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Sergey A. Siletsky, Ilya Belevich, Nikolay P. Belevich, Tewfik Soulimane, Mårten Wikström

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Time-resolved generation of membrane potential by $ba_3$ cytochrome $c$

oxidase from *Thermus thermophilus* coupled to single electron injection into the $O$

and $O_H$ states

Sergey A. Siletsky$^{a*}$, Ilya Belevich$^{c1}$, Nikolay P. Belevich$^{c}$, Tewfik Soulimane$^{b}$ and Märten

Wikström$^{c}$

$^a$Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University,

Moscow, Russian Federation

$^b$Department of Chemical Sciences and Bernal Research Institute, University of Limerick,

Ireland

$^c$Helsinki Bioenergetics Group, Institute of Biotechnology, P.O. Box 65, FI-00014, University of

Helsinki, Finland

1 Present address: Electron Microscopy Unit, Institute of Biotechnology, P.O. Box 56, FI-00014,

University of Helsinki, Finland

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* To whom correspondence should be addressed: Tel: +7 (495) 9395549 Fax: +7 (495) 9390338;
E-mail: siletsky@genebee.msu.su

Abbreviations: BNC, binuclear heme $a_3/Cu_B$ center; PLS, proton loading site; Rubpy, tris(2,2’-
bipyridyl) ruthenium; Tris, tris(hydroxymethyl)aminomethane; $\tau$, time constant; $\Delta\Psi$,
transmembrane electric potential difference; DM, (dodecyl L-D-maltoside).

**Running Title:** The $O\rightarrow E$ and $O_H\rightarrow E_H$ transitions of $ba_3$ from *T.thermophilus*
Summary

Two electrogenic phases with characteristic times of ~14 μs and ~290 μs are resolved in the kinetics of membrane potential generation coupled to single-electron reduction of the oxidized "relaxed" O state of ba₃ oxidase from T. thermophilus (O→E transition). The rapid phase reflects electron redistribution between Cu_A and heme b. The slow phase includes electron redistribution from both Cu_A and heme b to heme a₃, and electrogenic proton transfer coupled to reduction of heme a₃. The distance of proton translocation corresponds to uptake of a proton from the inner water phase into the binuclear center where heme a₃ is reduced, but there is no proton pumping and no reduction of Cu_B. Single-electron reduction of the oxidized "unrelaxed" state (O_H→E_H transition) is accompanied by electrogenic reduction of the heme b/heme a₃ pair by Cu_A in a "fast" phase (~22 μs) and transfer of protons in "middle" and "slow" electrogenic phases (~0.185 ms and ~0.78 ms) coupled to electron redistribution from the heme b/heme a₃ pair to the Cu_B site. The "middle" and “slow” electrogenic phases seem to be associated with transfer of protons to the proton-loading site (PLS) of the proton pump, but when all injected electrons reach Cu_B the electronic charge appears to be compensated by back-leakage of the protons from the PLS into the binuclear site. Thus proton pumping occurs only to the extent of ~0.1 H⁺/e⁻, probably due to the formed membrane potential in the experiment.
1. Introduction

The superfamily of terminal heme-copper oxidases includes cytochrome $c$ oxidase (COX) of mitochondria of eukaryotes, heme-copper oxidases of most aerobic prokaryotes, and the structurally related NO-reductases [1-3]. During turnover of these membrane-bound enzymes, electrons are transferred from cytochrome $c$ or quinol from the positive side and protons from the opposite (negative) side of the membrane to the catalytic oxygen reducing centre. As a result, reduction of dioxygen to water is coupled to generation of proton motive force. In addition, the main part of the heme-copper oxidases acquired the unique property of pumping an additional proton across the membrane per $e^-$ transferred to $O_2$ [4] with a doubling of energy-transduction efficiency.

Three major groups (A, B and C-type) of terminal heme-copper oxidases differ mainly in the structure of the input protonic paths [5, 6]. The most studied representatives of the A-type oxidases include the mitochondrial $aa_3$-type cytochrome $c$ oxidases, the bacterial enzymes like $aa_3$ oxidases from Paracoccus denitrificans and Rhodobacter sphaeroides, and the quinol oxidase $bo_3$ from Escherichia coli. In the A-family, two functional proton pathways lead from the N-side surface toward the catalytic site, the D and K channels. The D-channel provides all pumped protons throughout the catalytic cycle and two or three substrate protons [7]. The K-channel has been concluded to conduct only the remaining chemical proton(s) [8]. A third proton pathway (the H-channel) has been proposed to be responsible for transfer of the pumped protons in the mammalian cytochrome oxidase [9, 10], but see [11]; also [12].

The B-type oxidases have low sequence homology with the A-type, but they have a clearly related three-dimensional structure [13]. The most studied member of the B family is cytochrome $ba_3$ from Thermus thermophilus, which catalyses oxidation of a highly specific electron donor, cytochrome $c_{552}$ under conditions of limited oxygen supply. Similar to $aa_3$ oxidases, cytochrome $ba_3$ has four redox centers. The electron-accepting bimetallic copper site (Cu$_A$), and the binuclear center (BNC) composed of a high spin heme $a_3$ with a copper ion
nearby (CuB) are extremely similar for the two types of oxidases, while the electron-transferring low-spin heme $a$ of most A-type oxidases is replaced by heme $b$. The reduced binuclear centre of cytochrome $ba_3$ has several noticeable differences from the $aa_3$ oxidases with respect to interaction with NO and CO [14, 15]. The ferric heme $a_3$ in $ba_3$ oxidase does not react readily with H$_2$O$_2$ or any other typical heme $a_3$ ligands tested (cyanide, azide, sulfide), and the binuclear centre was postulated to be “closed” in the oxidized enzyme, opening up only after one-electron reduction [16].

Although cytochrome $ba_3$ lacks most of the highly conserved amino acid residues that form input proton channels in the A-family oxidases, it nevertheless generates an electric transmembrane potential under steady-state conditions, and pumps protons. In contrast to the A-type oxidases that typically pump 1 H$^+$ per e$^-$ transferred to O$_2$, cytochrome $ba_3$ typically pumps fewer protons per cycle [17-21]. The efficiency of the proton pump was only one half (H$^+/e^-$ $\sim$ 0.5) of that in $aa_3$ oxidase in conditions of saturation of the reaction substrates [17], but was increased up to $\sim$0.85+/0.11 in proteoliposomes when special care was taken to minimize generation of proton-motive force [21]. Out of three potential proton channels resolved in the structure of cytochrome $ba_3$ (D, K and Q channels) only the K-channel homologue has been identified as being involved in the transfer of both substrate and pumped protons [22]. This proton channel is referred to as the K-pathway analog, since it overlaps in space with the K pathway of the A-family oxidases [20, 22]. It remains unclear how the enzyme is able to pump protons and transfer substrate protons to the BNC without a functional D-pathway [23].

To resolve the steps of the oxidative phase of the catalytic cycle, a commonly used approach is to study the reaction of the CO-inhibited fully (four-electron) reduced enzyme with molecular oxygen after laser-induced flash photolysis of CO [24]. The second approach is based on injection of a single electron into the enzyme with help of photo-activated ruthenium derivatives [25-30] or by using a pulsed-radiolysis technique [31, 32]. These approaches can be
combined with time-resolved spectroscopic or electrometric techniques to identify the stages of electron and coupled proton transfers [19, 33, 34]. The electrometric technique has been successfully used to study different types of light-driven proteins [35-37] and different types of terminal oxidases [2, 18, 38-41].

