the onset of the voltage [13–16]. The latter kinetically followed, instead, the proton release into the chromatophore lumen and the reduction of cytochrome c_1 by FeS. Based on these observations, it has been suggested that the electrically unfavorable ET towards heme b_h is compensated by the redistribution of protons [13]. In this notion, the voltage generation was caused by the uptake/release of protons linked to the oxidation of b_h and to the reduction of cytochrome c_1 (see [13–16] for further details).

This concept of an electron/proton coupling in bc_1 has been supported by studies on *bf* in plants. Mutations of amino acid residues in cytochrome *f*, the analogue of cytochrome c_1, retarded both the reduction of cytochrome *f* and the generation of the transmembrane voltage without affecting the rate of cytochrome *b* reduction. Thereby the latter reaction was distinctly faster than the former two [17]. The linkage of the main electrogenic reaction in the bc_1 with the *oxidation* of cytochrome *b* (and not with its reduction) is also in line with a wealth of classical data on bc_1 operation in mitochondria, where the membrane potential blocks the oxidation of cytochrome *b* but not its reduction (see [18,19] for reviews).

The cited data on the operation of bc_1 in *Rb. sphaeroides* and *Rb. capsulatus* were obtained with the ubiquinone pool oxidized. In living bacterial cells the ubiquinone pool is usually half-reduced [20]. Under these more physiological conditions, however, the rate of cytochrome *b* oxidation is faster than those of its reduction, so that the flash-induced redox changes of cytochrome *b* can be neither detected nor compared with the $\Delta\psi$ generation.

Zn^{2+} is a well established inhibitor of the mitochondrial bc_1 [21,22]. Here we tested the effects of Zn^{2+} on the turnover of bc_1 in chromatophores of *Rb. capsulatus* under reducing conditions. Fig. 1 shows the effect of 50 μM Zn^{2+} on the flash-induced redox changes of heme b_h (as monitored at 561–570 nm), of the 'total' cytochrome *c* (cytochromes c_1+c_2, 551–570 nm), and on the $\Delta\psi$ generation (as monitored via electrochromic carotenoid band shift at 522 nm; the fast, here unresolved voltage jump reflected the charge separation in the RC, whereas the slower voltage onset was due to the turnover of bc_1). The respective traces were obtained in the absence of inhibitors of bc_1 (*con*, open circles), in the presence of Zn^{2+} (*Zn*, thick lines), of Zn^{2+} and antimycin A (*ant*, thin lines, oxidation of b_h was completely blocked), and of Zn^{2+}, antimycin A and myxothiazol (*myx*, dots, bc_1 switched off at both sides). To partly reduce the ubiquinone pool in our KCN-treated, but aerated chromatophore samples (as it was checked from the kinetics and extent of the carotenoid band shift, cf. [8]) we used the succinate/fumarate redox buffer in a ratio of 1:1.

