Rapid report

Flash-induced turnover of the cytochrome bc1 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

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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\(^{2+}\) \(\sim\) 100 \(\mu\)M 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. ß 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 reductases (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 a-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-
after FeS) and heme $b_1$. 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 $b_1$ (the FeS$_b$ state) into the ‘cytochrome $c_1$’ (FeS$_c$) position [3]. The second electron moves across the membrane, via hemes $b_1$ and $b_h$, to the other quinone-binding center, $N$, where an ubiquinone molecule is reduced to a semiquinone anion $Q_N^-$. Oxidation of the next ubiquinol at center $P$ leads, correspondingly, to the formation of the $Q_S$H$_2$ 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 phototropic bacteria (see [1,8,10] and references therein). In these preparations, a flash of light leads to the charge separation in the photosynthetic 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 $b_1$ 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 $hf$ 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 *Rh. sphaeroides* and *Rh. 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 *Rh. capsulatus* under reducing conditions. Fig. 1 shows the effect of 50 $\mu$M $Zn^{2+}$ 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 $b_{c_{1}}$ in chromatophores of *Rb. capsulatus*. Symbol code: circles, control traces; thick line, +50 μM ZnSO$_4$; thin line, +50 μM ZnSO$_4$ +5 μM antimycin A; dots, +50 μM ZnSO$_4$, +5 μM antimycin A, +5 μM myxothiazol. Arrows indicate the light flashes. Incubation medium for A–F: 25 mM HEPES, pH 7.5, 5 mM MgCl$_2$, 5 mM 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 $\Delta A_{552-570} = \Delta A_{552} - 1.34 \times \Delta A_{570} - 0.04 \times \Delta A_{522}$. 

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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 $\mu$M, (i) increased the amount of heme $b_h$ reduced after the flash and (ii) likewise slowed down the generation of $v_i$ and the cytochrome $c_1$ reduction. When added above 100 $\mu$M, 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 $b'f$ [22,23], we assume a multiple and unspecific Zn$^{2+}$ binding when added at a concentration >100 $\mu$M. Therefore we focus below on the extent 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 $\mu$M, (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 $\mu$M, 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 $b'f$ [22,23], we assume a multiple and unspecific Zn$^{2+}$ binding when added at a concentration >100 $\mu$M. Therefore we focus below on the effects of <100 $\mu$M Zn$^{2+}$. Fig. 1D–F show the effect of 50 $\mu$M 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 $\mu$M 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 $\mu$M). 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 bc1 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 silencing 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 $b_h$ 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 $b_h$ 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 $b_h$ 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$_b$→FeS$_c$ movement and the reduction of cytochrome $c_1$ by FeS$_c$ are $\geq 10^4$ [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$_b$↔FeS$_c$ equilibrium remains strongly shifted to the left after ubiquinol oxidation (say, with $K^{\text{eq}}_{\text{FeS}} = [\text{FeS}_b]/[\text{FeS}_c] > 100$). 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$_N^-$ semiquinone by heme $b_h$) allosterically promotes the FeS$_b$→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$_b$↔FeS$_c$ equilibrium to the right [28]. 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 $\mu$s (see [1] and references therein).
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