In the catalytic cycle of the typical $aa_3$ cytochrome oxidase the reduced binuclear center (the R state) binds $O_2$ to form a dioxygen adduct (the A state). The O-O bond is broken and dioxygen is reduced by four electrons, apparently in a single reaction step. Three electrons are taken from the binuclear center and a fourth from heme $a$, or alternatively (when heme $a$ is oxidized initially), from a local tyrosine residue yielding the $P_R$ or $P_M$ state, respectively [42]. The $P_R$ state is produced if the reaction starts from the fully reduced state and the enzyme has two additional electrons in Cu$_A$ and heme $a$ before starting the reaction. $P_R$ decays further into the F state coupled to uptake of one proton to the binuclear heme $a_3$/Cu$_B$ site. After arrival of a fourth electron from the Cu$_A$/heme $a$ pair and another proton, the reaction ends with the freshly formed oxidized $O_H$ state, which in the absence of electron donors relaxes into an oxidized ground state $O$ that may not be catalytically relevant.

The oxidative half of the catalytic cycle ($R\rightarrow A\rightarrow P_R\rightarrow F\rightarrow O_H$) is associated with pumping of two protons across the membrane, one pumped proton in each of the A→F and F→O$_H$ transitions [2, 38, 43]. Additionally, each of these two transitions is accompanied by uptake of a “chemical” proton to the BNC from the N phase. Then the cycle continues with the reductive phase, in which two electrons (and two protons) are delivered to the heme $a_3$–Cu$_B$ site to reduce it back to the R state via a one-electron reduced intermediate ($E_H$); and two additional protons are pumped across the membrane [43-45].

Immediate reduction of the resting $O$ state and freshly formed $O_H$ state of $aa_3$ oxidase can be accomplished experimentally using the Rubpy complex [38, 43, 45, 46]. The $O_H\rightarrow E_H$ transition has been shown to result in final electron delivery to the optically invisible Cu$_B$ site, and pumping with a stoichiometry of one proton across the membrane with formation of the
one-electron reduced $E_H$ state \([41, 43, 47]\), whereas the reduction of the “relaxed” state (O) is not accompanied by fast electron transfer into the BNC, and is not linked to proton pumping \([38, 46]\). It is assumed that the transition of state $O_H$ into O could be associated with hydration of BNC \([48]\) or with a slow uptake of a proton in absence of a reductant \([49]\). However the structural difference between the O and $O_H$ states is still not known exactly \([50, 51]\).

The catalytic cycle of representatives of family B cytochrome oxidases has been far less studied. In our previous work on $ba_3$ oxidase from \textit{T. thermophilus} \([19]\), the corresponding intermediates of the binuclear center during single-turnover oxidation of fully-reduced $ba_3$ cytochrome oxidase by molecular oxygen were monitored by time-resolved optical spectroscopy, and individual electrogenic steps were resolved by studying the parallel kinetics of membrane potential generation. Similar to the A-type cytochrome oxidases, the oxidative phase of cytochrome $ba_3$ consists of four catalytic intermediates \([19]\). The chemical nature and the spectral properties of the intermediates, corresponded in time to the three compounds (A, P and $O_H$) and reproduced the general features of the $aa_3$-type oxidases, but the intermediate corresponding in time to the F state has a spectrum identical to that of the P state \([19, 52, 53]\). In addition, it was concluded that the $P_R\rightarrow F$ transition is not coupled to proton pumping, since the electrogeneity of the transition, corresponding in time to the $P_R\rightarrow F$ transition of $aa_3$ oxidase, was smaller than that of the $F\rightarrow O$ transition (~36% vs. 54%) \([19]\). As in the case of $aa_3$ oxidase, the amplitude of the back-flow reaction in cytochrome $ba_3$ was used to estimate the amount of charge transferred through the partial steps of the reaction, and the $F\rightarrow O$ transition was supposed to be as efficient in pumping of proton as the corresponding transition in the A-type cytochrome $c$ oxidases \([19]\).

Later, von Ballmoos \textit{et al.} studied the effect of high pH on the oxidative phase of the catalytic cycle of $ba_3$ oxidase and concluded that for the two electrons coming to the BNC in the $P_R\rightarrow F\rightarrow O$ transitions not two but only one proton is transferred across the membrane \([54]\). According to their interpretation, the first kinetic phase, corresponding in time to the $P_R\rightarrow F$
transition of $aa_3$ oxidases, represents transfer of a proton not into BNC, but into the proton-loading site (PLS), and both the $P_R\rightarrow F$ and $F\rightarrow O$ transitions proceed at the same time in a millisecond phase.

The reductive phase of the catalytic cycle of $ba_3$ oxidase is still poorly understood and a time-resolved study of the charge transfer events is required. Earlier, in an optical study of the $O_H\rightarrow E_H$ transition we have confirmed the presence of both $O_H$ and $O$ intermediates in cytochrome $ba_3$ [55]. In the current work, we have resolved the kinetics of charge translocation and the parallel steps of electron transfer through the redox-centres coupled to the single-electron injection into the oxidized relaxed state (the $O\rightarrow E$ transition) and the nonrelaxed $O_H$ state (the $O_H\rightarrow E_H$ transition) of this enzyme.
2. Material and methods

2.1. Enzyme preparation and reconstitution into phospholipid vesicles.

Cytochrome $ba_3$ was isolated from *T. thermophilus* HB8 cells as described in [56]. The enzyme was reconstituted into vesicles by the Bio-Beads method (SM-2 adsorbent; Bio-Rad, Hercules, CA) as described in [34], except that the concentration of oxidase during reconstitution was increased to 8-10 μM.

2.2. Electron injection.

Single-electron injection into the O state (O→E transition) was carried out in the presence of the photo-activatable dye tris-bipyridyl ruthenium (Rubpy) and its sacrificial donor, aniline [28]. At low ionic strength, Rubpy produces a non-covalently bound complex with $ba_3$ oxidase at close distance to CuA. The reaction was initiated by a laser flash (BrilliantB; Quantel, Les Ulis, France; frequency-doubled YAG, 532 nm, pulse energy 40-120 mJ). A laser flash excites the molecule of Rubpy to an $E_m$ of about ~1.5 V, which then donates an electron to the oxidase in less than 0.5 μs. Aniline reduces the oxidized molecule of Rubpy preventing return of the injected electron, and favoring unidirectional reduction of the oxidase.

The small quantum efficiency of the electron injection assures that either one or no electron is injected [18, 55]. The injected electron stays on the molecule injected without equilibration with other oxidase molecules on the time scale of the measurements. The vectorial charge movements associated with electron injection and the induced proton transfer steps were followed by time-resolved electrometry in phospholipid vesicles inlaid with the enzyme. The results are compared with data on the trajectory of the injected electron through the redox centers of the enzyme monitored by time-resolved optical spectroscopy.

2.3. Time-resolved measurement of electric potential generation.
The development of electric potential across the vesicle membrane was monitored by an electrometric technique [57], as adapted from time-resolved experiments with \textit{aa}_3-type cytochrome oxidase [38, 58, 59]. Details of the sample preparation and the methodology can be found in [19, 34, 41].

2.4. \textit{Time-resolved spectrophotometric measurements.}

Time-resolved multi-wavelength absorption changes were followed by a home-constructed CCD-based instrument, which allows recording of 110 surfaces of absorption changes with a time resolution up to 1 \(\mu\)s between the spectra. The resulting surfaces of optical changes were collected with resolutions of 1 and 16 \(\mu\)s between the spectra and merged together to generate the surface of optical changes for the complete reaction. This setup has been described in detail [43, 47, 55].