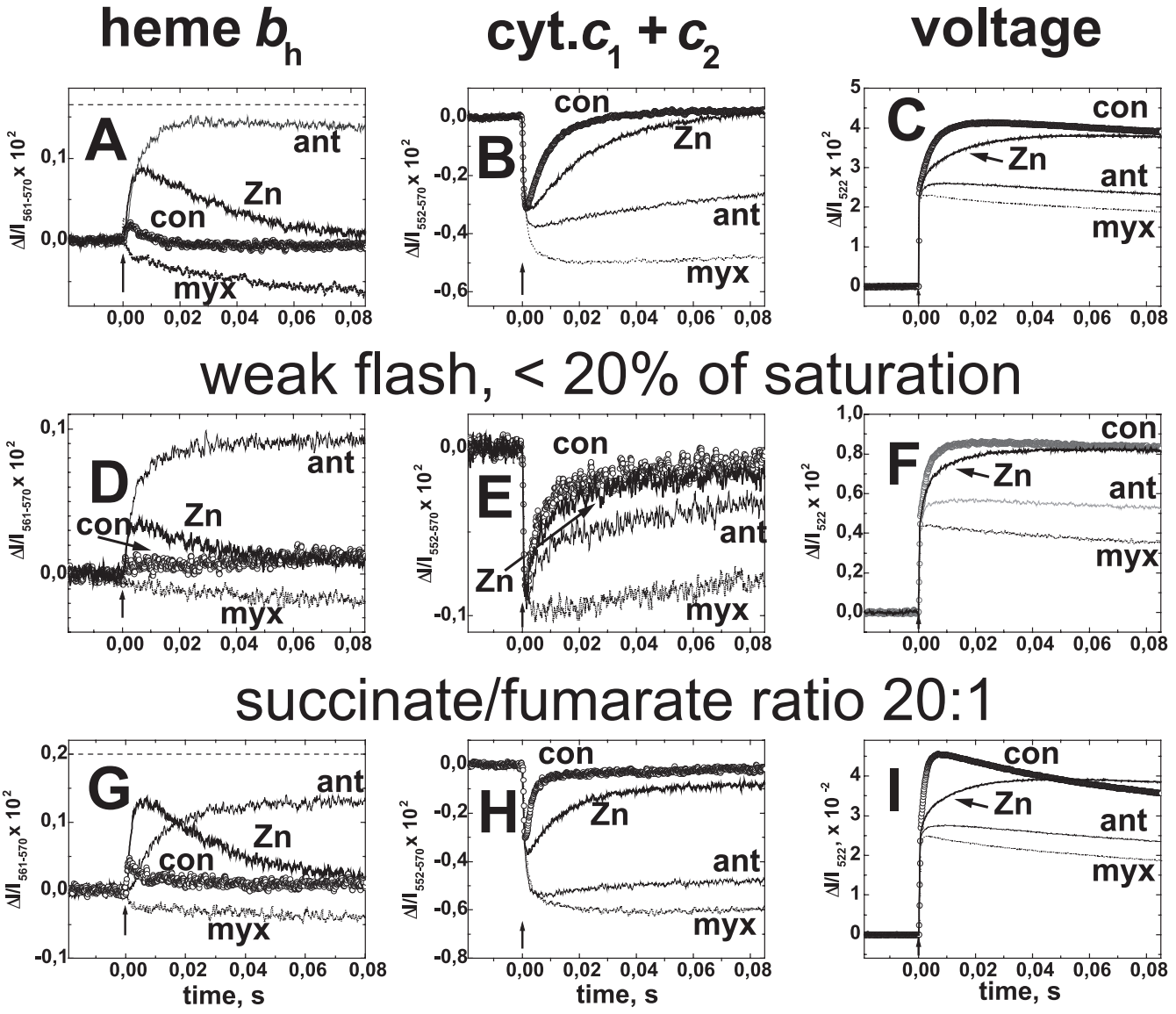

Fig. 1. The effect of Zn^{2+} on the turnover of bc_1 in chromatophores of *Rb. capsulatus*. Symbol code: circles, *con*trol traces; thick line, +50 μM *Zn*SO_4; thin line, +50 μM $ZnSO_4$, +5 μM *ant*imycin A; dots, +50 μM $ZnSO_4$, +5 μM antimycin A, +5 μM *myx*othiazol. Arrows indicate the light flashes. Incubation medium for A–F: 25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 5 μM TMPD, 5 μM oligomycin, 2 mM KCN, 2 mM potassium succinate, 2 mM potassium fumarate. In G–I 8 μM methylene blue was added as an additional redox mediator and 100 μM potassium fumarate were used instead of 2 mM. The dashed lines in A and G indicate the total amount of heme b_h reduced in the presence of antimycin A (as estimated after two saturating light flashes on an aerated sample at the succinate/fumarate ratio of 1:1 for each batch of chromatophores; note that at the succinate/fumarate ratio of 20:1 (G) the b_h heme seemed to be partially pre-reduced in the dark). Procedures of cell growing (*Rb. capsulatus*, strain B10), chromatophore isolation and spectrophotometric measurements are described in [16]. Repetitive signals measured at 0.08 Hz were averaged eight times for voltage traces and 16 times for heme b_h and cytochrome *c* traces, respectively. The voltage traces were followed at 522 nm. The absorbance transients of heme b_h, $\Delta A_{561-570}$, were obtained as $=\Delta A_{561}-1.08\times\Delta A_{570}$ (to normalize the contribution of P^+ on these wavelengths)$-0.15\times\Delta A_{552-570}$ (to account for the spectral contribution of cytochromes *c* at 561 nm)$-0.01\times\Delta A_{522}$ (to account for the spectral contribution of the electrochromic band shift). Correspondingly, the redox changes of the total cytochrome *c*, $\Delta A_{552-570}$, were measured as $\Delta A_{552-570}=\Delta A_{552}-1.34\times\Delta A_{570}-0.04\times\Delta A_{522}$.

