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Electron and proton transfer in the ba_3 oxidase from *Thermus thermophilus*

Irina A. Smirnova · Dmitry Zaslavsky · James A. Fee · Robert B. Gennis · Peter Brzezinski

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Abstract The ba_3-type cytochrome *c* oxidase from *Thermus thermophilus* is phylogenetically very distant from the aa_3–type cytochrome *c* oxidases. Nevertheless, both types of oxidases have the same number of redox-active metal sites and the reduction of O_2 to water is catalysed at a haem a_3-Cu_B catalytic site. The three-dimensional structure of the ba_3 oxidase reveals three possible proton-conducting pathways showing very low homology compared to those of the mitochondrial, *Rhodobacter sphaeroides* and *Paracoccus denitrificans* aa_3 oxidases. In this study we investigated the oxidative part of the catalytic cycle of the ba_3-cytochrome *c* oxidase using the flow-flash method. After flash-induced dissociation of CO from the fully reduced enzyme in the presence of oxygen we observed rapid oxidation of cytochrome *b* ($k \cong 6.8\times10^4$ s^{-1}) and formation of the peroxy (P_R) intermediate. In the next step a proton was taken up from solution with a rate constant of $\sim1.7\times10^4$ s^{-1}, associated with formation of the ferryl (F) intermediate, simultaneous with transient reduction of haem *b*. Finally, the enzyme was oxidized with a rate constant of ~1,100 s^{-1}, accompanied by additional proton uptake. The total proton uptake stoichiometry in the oxidative part of the catalytic cycle was ~1.5 protons per enzyme molecule. The results support the earlier proposal that the P_R and F intermediate spectra are similar (Siletsky et al. *Biochim Biophys Acta* 1767:138, 2007) and show that even though the architecture of the proton-conducting pathways is different in the ba_3 oxidases, the proton-uptake reactions occur over the same time scales as in the aa_3-type oxidases.

Keywords Cytochrome ba_3 · Proton uptake · Electron transfer · Respiration · Haem-copper

Abbreviations and Definitions

A	a ferrous-oxy adduct
DM	*n*-dodecyl-β-D-maltoside
P_R	the peroxy intermediate
F	oxo-ferryl intermediate
O	fully-oxidized enzyme
R	fully-reduced enzyme
Cu_A	copper A
Cu_B	copper B
k	rate constant
τ	time constant (1/*k*)

Introduction

The ba_3-cytochrome *c* oxidase from the extremely thermophilic eubacterium *Thermus thermophilus* is expressed at low oxygen concentrations. The enzyme is an integral

Smirnova and Zaslavsky contributed equally to the work described in this paper.

I. A. Smirnova · D. Zaslavsky
A.N.Belozersky Institute of Physicochemical Biology,
Moscow State University,
Moscow 119899, Russia

J. A. Fee
Department of Molecular Biology, The Scripps Research Institute,
La Jolla, CA 92037, USA

R. B. Gennis
Department. of Biochemistry, University of Illinois,
Urbana, IL 61801, USA

P. Brzezinski (✉)
Department of Biochemistry and Biophysics,
The Arrhenius Laboratories for Natural Sciences,
Stockholm University,
106 91 Stockholm, Sweden
e-mail: peterb@dbb.su.se

membrane protein, which catalyses reduction of oxygen to water by ferro-cytochrome c_{552}, conserving a fraction of the free energy of this reaction in the form of a transmembrane electrochemical proton gradient (for review on oxidases in general, see Belevich and Verkhovsky 2008; Brunori et al. 2005; Brzezinski and Ädelroth 2006; Brändén et al. 2006; Hosler et al. 2006; Pereira et al. 2001; Wikström and Verkhovsky 2006, 2007). Presumably, the enzyme pumps protons with a stoichiometry ~0.5 H^+/e^- (Kannt et al. 1998; see also Siletsky et al. 2007), i.e. lower than that of the aa_3-type oxidases (~1 H^+/e^-). The three-dimensional structure of the ba_3-cytochrome *c* oxidase from *T. thermophilus*, determined to a resolution of 2.4 Å (Soulimane et al. 2000) shows that the enzyme consists of three subunits, I, II and IIa forming 15 transmembrane helices, and a small periplasmic domain. Like the aa_3-type oxidases, the enzyme holds four redox-active metal sites. Three of these are found in subunit I; haem *b* and a binuclear centre consisting of a high-spin haem a_{s3} and Cu_B. Haem *b* has a low-spin iron and two axial histidine residue ligands. Haem a_{s3} has the iron in the plane of the haem and the distance to the axial His384 ligand is considerably larger than in the aa_3 oxidases from *Rhodobacter sphaeroides*, *Paracoccus denitrificans* and mitochondria from bovine heart (Iwata et al. 1995; Ostermeier et al. 1997; Svensson-Ek et al. 2002; Tsukihara et al. 1996; Yoshikawa et al. 1998). Furthermore, haem a_{s3} differs slightly from haem *a* in that it has a formyl group at C8 and a hydrophobic hydroxyethylgeranylgeranyl moiety on C2. One of the three histidine ligands of Cu_B (His233) is covalently bound to Tyr 237.