The following extinction coefficients for absorption changes in the band of reduced heme \(b\) (560-574 nm) and heme \(a_3\) (614-658 nm) were used: 19000 \(\text{mM}^{-1}\text{cm}^{-1}\) and 6300 \(\text{mM}^{-1}\text{cm}^{-1}\), respectively [55, 60]. The extent of oxidoreduction of Cu\textsubscript{A} was calculated based on the extinction coefficient 810 \(\text{mM}^{-1}\text{cm}^{-1}\) (790-710 nm; deduced from the redox spectrum of the enzyme) [55].

2.5. \textit{Data analysis.}

The experimental kinetics were fitted with the help of a model of sequential (serial) reactions [34, 37, 61]. MATLAB (The Mathworks, South Natick, MA) was used for data analysis and presentation. For comparison with some recent experiments where the fit of the electrometric traces was performed by the sum of the exponential terms [38, 39, 62], the amplitudes of the corresponding components can be obtained by recalculation according to work in [61, 63].
3. Results

3.1. Charge transfer kinetics coupled to the O→E transition

Fig. 1 shows the kinetics of membrane potential generation by cytochrome ba$_3$ from T. thermophilus starting from the oxidized resting state (O). In response to the laser flash, Rubpy injects an electron in a non-electrogenic reaction to the copper center, Cu$_A$. Then, the electron is redistributed between the redox centers according to the intrinsic redox potentials, which is accompanied by generation of an electric potential difference across the membrane of the proteoliposomes in which the ba$_3$ is embedded. To exclude accumulation of the one-electron reduced form of enzyme (E) prior to the electron injection, and in order to prevent a possible photoinduced slow electron current [64], the Rubpy and aniline were added to the sample immediately before the laser flash, and all manipulations were performed in the dark, following similar measurements on aa$_3$ cytochrome oxidase from P. denitrificans [46, 65]).

The generation of ΔΨ corresponds to transfer of negative charge towards the inside of the proteoliposomes, or of positive charge in the opposite direction. The flash-induced photopotential increases with two electogenic phases with characteristic times of ~14 µs and ~290 µs and relative amplitudes of ~56% and 44% of the total response (Fig. 1A, Table 1). This is followed by a passive discharge of the membrane and return of the electric potential to the initial level on the time scale of several seconds (not shown). Approximation of the kinetics of ΔΨ generation with three components does not lead to an improvement in the graph of residuals (see inset in Fig. 1A).

Incubation with ferricyanide before the flash does not lead to noticeable changes of the kinetics of the membrane potential generation (Fig. 1B), which means that the enzyme is fully converted to the oxidized state before the flash in these conditions. Performing a series of laser flashes with intervals of 0.2 s in absence of ferricyanide is accompanied by gradual decrease of the overall amplitude of the response. In addition to the first flash, Fig. 1B shows the electrometric response upon the 10th and 20th laser flash in these conditions. The exposition of
the liposome-reconstituted ba3 oxidase to several laser pulses leads to the appearance of an additional electrogenic phase with \( \tau \sim 950 \mu s \) (\(~10\%\) amplitude after twenty flashes) most likely caused by the accumulation of the single-electron form (state E). In contrast to cytochrome aa3 from \( P.\)denitrificans \([46, 66]\), the changes in response of ba3 oxidase upon multiple flashes are not very significant due to the small quantum yield \([55]\).

In an earlier study, we used optical spectroscopy to resolved the kinetics on the electron transfer through the hemes caused by the electron injection into the O state of ba3 oxidase \([55]\). Now we expanded these results to the near infra-red range to follow CuA for more reliable description of the electron redistribution between the redox-centres. Fig. 2 shows the time-resolved electron transfer through the redox centers measured by optical spectroscopy in response to the injection of an electron into the oxidized O state.

The initial decrease of absorption at 820 nm reflects the reduction of CuA by Rubpy. The rise of absorption after the flash at 820 and 560 nm (\( \tau \sim 11 \mu s \)) corresponds to the fast phase of \( \Delta \Psi \) generation (\( \tau \sim 14 \mu s \)) and reflects electron transfer from CuA to heme b. There is no parallel change of absorbance at the characteristic band of heme a3, i.e. heme a3 is not reduced during the fast reaction component. A subsequent decrease of optical density at 560 nm (\( \tau \sim 230 \mu s \)) correlates with the slow component of the kinetics of membrane potential generation (\( \tau \sim 290 \mu s \)), and corresponds to partial reoxidation of heme b (Table 1). In parallel, there is an increase of the absorption band of reduced heme a3 (614 nm minus 658 nm), and in the near infrared band of oxidized CuA (Fig. 2, Table 1). Thus the slow component of \( \Delta \Psi \) generation takes place concomitantly with partial reduction of heme a3 by heme b and CuA.

Based on the extinction coefficients for reduction of heme b and heme a3 are 19000 (see section 2.4.) \(~0.48 \mu M\) of heme b is reduced during the fast phase. In the slow component, reduction of \(~0.22 \mu M\) of heme a3 is accompanied by oxidation of \(~0.11 \mu M\) heme b, while the remaining part of the reducing equivalents for reduction of heme a3 is provided by the oxidation of CuA. This is in agreement with the equilibrium electron redistribution between heme b and
heme $a_3$ at the end of the slow phase reported earlier ($K_{eq}=0.37$, [32, 55]). There is no indication of electron transfer to Cu$_B$ during this transition.

3.2. Charge transfer kinetics coupled to the $O_H\rightarrow E_H$ transition

Before starting measurements, the enzyme was initially fully reduced in the presence of carbon monoxide to form a stable CO complex of heme $a_3$. After that, a pulse of oxygen-saturated water was added to the reaction cell, immediately followed by a series of laser flashes with a frequency of 5 Hz. Upon the first laser flash, the enzyme enters into the oxidative phase of the catalytic cycle and the fully oxidized $O_H$ state forms, as described earlier [43, 45, 55]. Subsequent flashes at intervals of 200 ms excite Rubpy and initiate the $O_H\rightarrow E_H$ transition. The resulting amplitude of the photoelectric response is considerably smaller than after the first flash (not shown) due to the low quantum efficiency of the electron injection. For the same reason, the kinetics of membrane potential generation were similar in shape and amplitude for tens of flashes fired subsequently.

Fig. 3 shows a typical photoelectric response coupled to the electron injection from Rubpy into the activated $O_H$ state. The experimental trace of the kinetics is shown together with a fit to the data (Fig. 3, main panel). The kinetics of generation of $\Delta \Psi$ can be reasonably modeled with three components: "fast", "middle" and "slow" with the time constants (inverse rate constants) of 22 $\mu$s, 185 $\mu$s and 790 $\mu$s, respectively, and relative amplitudes of ~33%, 42% and 25% (Table 2). An approximation with two components yields a much worse fit (Fig. 3 inset), whereas a fit with four transitions does not lead to an improvement in the graph of residuals (not shown).

The typical values of the amplitude of the photoelectric response were in the range of 1.5-2.5 mV, which is about 1.5-2 times higher than for the injection into the O state. The estimated quantum yield of electron injection into both $O_H$ and O states was approximately 2.5%, which is several times smaller than for cytochrome $aa_3$ from $P.denitrificans$ in the same
conditions [43]. This fact can probably be explained by a lower affinity of Rubpy to the cytochrome c552 binding site in ba3 oxidase, where binding to c552 is based mainly on hydrophobic interactions.

In order to describe the nature of the charge transfer events in each phase it is necessary to compare the electrometric data with the kinetics of electron transfer. Fig. 4A shows the kinetics of the optical changes. The traces at 560 nm minus 574 nm, and 614 nm minus 658 nm show reduction and subsequent reoxidation of heme b and heme a3, respectively, whereas the trace at 830 nm reflects reduction of CuA by Rubpy (initial decrease of absorption) followed by increase of absorption due to its subsequent oxidation by heme b.