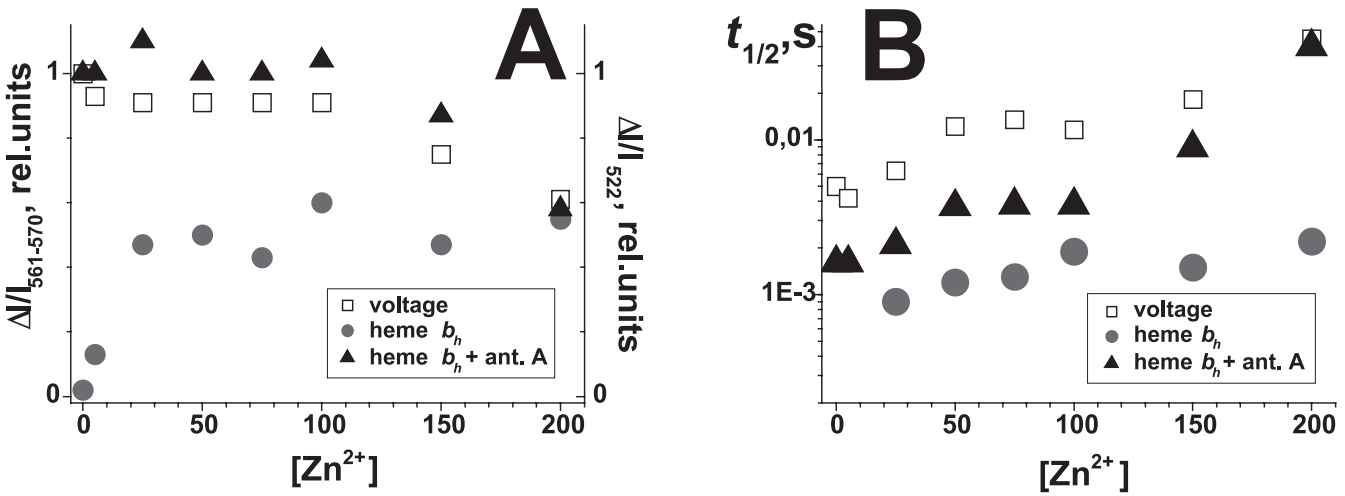

Fig. 2. Dependence of the partial reactions in bc_1 of *Rb. capsulatus* on the concentration of Zn^{2+}. ●, heme b_h reduction in the absence of antimycin A; ▲, heme b_h reduction in the presence of antimycin A; □, electrogenic reaction in the bc_1 as determined by subtracting the voltage transients obtained in the presence of antimycin A and myxothiazol from those measured in the absence of these inhibitors, cf. Fig. 1. Experimental conditions as in Fig. 1A–G. For different Zn^{2+} concentrations different samples were used. The size of the symbols corresponds roughly to the error of the measurement in each particular sample (cf. the noise of the signals in Fig. 1A–C). (A) Relative extents of the partial reactions of bc_1 reached at 300 ms time interval after the flash. In the case of heme b_h reactions, the amount of heme b_h reduced after a saturating flash in the presence of antimycin A and in the absence of Zn^{2+} (see the thin line in Fig. 1A) was taken as 1 (the left *Y*-axis). In the case of the voltage, the extent of electrogenic reaction as measured in the absence of Zn^{2+} was taken as 1 (the right *Y*-axis). (B) Half-times of the same partial reactions.

From the kinetic traces in Fig. 1 and from the respective concentration dependence in Fig. 2 one can see that Zn^{2+}, being added at <100 μM, (i) increased the amount of heme b_h reduced after the flash and (ii) likewise slowed down the generation of $\Delta\psi$ and the cytochrome c_1 reduction. When added above 100 μM, Zn^{2+} caused diverse effects: besides slowing dramatically the heme b_h reduction in the presence of antimycin A (see Fig. 2), it even retarded the cytochrome *c* oxidation by the RC (not documented). By analogy with the effect of other divalent cations on bc_1 and *bf* [22,23], we assume a multiple and unspecific Zn^{2+} binding when added at a concentration >100 μM. Therefore we focus below on the effects of <100 μM Zn^{2+}. Fig. 1D–F show the effect of 50 μM Zn^{2+} under conditions where the intensity of the actinic flash was attenuated, so that only 1/5 of the RC were excited and only about half of bc_1 turned over (the RC:bc_1 ratio was approx. 3:1 in our chromatophores). Under such single turnover conditions, the reduction of cytochrome c_1 still lagged behind b_h reduction and correlated with the $\Delta\psi$ generation. According to Fig. 1G–I the effect of 50 μM Zn^{2+} increased upon further reduction of the ubiquinone pool, i.e. at a succinate/fumarate ratio of 20:1. It is noteworthy that the kinetic traces obtained in the presence of myxothiazol alone coincided with those obtained in the presence of myxothiazol and antimycin A (not documented). This observation ruled out the possibility of a heme b_h reduction via center *N* after a flash in the presence of Zn^{2+}.