Subunit II has one transmembrane helix and a periplasmic domain composed of a 10-stranded β-barrell. It holds a *di*-nuclear Cu_A centre, which is the primary electron acceptor of the oxidase. Subunit IIa is a small polypeptide (34 amino acid residues) of one transmembrane helix, which corresponds in space to helix 1 of subunit II in the aa_3-type oxidases (Soulimane et al. 2000).

The ba_3-cytochrome *c* oxidase shows little sequence homology to other cytochrome *c* oxidases of which the structure is known (from *P. denitrificans*, *R. sphaeroides* and bovine heart mitochondria). Like in other terminal oxidases, the catalytic binuclear centre is located in a middle of the membrane, which requires proton-conducting pathways for delivery of substrate protons necessary for oxygen reduction. In addition, there must be proton pathways, which facilitate transfer of "pumped" protons across the membrane.

The haem-copper oxidases have been classified into three families depending on their proton-pathway architecture (Pereira et al. 2001). While the mitochondrial, *R. sphaeroides* and *P. denitrificans* aa_3 oxidases all belong to the type A enzymes, the ba_3 cytochrome *c* oxidase belongs to the type B family. Three possible proton pathways have been proposed in the ba_3-cytochrome *c* oxidase from *T. thermophilus*. One of them has a spatial location equivalent to the K pathway in the type A oxidises (Fig. 1) and it is primarily used for proton uptake in the ba_3 cytochrome *c* oxidase (Gennis et al., unpublished data). In this pathway the highly conserved residues in the type A oxidases, $Thr359^R$ and $Lys362^R$ (the superscript R is given for the *R. sphaeroides* oxidase numbering), are replaced in the ba_3 enzyme by Ser309 and Thr312. Other residues of this pathway include Tyr244 (not shown in Fig. 1), Tyr248 and structural water molecules connected to Tyr 237, which is covalently bound to the histidine ligand of Cu_B. In the lower part of this pathway Asp517, Glu15(II) (not shown in Fig. 1) and Ser261 provide the proton connection to the surface.

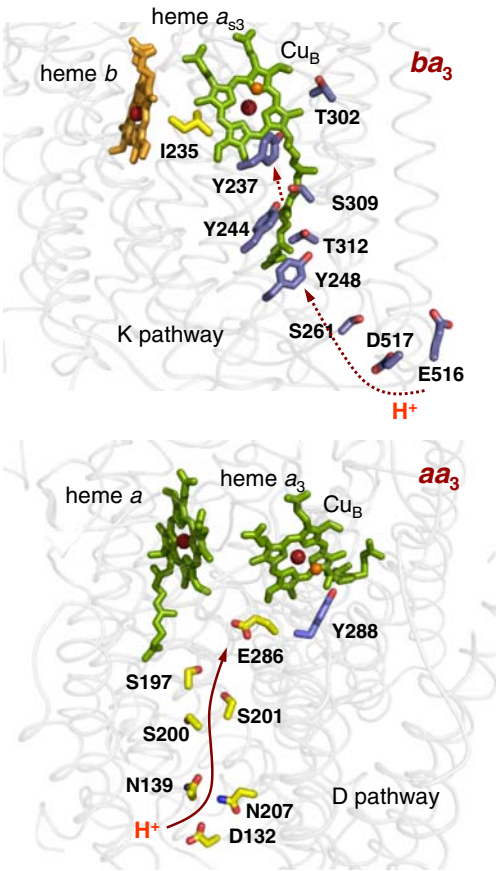

Fig. 1 Proton-conducting pathways in ba_3 cytochrome *c* oxidase from *T. thermophilus* (structure 1EHK in the PDB data bank; K pathway) and aa_3 cytochrome *c* oxidase from *R. sphaeroides* (structure 1M56 in the PDB data bank; D pathway). Only pathways that are used in the oxidative half of the catalytic cycle are shown. Haems of *a*-type and *b*-type are shown in *green* and *light orange*, respectively. Iron atoms of the haems and Cu_B are shown in *red* and *orange*, respectively. Oxygen atoms are *red* and nitrogen atoms *blue*. The residue Ile235 in the ba_3 oxidase structure occupies the same position as residue Glu286 of the aa_3 oxidase. *Red arrows* indicate proton transfer along the pathways. Water molecules of the pathways are not included

The other proton pathway with a location equivalent to that of the D pathway in the type A oxidases can be traced from the protein surface (Glu17) via Tyr91, Thr21 and two structural water molecules to an internal cavity 12.6 Å away from the catalytic site. A key residue of the D pathway of type A oxidases, $Glu286^R$ (see Fig. 1), is not found in the ba_3 enzyme. Instead, protons may be transferred to the catalytic site either directly from this cavity or via residues Thr81, Thr394 and Ser391, shared with a third proton pathway called the Q pathway. The Q pathway starts at Glu254 and leads via two structural water molecules and Thr396, the carbonyl oxygen of Leu392 and Ser391 to the junction with the D pathway mentioned above.