Due to the much better signal-to-noise ratio, the electrometric data appears more reliable than the light absorption data with respect to the number of involved kinetic components. Moreover, major electrogenic charge transfer steps may be accompanied by weakly manifested changes in the optical spectra [39]. Therefore, we have analyzed the surface of optical absorbance changes based on the kinetic data from measurements of the electrogenic steps. Fig. 4B shows corresponding transient spectra of three kinetic components. The results are in rough agreement with earlier conclusions [55].

The spectrum of the fast phase has sharp peaks at 561 nm and 611 nm due to reduction of heme b, and heme a3, respectively, and a wide peak centered at 810 nm that can be assigned to oxidation of CuA (Fig. 4B) [67]. The ratio of amplitudes at the characteristic wavelengths shows that the electron is fully transferred from CuA and redistributed between hemes b and a3 in proportions of 55% and 45%, respectively. In complete contrast to electron injection into the O state (see above; [55]), the fast phase of membrane potential generation thus correlates with reduction of both hemes b and a3.

The spectrum of the second component (the "middle" phase) suggests a small (~10%) reoxidation of heme b by CuB (since there is no discernible reduction of heme a3). However, this phase makes maximum contribution to the generation of membrane potential (Table 2). The
spectrum of the "slow" phase includes troughs at 560 and 613 nm reflecting reoxidation of both hemes by CuB, which is now the final acceptor of the injected electron. It is evident that during this phase, both hemes $a_3$ and $b$ are fully reoxidized with parallel kinetics (Fig. 4A). Noticeably, in case of the 3-component fit, the spectrum of the slow phase contains the well-defined charge transfer band (with maximum around 660 nm), that is more symmetrical now (as compared to [55]), and is very close to the band in the stationary spectrum of the oxidized heme $a_3$. In contrast to the O state, the absorbance at 830 nm is not changed in the submillisecond time range during the $O_{H} \rightarrow E_{H}$ transition (Fig. 4), which confirms involvement of CuA in the reaction only during the fast phase (Table 2).

The electron transfer from heme $b$ to the BNC occurs parallel to the plane of the membrane and does not contribute to the electrogenicity. Hence, the fast electrogenic component is associated with the electron transfer from CuA to the heme $b$/heme $a_3$ pair, while the middle and slow components reflect electrogenic proton translocation that is coupled to oxidation of heme $b$ and heme $a_3$ by the CuB site. For comparison, Table 2 also shows the corresponding data for the $aa_3$ oxidase from $P.\text{denitrificans}$. As one can see, the main differences of the $ba_3$ oxidase from the $aa_3$ are that: 1) electron transfer in the fast phase occurs to both hemes, 2) the electrogenic fast phase of electron transfer from CuA to the inside of the protein has a much greater contribution in the $ba_3$ oxidase, and therefore the total contribution of the other phases associated with the transfer of protons, is significantly smaller, 3) an additional electrogenic phase (with $\tau \sim 2.5$ ms), reflecting the release of a proton to the P side, is not observed in $ba_3$. 
4. Discussion.

4.1. Charge transfer kinetics coupled to the $O \rightarrow E$ transition

It is known that the resting fully oxidized (as isolated) O-state of $aa_3$ cytochrome oxidase is not competent in transmembrane proton pumping and rapid electron transfer. With cytochrome $aa_3$ from $P. denitrificans$ (and from bovine [28, 38]) it was shown that the fast phase in the electron injection experiment consists only of electron transfer from Cu$_A$ to the low-spin heme, without any reduction of the high-spin heme, which occurs much later in time (Figure 5, [28, 46, 65, 68]).

In contrast, injection of an electron into the fully oxidized resting state O of $ba_3$ oxidase leads not only to electron transfer from Cu$_A$ to the low-spin heme, but also to additional electron equilibration between the low-spin heme $b$, Cu$_A$ and heme $a_3$ (Figs. 2, 5; [32, 55]). The kinetics of membrane potential generation shows two electrogenic phases with relative amplitudes of 56% and 44% and characteristic times of $\sim 14 \mu s$ and $\sim 290 \mu s$, confirming a preliminary report [55].

Electron injection into the "as isolated" $ba_3$ oxidase, incubated in the light with Rubpy and aniline prior to the laser flash resulted in three electrogenic components [16], two of which ($\sim 20 \mu s$ and $\sim 300 \mu s$) are much like those found in the present work, while the third one is much slower ($\sim 10 ms$) and smaller in amplitude ($\sim 15\%$). In the present work we did not resolve this latter component. Continuous illumination of the O state with a series of laser flashes leads to appearance of an electrogenic phase with $\tau \sim 950 \mu s$ ($\sim 10\%$ on the 20-th flash) most probably due to accumulation of the single-electron form (state E). Though the rate constant of this component is almost $\sim 10$ times slower than in case of $E \rightarrow R$ transition of $aa_3$ oxidase from $P. denitrificans$ and is reminiscent of the data obtained for the K-channel mutant (K354M) of the $aa_3$ oxidase [46], it is still about ten times faster than the $\sim 10 ms$ electrogenic phase cited above.

Similar to [32], where a pulse radiolysis technique for electron injection was used, we observe partial electron transfer from Cu$_A$ to heme $b$ during the fast phase of the reaction (Figs.
This is in contrast to cytochrome oxidase from mitochondria, where the electron transfer from Cu\textsubscript{A} to the low-spin heme was almost complete in these conditions [69]. This is a property of the relaxed O state of the BNC of cytochrome \textit{ba3} since, for example, in the case of the P to F transition both cytochrome \textit{ba3} and mitochondrial COX show complete electron transfer from Cu\textsubscript{A} to the low-spin heme [19].

The observed rate constant of the fast phase is the sum of the constants for the forward and reverse reactions. From the equilibrium constant $K_{\text{eq}} \approx 0.53$ [32] one can estimate the rate constants of the forward ($k_1$) and reverse ($k_{-1}$) electron transfer between Cu\textsubscript{A} and heme \textit{b} as $k_1 = 33 \times 10^3$ and $k_{-1} = 62 \times 10^3$ s\textsuperscript{-1}, respectively. For the slow phase, the $K_{\text{eq}}$ for electron transfer from heme \textit{b} to the heme \textit{a3} is ~0.37 [32], and the observed time constant 230 \textmu s gives the rate constants of the forward ($k_2$) and reverse ($k_{-2}$) electron transfers as $k_2 = 0.12 \times 10^3$ and $k_{-2} = 3.2 \times 10^3$ s\textsuperscript{-1}.

From the above results follows that the electron transfer from Cu\textsubscript{A} to the low-spin heme contributes to both the fast and slow electrogenic phases. However, the slow electrogenic phase cannot be explained by the electron redistribution alone, because the electron transfer from heme \textit{b} to heme \textit{a3} is not electrogenic [13], and the contribution of electron transfer from Cu\textsubscript{A} to BNC via heme \textit{b} during this phase is small (Table 1). Obviously, the slow electrogenic phase includes proton transfer coupled to the reduction of heme \textit{a3}. In this reaction, the electrogenic uptake of a proton to the BNC raises the $E_m$ of heme \textit{a3} which initiates the second electron re-equilibration phase between the Cu\textsubscript{A}/heme \textit{b} pair and heme \textit{a3}. The ratio of amplitudes of the slow and fast electrogenic phases can now be used to approximate the distance across the membrane dielectric by which the proton transfer occurs,

$$A_{\text{slow}}/A_{\text{fast}} = [(s*d + r*(1-d) + p)/f*d]$$

Eq.1

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where \( f \) and \( s \) are the fractions of primary electron transfer from Cu\(_A\) to the low-spin heme in the fast and slow phases, respectively; \( d \) is the “dielectric depth” of the low-spin heme from Cu\(_A\); \( p \) is the number of protons pumped in the transition, \( r \) is the fraction of heme \( a_3 \) reduced during the slow phase, and \( r^*(1-d) \) corresponds to the substrate proton transfer from the inner water phase coupled to the reduction of heme \( a_3 \).