Our data revealed that the presence of Zn^{2+} introduced a new bottleneck in the oxidoreduction of bc_1. The bottleneck was more pronounced at higher quinol/quinone ratio (cf. the top and bottom rows in Fig. 1). The binding of Zn^{2+} retarded the oxidation of heme b_h, causing its 'over-reduction'. In contrast to the complete block of heme b_h oxidation by antimycin A, the Zn^{2+}-imposed block was leaky. The bc_1 remained functional, and the extent of the transmembrane voltage was similar in the presence and absence of Zn^{2+} (<100 μM). Thereby the onset of heme b_h reduction in the presence of different Zn^{2+} concentrations was approx. 10 times faster that the reduction of cytochrome *c* and the onset of the voltage. Such a kinetic discrepancy might have been due to multiple bc_1 turnovers contributing to $\Delta\psi$ generation and cytochrome c_1 reduction. This was checked

by using an attenuated flash of light that allowed a single turnover only of some bc_1 complexes. Under these conditions the kinetic discrepancy remained (see Fig. 1D–F). It is noteworthy that the rate of cytochrome *c* reduction and of voltage generation correlated with those of heme b_h oxidation (this is most clearly seen in Fig. 1G–I). In our opinion, these observations present the most dramatic evidence in favor of our earlier suggestion on the electrical silence of heme b_h reduction. Instead the electrogenicity seems to correlate with the heme b_h oxidation and the reduction of cytochrome c_1 [13–16].

Why is the reduction of heme b_h non-electrogenic? We weigh currently the following possibilities. (A) The reduction of b_l can be coupled with its protonation from the lumen, as discussed in [6]. Such a proton displacement (along a water chain as revealed by the X-ray structure [6]) would be especially favorable under coupled conditions (the proton release from center *P* seems to be electrogenic [24]). (B) The ET between b_l and b_h might be electrically silenced by proton redistribution in the space between two hemes. Some non-crystallizable water is expected to be present in this space to allow ubiquinone exchange between centers *P* and *N*. The protonic relaxation might serve as a prerequisite for the ET via heme b_l to heme b_h [25] and could compensate this ET electrically.

But how can the electrogenic reaction be kinetically coupled with the reduction of cytochrome c_1? The rotary mobility of the FeS domain might provide an answer. According to various estimates, the intrinsic rate constants of both the $FeS_b \rightarrow FeS_c$ movement and the reduction of cytochrome c_1 by FeS_c are $\geq 10^5$ [1,26]. The slower cytochrome c_1 reduction already on the first bc_1 turnover (as compared to the cytochrome *b* reduction, see Fig. 1D–F) indicates that the electron released by ubiquinol 'sticks' for milliseconds at FeS_b (see also [15,22]). Most likely, the $FeS_b \leftrightarrow FeS_c$ equilibrium remains strongly shifted to the left after ubiquinol oxidation (say, with $K_{eq}^{FeS} = [FeS_b]/[FeS_c] > 100$). The absence of a fast electrogenicity indicates that both protons, which are released at the oxidation of ubiquinol, remain bound to FeS and cytochrome *b*, respectively. The observed kinetic coupling between the oxidation of b_h, the reduction of cytochrome *c* and the generation of $\Delta\psi$ could be rationalized by the *single* assumption that the protein reorganization coupled to a redox event in center *N* (e.g. to a reduction of Q_N to a $Q_N^{\bullet-}$ semiquinone by heme b_h) allosterically promotes the $FeS_b \rightarrow FeS_c$ transition. This assumption finds support in a recent observation that in the bc_1 of *Rb. sphaeroides* the binding of antimycin A (a semiquinone analogue [27]) to center *N* shifts the $FeS_b \leftrightarrow FeS_c$ equilibrium to the right [28][1]. If our assumption is correct, then the oxidation of heme b_h leads to the following events (see [29] for a hypothetical structural scheme): (i) the reduced and protonated FeS domain moves towards cytochrome c_1, reduces the latter and releases its proton; (ii) the proton(s), which were electrostatically compensating the electron at cytochrome *b*, are electrogenically released at the *p*-side as well; (iii) quinone reduction in center *N* leads to the proton binding from the *n*-side. Hence, several electrogenic proton transfer reactions, *all* of them kinetically coupled with the oxidation of heme b_h, might contribute to the voltage generation by bc_1.

Two Zn^{2+}-binding sites have been revealed in the X-ray structure of mitochondrial bc_1, both close to center *P* [30]. The suggested conformational coupling allows to understand how the binding of zinc ion(s) close to center *P* could retard the oxidation of heme b_h in center *N*. Zn^{2+} can decrease the mobility of the FeS headpiece and/or block the proton release channel(s). Studies of the mutants with replaced putative Zn^{2+} ligands might help to discriminate between these possibilities.

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[1] Not surprisingly, in the presence of antimycin A the reduction of cytochrome c_1 is much faster than in its absence and proceeds at < 100 μs (see [1] and references therein).

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