The parameters \( f \), \( s \) and \( r \) in eq.1 can be calculated from the equilibrium constants \( K_{1eq} \) and \( K_{2eq} \) and are found to be \( \sim 0.35 \), \( \sim 0.07 \) and \( \sim 0.11 \), respectively. We may initially assume that \( p=0 \) since the O to E transition is not associated with proton pumping in cytochrome \( aa_3 \) (see Introduction). Eq. 1 then yields \( d \sim 0.36 \) which agrees very well with the crystal structure and analyses published earlier [62, 70-72], thus also confirming the absence of significant proton pumping.

This model is the simplest explanation of the charge transfer events but it is based on the assumptions that the transfer of the substrate proton, coupled with the reduction of heme \( a_3 \), takes place directly to a group in the binuclear center, and that the dielectric permittivity is roughly the same throughout the thickness of the membrane. As we compare kinetics of enzyme in detergent solution and in proteoliposomes, it is important to note that the equilibrium constants of intramolecular electron transfer between the redox centers are identical in these two conditions.

If we assume the transfer of the electron from Cu\(_A\) to the low-spin heme \( b \) as a \( d \) charge unit, and the distance of proton transfer perpendicular to the membrane coupled to the reduction of the heme \( a_3 \) by electron transfer from heme \( b \) to heme \( a_3 \) during the slow electrogenic phase as \( x \) charge units, then from Eq.1 the value of \( x \) is

\[
Eq. 2 \quad x = \frac{[(A_{slow}/A_{fast})]f^*(d-s)d}{r}
\]
Table 3 shows the extent of transmembrane proton transfer $x$ for cases of $d$ in the range 0.4 to 0.33. In the case of $d=0.4$ the magnitude of the transmembrane proton transfer is slightly larger than the distance from the aqueous N-phase to the BNC. In the case of $d=0.33$ the magnitude of proton transfer is less than that distance (~0.58). The analysis shows that the best value for $d$ is about 0.36, which is in close agreement with the theoretical estimates for the Cu$_A$ heme $a$ and Cu$_A$ heme $a_3$ electrogenic distances in the $aa_3$ oxidases (~0.38 and 0.37, respectively [71]). Altogether, the data shows that reduction of heme $a_3$ during the $O\rightarrow E$ transition is accompanied by a substrate proton transfer from the inner aqueous phase to the BNC, and is not coupled to proton pumping (Fig. 5).

Despite the dissimilarities, the $O$ states of both $aa_3$ and $ba_3$ oxidases are thus incapable of pumping protons. As we have shown recently [55], the notion of two functionally distinct states of the fully oxidized enzyme, the relaxed $O$ and the metastable $O_H$ states, previously deduced for the proton-pumping $aa_3$-type enzymes, also applies on the aberrant $ba_3$ cytochrome $c$ oxidase from $T. thermophilus$, and the $O_H$ states behave very differently in the two types of enzymes in time-resolved spectroscopy measurements. A time-resolved study of charge translocation during the $O_H\rightarrow E_H$ transition in cytochrome $ba_3$ is required to answer the crucial question of the capability of the $O_H$ state of $ba_3$ to pump protons, and to elucidate the phenomenon of variability of proton pump efficiency of B-type cytochrome oxidases.

4.2. Charge transfer kinetics coupled to the $O_H\rightarrow E_H$ transition

The time-resolved study of the $O_H\rightarrow E_H$ transition of $aa_3$ oxidase from $P. denitrificans$ revealed four phases of $\Delta \Psi$ generation [43]. The fast electrogenic component (10 $\mu$s, ~12% of amplitude) was attributed to transfer of an electron from Cu$_A$ to heme $a$ in ~70% of the enzyme population; the remaining Cu$_A$ oxidation occurring in conjunction with the slower phases. Only this electrogenic phase is observed after injection of the electron into the "resting" state O [46]. The 150 $\mu$s component (~42% of amplitude) is associated with electron transfer from the
CuA/low-spin heme \(a\) pair to the high-spin heme \(a_3\) and to the transfer of a pumped proton through the D-channel from the inner water phase into a proton-loading site (PLS) near the binuclear center. As a result, 60% of the electron is found at heme \(a_3\), while 40% remains at heme \(a\) [43]. The 800 \(\mu\)s component (~30% of amplitude) was attributed to electron transfer from heme \(a\) (40%) and heme \(a_3\) (60%) to CuB, and to uptake of a chemical proton into the binuclear centre through the K-channel. Release of the pumped proton from the PLS into the outer water phase was assumed to occur during the slowest 2.5 ms phase (~16% of amplitude).

Fig. 6 shows our current interpretation of this data, which differs slightly from the original in that loading of the PLS requires electron transfer to the BNC, and does not occur on mere reduction of heme \(a\) (see [12]). A substantial part of the electrogenicity of the 150 \(\mu\)s phase is, instead, attributed to proton transfer in the K-channel, which is postulated to be necessary to allow electron transfer to heme \(a_3\). Consistent with this, the 150 \(\mu\)s phase is absent in the K354M mutant form of the *Paracoccus aa3* enzyme [45].

Electron injection into the O\(_H\) state of *ba3* enzyme incorporated into phospholipid vesicles causes a response that is qualitatively reminiscent of the corresponding case with *aa3* (Table 2), showing three kinetic phases (fast, middle and slow), albeit a fourth phase found for *aa3* (\(\tau\approx2.5\) ms) is missing. Again similar to the *aa3* oxidase, the rate constants of the three phases in the optical and electrometric measurements are very close to one another. Yet, a quantitative inspection reveals substantial differences.

### 4.2.1. Three time-resolved reaction components

In contrast to the O\(_H\)→E\(_H\) transition of *aa3* oxidase (and to the O→E transition of cytochrome *ba3*), the first time-resolved component (\(\tau_1\approx22\) \(\mu\)s, the "fast" phase) is characterized by complete movement of the injected electron from Cu\(_A\) to both hemes (Table 2). Despite this, the apparent rate constant of the phase correlates with the rate constant of electron transfer from Cu\(_A\) to heme \(b\) (i.e. to the \(k_1\approx0.34\times10^5\) s\(^{-1}\)). This indicates that electron transfer between the hemes is much
faster than that between CuA and the low-spin heme, similar to other terminal heme-copper oxidases, where it can occur on the nanosecond timescale [73]. A few faster observed rate constants of the fast electrogenic phase in the aa3 oxidase (and in the O→E transition of the ba3 oxidase, see above) may be explained by the reversibility of the electron transfer, and accordingly, the sum of the constants of forward and reverse reactions of the electron transfer between CuA and low-spin heme.

The two following submillisecond components (middle and slow, with τ~185 µs, and τ~790 µs) include full reoxidation of the low and high-spin hemes. Due to complete oxidation of CuA and of both hemes, the final acceptor of the injected electron is CuB as it is in the aa3 oxidase (Fig. 6) [43, 55].

The contribution of the fast electrometric component to the total response is ~33%. Unlike aa3 oxidase from P.denitrificans it represents 100% electron transfer from CuA to the heme area. Because the structure implies that the heme groups are located at almost the same distance from the surface of the membrane [13, 70], the amplitude of the fast electrogenic phase can also be used to assess the electrogenicity of the other phases (Table 4).

Table 4 shows the results of the estimated charge transfer events in the O_H→E_H transition assuming the electrogenic distance between CuA and heme b to be ~0.36, as just validated (see above), and comparing to the corresponding transition of the aa3 oxidase from P.denitrificans [43]. The total number of charges transferred across the membrane in the ba3 oxidase is only about one half of those in the aa3 oxidase, suggesting that the O_H→E_H transition in ba3 oxidase may not overall be coupled to significant proton pumping across the membrane. This interpretation would also explain the absence of the 2.5 ms phase in ba3, which was assigned to release of the pumped proton from PLS to the outer water phase in the aa3 enzyme [43].

Though the overall amplitude of the middle and slow electrogenic phases (~0.73) are in rough agreement with the suggestion that they mostly reflect transfer of a single ("substrate")
proton from the N-side of the membrane to the BNC, the mechanism appears not to be as simple as predicted for the O→E transition. First, the total electrogenicity of the middle and slow phases is still somewhat higher than they are expected to be assuming complete lack of proton pumping. More important, electron injection in the O_H state produces two major protonic electrogenic phases, similar to the O_H→E_H transition of the proton-pumping aa_3 oxidase, and final transfer of the electron to Cu_B. From this the possibility emerges that the proton pump may be initially loaded by protonation of the PLS in the ba_3 oxidase, followed by backflux (leakage) of the proton to the BNC so that very little net pumping is achieved in the overall reaction.

In aa_3-type oxidases [43, 61, 74] reduction of the high-spin heme occurs only slowly after electron equilibration between Cu_A and the low-spin heme, and is limited by proton uptake into the binuclear center [75]. In ba_3, however, the electron is already transferred to heme a_3 during the 20 μs phase in approximately one half of the population. This suggests that the Em of heme a_3 is raised already in this early state, relative to the case with the aa_3-type enzymes (cf. below).

4.2.2. Protonation state of the BNC in the O_H form of cytochrome ba_3

As already noted, there is substantial reduction of heme a_3 in the fast phase indicating a redox potential as high as that for heme a, which is very different from the case of the aa_3 enzymes where heme a_3 is reduced only in the next phase coupled to proton uptake. In the aa_3 enzymes the structure of the BNC in the O_H state is likely to be Fe[III]-OH^- Cu[II] tyr-O^- ↔ Fe[III]-OH^- Cu[II] tyr-O^* [8]. For ba_3 it is known that the earlier F state has the same optical spectrum as state P, which indicates that the OH^- ligand of Cu_B is retained in state F, and that the proton taken up in the P to F transition has instead protonated the tyrosinate to tyrosine. The F state of ba_3 may thus have the structure Fe[IV]=O^2- Cu[II]-OH^- tyr-OH and reduction to O_H may yield Fe[III] Cu[II]-OH^- tyr-O^- with a relatively high-potential heme a_3. Although the ligand structure looks the same as for aa_3, the OH^- may not bind to heme iron due to the substantial differences between the structures and ligand reactivity of the BNC in ba_3 and aa_3 enzymes (see Introduction). By contrast, Cu_B is low
potential due to the OH$^-$ ligand that is not shared with the heme iron. Reduction to the E$^\text{H}$ state would thus reduce the Cu$_B$ either coupled to protonation of its hydroxide ligand to water, or – perhaps more likely - to protonation of the tyrosinate and a shift of the OH-ligand to become the distal ferric heme ligand, i.e. Fe[III]-OH$^-$Cu[I] tyr-OH.

4.2.3. The middle component

The middle electrogenic phase of the $ba_3$ enzyme has the largest amplitude (~ 42%, tables 1,2). In parallel, there is only a slight (~10%) oxidation of heme $b$. This is in contrast to the 150 $\mu$s component of $aa_3$ oxidase [43], where the electron, previously distributed between Cu$_A$ and heme $a$ is shifted significantly to heme $a_3$, resulting in a complete oxidation of Cu$_A$ (~30%), reduction of 60% of heme $a_3$ with ~40% of the electron density remaining at heme $a$ (Table 2; Fig. 6).

While the 150 $\mu$s component of $aa_3$ oxidase includes both the electron transfer and the coupled proton translocation from the N phase to the PLS through a large part of the membrane (~80% of membrane dielectric thickness ([43]; Fig. 6), the proton translocation during the middle phase of $ba_3$ oxidase is kinetically distinct from the reduction of the heme groups by Cu$_A$ during the fast phase. It is delayed, and does not limit the rate of reduction of heme $a_3$. The obvious explanation for this is a higher redox-potential of heme $a_3$ in the O$_H$ state, as just discussed. By analogy with the pumping mechanism of the $aa_3$ oxidases, it seems logical to suggest that during the middle phase the proton is first transferred to the PLS (Fig. 7), whereas the slow electrogenic phase is associated with protonation of BNC. Assuming again a value of 0.36 for the electrogenicity of the electron transfer from Cu$_A$ to heme $b$, the amplitude of the middle electrogenic phase in $ba_3$ oxidase corresponds to proton transfer over a distance of about 0.36 x 0.42/0.33 = 0.46 of the membrane dielectric thickness. By analogy with $aa_3$ we may assume that this corresponds to proton translocation from the aqueous N-side to the PLS in response to the 45% reduction of heme $a_3$ in the fast phase. In addition we have in the slow phase 10% oxidation of heme $b$ apparently by Cu$_B$, since there is no net reduction of heme $a_3$ in this phase. The former amounts to 0.45*y translocated.
charges where \( y \) is the dielectric distance between the N-side and the PLS. The 10% oxidation of heme \( b \) by \( \text{Cu}_B \) is assumed to be linked, first, to uptake of protons from the N-side to the PLS (i.e. \( 0.1 \times y \) charges translocated), followed by uptake of protons from the N-side to the BNC when the electrons reach \( \text{Cu}_B \) (i.e. \( 0.1 \times 0.64 = 0.06 \) charges translocated), and consequent release of the 10% protons in the PLS to the P-side (\( 0.1 \times [1-y] \)). Since the total charge translocation of the middle phase is 0.46, we can solve with respect to \( y \) and find that \( y = 0.67 \), in fair agreement with the expectation that the PLS lies immediately on the P-side of the heme groups [76, 77].

Direct proton transfer to the BNC in the middle electrogenic phase is very unlikely for the following reasons. If there is a single \( \text{OH}^- \) ligand in the binuclear site located on the cupric \( \text{Cu}_B \), and which becomes protonated on reduction of heme \( a_3 \), it would be difficult to understand why there is such a long delay until heme \( a_3 \) is reoxidized by \( \text{Cu}_B \). If the single \( \text{OH}^- \) ligand is initially at the ferric heme \( a_3 \), the fast reduction to ferrous heme (without proton uptake) would require a switch of the \( \text{OH}^- \) group to coordinate \( \text{Cu}_B \). However, if protonation of the \( \text{OH}^- \) ligand of \( \text{Cu}_B \) occurs during the middle phase, it is again difficult to understand that the electron at heme \( a_3 \) is not immediately transferred to the copper but it rather delayed to the slow phase.

4.2.4. The slow component

The last phase of the reaction includes complete oxidation of both hemes by \( \text{Cu}_B \). This indicates that the \( E_m \) of \( \text{Cu}_B \) is raised considerably during this phase, which can only be explained by arrival of a chemical proton to the catalytic site, which is similar to the 0.8 ms phase observed during the \( \text{O}_H \rightarrow \text{E}_H \) transition of cytochrome \( \text{aa}_3 \) (Fig. 6). The amplitude of the slow electrogenic phase is about 25%, corresponding to an electrogenic distance of about \( 0.36 \times 25/33 = 0.27 \) (Table 4).

The slow phase includes 45% oxidation of heme \( b \) and 45% oxidation of heme \( a_3 \) by \( \text{Cu}_B \). Above, we concluded that the PLS was 45% protonated after the middle phase. A basic interpretation of the slow phase would be to suggest, in accordance with the proposal for the middle phase, that initial 45% electron transfer from heme \( b \) to heme \( a_3 \) would load the PLS from the N-
side (0.45*0.67 = 0.3 charges translocated), bringing the PLS to be 90% loaded, and heme $a_3$ to have 90% of the injected electrons. Since we already have 0.3 translocated charges and the observed extent of the slow phase is 0.27 charges, it follows that the transfer of the electrons from heme $a_3$ to $Cu_B$ is completely charge-compensated by leakage of the protons in PLS back to the BNC (0.9*0.03 = 0.03 charges), which when subtracted from 0.3 yields the observed 0.27 charges translocated in the slow phase.

A key question with this proposed mechanism is why, in the slow phase, all protons loaded in the PLS would leak back to compensate the charge in the BNC instead of being pumped out into the aqueous P-phase. The proton-pumping efficiency of the B-type enzymes is known to be low (see Introduction), and experiments have suggested that the B type enzymes pump protons with high stoichiometry only when there is no opposing proton-motive force (pmf), in other words when little or no work is being done [21]. This implies that it is a pmf-driven proton leak that renders the $ba_3$ enzyme a poor proton pump. The experimental conditions in our experiments here preclude measures to dissipate the protonmotive force, and thus such a leak may indeed take effect in the slow phase of the $O_H\rightarrow E_H$ transition when prior charge translocation has generated a significant membrane potential. As shown schematically in Fig. 7, proton-pumping is indeed poor in this type of experiment albeit not completely absent (as in the case of state O), and retaining the key mechanistic step of transferring the proton to the PLS in the middle phase.

5. Conclusions.

Electron injection into the fully oxidized resting state O of $ba_3$ cytochrome oxidase from $T.thermophilus$ leads to electron transfer from $Cu_A$ to the low-spin heme, and to partial electron transfer from the low-spin heme $b$ to heme $a_3$ producing two electrogenic phases. The fast electrogenic phase reflects electron transfer from $Cu_A$ to heme $b$, while the slow phase includes electron equilibration from the $Cu_A$/heme $b$ pair to heme $a_3$ and coupled electrogenic proton transfer. The amplitude of the vectorial proton transfer corresponds to uptake of a substrate
proton from the inner water phase to the BNC. The data indicates that the electrogenic distance from the membrane surface to BNC in cytochrome \( ba_3 \) is close to that in the \( aa_3 \) oxidases and that the electron injection into the resting O state of cytochrome \( ba_3 \) is not accompanied by proton pumping.

The \( O_H \rightarrow E_H \) transition is accompanied by electrogenic reduction of heme \( b \) by \( Cu_A \) through the "fast" phase and transfer of protons during the "middle" and "slow" electrogenic phases coupled to the electron redistribution between the heme \( b/heme \ a_3 \) pair and \( Cu_B \). The time constants of these phases are very close to those in the \( aa_3 \) oxidases but the total amplitude of the protonic phases is only about one half of that in \( aa_3 \) oxidase. The different kinetic behavior of the \( O_H \) state compared to state O suggests that the "middle" electrogenic phase may be associated with loading of the proton pump. However, very little proton-pumping takes place overall, because this proton appears to leak back to the BNC in the "slow" electrogenic phase annihilating the charge of the electron that has moved to \( Cu_B \). This model helps to explain mechanistically how the proton-pumping efficiency of the \( ba_3 \) enzyme is poor due to proton back-leakage in the presence of a proton-motive force, as predicted (21).

**Acknowledgments**

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Figure Legends.

Figure 1.

A: Generation of transmembrane electric potential difference $\Delta \Psi$ by the oxidized $ba_3$ cytochrome oxidase (O state) incorporated into proteoliposomes in response to electron injection from Rubpy (average of 3 individual responses). Conditions: Tris (pH 8), 5 mM; Rubpy, 1 mM; aniline, 20 mM, 2mM ferricyanide. The main panel shows the experimental trace and the fit with a sum of exponentials (Table 1); Inset: The residuals are shown for two types of approximation, by two or three components.

B: Flash-induced generation of transmembrane electric potential difference $\Delta \Psi$ by the oxidized $ba_3$ cytochrome oxidase (O state) incorporated into proteoliposomes. Conditions: Tris (pH 8), 5 mM; Rubpy, 1 mM; aniline, 20 mM. The cytochrome oxidase is exposed to several laser pulses with a frequency of 5 Hz. The electrogenic response curves after the 1st, 10th and 20th flashes are shown. The dotted line shows the electrogenic response curve after the 1st flash in the presence of 2 mM ferricyanide. The curves in the presence and absence of ferricyanide are normalized to amplitude.

Figure 2.

Kinetics of light-induced absorbance changes of the relaxed O state of $ba_3$ cytochrome oxidase in response to electron injection from Rubpy. Conditions: cytochrome $ba_3$, 50 μM; Tris (pH 8), 2 mM; DM, 0.05%; aniline, 20 mM; Rubpy, 200 μM. Characteristic traces at three wavelengths: 560 nm minus 575 nm (heme $b$), 614 nm minus 658 nm (heme $a_3$), and 820 nm (CuA) were extracted from the surface of optical changes, and are shown together with the corresponding fits (Table 1 or see [55], for details).
Figure 3.

Generation of transmembrane electric potential by the oxidized non-relaxed $ba_3$ cytochrome oxidase from *T.thermophilus* coupled to the $O_H\rightarrow E_H$ transition.

Conditions: 3mM Tris (pH 8), 200 uM Rubpy, 20 mM aniline, 50 μg/ml catalase, 30 mM Glucose, 25 μM hexa amine ruthenium, 1 mM ascorbate, 100% CO, 4mg/ml glucose oxidase.

The shown kinetics of $\Delta\Psi$ generation is an average of 22 individual responses. The main panel shows the experimental trace together with the fit with a sequential reaction model (Table 2).

Inset: The residuals are shown for two type of approximation by two or three components.

Figure 4.

The kinetics of electron transfer through the redox-centres after electron injection into the pulsed form of the oxidized cytochrome $ba_3$ from *T.thermophilus* (the $O_H\rightarrow E_H$ transition).

A: Light-induced absorbance changes in response to the electron injection from Rubpy. Traces at three characteristic wavelengths: 560 nm (heme $b$), 614 minus 658 nm (heme $a_3$), and 830 nm (Cu$\text{A}$) extracted from the surface of optical changes are shown together with the global fit of the surface based on the rate constants obtained from the electrometric measurements (Table 2).

B: Kinetic transient absorption spectra of the phases obtained by a global fit of the absorbance change surface based on the rate constants obtained from the electrometric measurements.

Conditions: 2 mM Tris (pH 8); 0.05% DM; 20 mM aniline; 7 μM TMPD was first made anaerobic on a vacuum line, and then fully reduced by 1 mM potassium ascorbate. Then, anaerobic fully reduced oxidase was mixed (1:1) in the stopped flow module with oxygen-saturated buffer (2 mM Tris (pH 8); 0.05% DM; 20 mM aniline; 400 μM Rubpy). The final concentration of $ba_3$ oxidase was ~32 μM.

Figure 5.
Single-electron injection into the oxidized O state of the $aa_3$ cytochrome oxidase from $P.dentirificans$ (a) and $ba_3$ oxidase from $T.thermophilus$ (b).

The redox-active cofactors are indicated. The rhombus and square represent hemes $a$ (or $b$) and $a_3$, respectively. The circle above the hemes represent Cu$_A$, while the circle next to heme $a_3$ represents Cu$_B$. The hexagon represents the proton loading site (PLS). The green and blue arrays indicate electron and proton transfer events during the subsequent reaction step. The degree of reduction of the redox-centres is estimated based on the values of corresponding equilibrium constants from [32, 68].

**Figure 6.**

Single-electron injection into the oxidized O$_H$ state of the $aa_3$ cytochrome oxidase from $P.dentirificans$.

The redox-active cofactors are indicated. The rhombus and square represent hemes $a$ and $a_3$, respectively. The circle above the hemes represent Cu$_A$, while the circle next to heme $a_3$ represents Cu$_B$. The hexagon represents the proton loading site (PLS). The green and blue arrays indicate electron and proton transfer events during the subsequent reaction step. The degree of reduction of the redox-centres is estimated based on the values of corresponding equilibrium constants from [43]. Note that the PLS is loaded in the 150 us phase to an extent only matching the electron transfer to heme $a_3$, and that the observed electrogenicity includes proton transfer in the K-pathway involving lysine-354, which differs from the original interpretation (see [12], and the text).

**Figure 7.**

Single-electron injection into the oxidized O$_H$ state of the $ba_3$ oxidase from $T.thermophilus$.

The redox-active cofactors are indicated. The rhombus and square represent hemes $b$ and $a_3$, respectively. The circle above the hemes represent Cu$_A$, while the circle next to heme $a_3$ represents
CuB. The hexagon represents the proton loading site (PLS). The green and blue arrays indicate electron and proton transfer events during the subsequent reaction step. The degree of reduction of the redox-centres is estimated based on the data of the absorbance changes. The thickness of the blue arrows are proportional to the number of protons transferred at this stage. The possible mechanism of the articulation of electron and proton transfer steps are shown (for details, see the Discussion).

References


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Figure 1
Figure 2

[Graph showing absorbance over time with peaks for heme a, heme b, and CuA]
Figure 3
Figure 4
Figure 5

(a) Cu$_A$ 100% → PLS → heme $a_3$ → Cu$_B$ 12 µs → 33% → 67%

(b) Cu$_A$ 100% → PLS → heme $a_3$ → Cu$_B$ 14 µs → 65% → 35% → H$_{chem}$ → 58% → 31% → 11%
Figure 6
Table 1. The components of the kinetics of membrane potential generation and changes of absorbance coupled to the O→E transition of $ba_3$ oxidase from *T.thermophilus*

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<tr>
<td>−</td>
<td>0.0000</td>
<td>0.0014</td>
</tr>
<tr>
<td>−0.0036</td>
<td>0.0012</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

*The time-resolved electrometric measurements were repeated more than 15 times with four different samples.

**The absorbance changes at the characteristic wavelength were extracted from the surface of absorbance spectra.
**Table 2.** Time-resolved steps of the kinetics of membrane generation and changes of absorbance coupled to the $\text{O}_2 \rightarrow \text{E}_1$ transition of $ba_3$ oxidase from *T. thermophilus* and $aa_3$ oxidase from *P. denitrificans*

<table>
<thead>
<tr>
<th>Object</th>
<th>Generation of membrane potential</th>
<th>Changes of absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Electrogenic phases</td>
<td>Time constant $\tau$, $\mu$s</td>
</tr>
<tr>
<td></td>
<td>silent</td>
<td>~0.5</td>
</tr>
<tr>
<td>$ba_3$ oxidase from <em>T. thermophilus</em></td>
<td>fast</td>
<td>22 +/-7.2</td>
</tr>
<tr>
<td></td>
<td>middle</td>
<td>185 +/-48</td>
</tr>
<tr>
<td></td>
<td>slow</td>
<td>790 +/-152</td>
</tr>
<tr>
<td></td>
<td>silent</td>
<td>~0.5</td>
</tr>
<tr>
<td>$aa_3$ oxidase from <em>P. denitrificans</em></td>
<td>fast</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>middle</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>slow</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>proton release</td>
<td>2600</td>
</tr>
</tbody>
</table>

* data from [43]
*The time-resolved electrometric measurements were repeated more than 20 times with different samples.
Table 3. The estimated charge transfer events and the total electrogenicity for the population of enzyme in which the electron is transferred from CuA to heme $a_3$ through the $O\rightarrow E$ transition varying the assumed electrogenic distance between CuA and heme $b$

<table>
<thead>
<tr>
<th>Electrogenic event</th>
<th>$\tau$, $\mu$s</th>
<th>Charges across the membrane calculated for various values of electrogenic distance between CuA and heme $b$ (from 0.4 to 0.33 of the membrane dielectric thickness)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>electron transfer</td>
<td>14</td>
<td>0.40 0.39 0.38 0.36 0.34 0.33</td>
</tr>
<tr>
<td>proton transfer</td>
<td>290</td>
<td>0.69 0.68 0.66 0.63 0.59 0.58</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1.09 1.07 1.04 0.99 0.93 0.91</td>
</tr>
</tbody>
</table>

* The data should be considered only as showing a qualitative trend, because the actual dielectric inhomogeneity of the protein is difficult to quantify. The standard deviations for the estimations is less than ~15% given the errors in electrometric measurements and the errors of determination of the equilibrium constants $K_{1eq}$ and $K_{2eq}$ in [78] and [55].
Table 4 Time-resolved steps of the kinetics of membrane generation coupled to the $O_H \rightarrow E_H$ transition

<table>
<thead>
<tr>
<th>Electrogenic phase</th>
<th>Cytochrome $b_{a3}$ from <em>T.thermophilus</em></th>
<th>Cytochrome $aa_{3}$ from <em>P.denitrificans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau$, $\mu$s</td>
<td>$\alpha$, %</td>
</tr>
<tr>
<td>fast</td>
<td>22</td>
<td>33</td>
</tr>
<tr>
<td>middle</td>
<td>185</td>
<td>42</td>
</tr>
<tr>
<td>slow</td>
<td>790</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>total</td>
<td>100</td>
<td>1.09</td>
</tr>
</tbody>
</table>

* data from [43]

$\tau$, time constant (inverse rate constant);

$\alpha$, electrogenic amplitude

$^a$ The amount of charges transferred across the membrane during the kinetic component calculated based on the value of electrogenecity of the electron transfer from Cu$_A$ to heme $a$ to be 0.36 of the thickness of the membrane dielectric and the corresponding contribution (electrogenic amplitude) of the component.

$^b$ The amount of charges transferred across the membrane during the kinetic component calculated based on the value of electrogenecity of the electron transfer from Cu$_A$ to heme $a$ to be 0.36 of the thickness of the membrane dielectric and the corresponding contribution (electrogenic amplitude) of the component [71, 72].
Conflict of interest.

The authors declare no conflict of interest.
Highlights:

Cytochrome $ba_3$ from *T.thermophilus* is a B type heme-copper oxidase

The kinetics of membrane potential generation were studied by electron injection

The “relaxed” O and “unrelaxed” $O_H$ states were found to react very differently

A leak of the pumped proton was found in the reaction of the $O_H$ state

The reaction of the O state involved no proton-pumping step