The flow-flash technique has been used extensively in studies of the reaction of terminal oxidases with O_2. When using this technique a solution of the enzyme with CO bound at the catalytic site (haem a_3) is mixed with an oxygenated buffer in a stopped-flow apparatus. A short time after mixing the reaction of the reduced enzyme with oxygen is initiated by flash-induced dissociation of the blocking CO ligand. Oxygen binding and the subsequent step-wise reduction of O_2 to H_2O can be followed in time using various spectroscopic techniques (for reviews, see Brändén et al. 2006; Einarsdóttir 1995; Ferguson-Miller and Babcock 1996; Namslauer and Brzezinski 2004). In the aa_3-type oxidases, e.g. from *R. sphaeroides*, initially oxygen binds to the reduced haem a_3 forming state A ($k \cong 1\times10^5\ s^{-1}$). In the next step the O–O bond is broken and an electron is transferred from haem *a* to the catalytic site forming the "peroxy", P_R state ($k \cong 2\times10^4\ s^{-1}$). Next, a proton is taken up from solution to the catalytic site forming the ferryl state, F, simultaneous with fractional electron transfer from Cu_A to haem *a* ($k \cong 1\times10^4\ s^{-1}$). Finally, the fully-oxidized enzyme (O) is formed concomitantly with proton uptake ($k \cong 0.8\times10^3\ s^{-1}$). During the reaction, protons are pumped in the P_R to F and F to O transitions (Faxén et al. 2005; Jasaitis et al. 1999; Wikström and Verkhovsky 2002).

Very recently, results of a study of the reaction of the reduced ba_3-cytochrome *c* oxidase with oxygen were published (Siletsky et al. 2007). In that study optical absorption spectra of the reaction intermediates were obtained and vectorial charge translocation reactions were monitored. The authors could not detect the $P_R \rightarrow F$ transition spectroscopically and concluded that these two intermediate states had the same optical absorption spectra. This conclusion was based on observation of haem *b* reduction simultaneously with the putative $P_R \rightarrow F$ transition and internal charge transfer. In the present study we investigated rapid electron and proton transfer in the ba_3-cytochrome *c* oxidase during the oxidative part of the catalytic cycle using the flow-flash technique and at the same time we measured proton uptake linked to the oxidation reaction. We observed proton uptake over the same time scale as that of haem *b* reduction, which indicates that the F state is indeed formed on that time scale. Furthermore, the data presented by Siletsky et al. (2007) were interpreted to suggest that no proton pumping occurred during the P_R to F transition while one proton was pumped during the F to O transition. When determining the proton-pumping stoichiometry it is essential to know in which steps substrate protons are taken up and the stoichiometry of the proton uptake. In the present study we show that approximately equal amounts of substrate protons (~0.7) are taken up from solution during each of the P_R to F and the F to O transitions. These protons are transferred with rates similar to those observed with the aa_3-type oxidases in spite of the very different proton-conducting pathway architecture and the absence of the key residue $Glu286^R$.

Materials and methods

Cell growth and enzyme purification

Cultivation of *T. thermophilus* HB8 and purification of the ba_3-cytochrome *c* oxidase were performed as described in (Chen et al. 2005; Keightley et al. 1995).

Electron transfer and proton uptake measurement

The cytochrome ba_3 samples were prepared in anaerobic cuvettes as described in (Ädelroth et al. 1997, 1998). Completely reduced (i.e. with four electrons/enzyme) cytochrome ba_3 in 100 mM HEPES–NaOH (pH 7.5), 0.1% dodecyl-β-D-maltoside was obtained by addition of 2 mM sodium ascorbate and the redox mediator 5 μM phenazine methosulphate (PMS) after replacement of air for nitrogen on a vacuum line. Then, nitrogen was exchanged for carbon monoxide.

Flow-flash experiments were performed using a locally modified stopped-flow apparatus (Applied Photophysics, DX-17MV) as described in (Ädelroth et al. 1997). The enzyme and O_2-containing solutions were mixed with an oxygen-saturated solution (~1.2 mM oxygen) at a ratio of 1:5 resulting in a final oxygen concentration of ~1 mM. The reaction of the enzyme with oxygen was initiated by flash photolysis of the enzyme–CO complex (10 ns; 200 mJ; 532 nm, Nd-YAG laser from Spectra Physics). The kinetics was monitored at different single wavelengths (see figures). The traces at different wavelengths were fit to a sum of kinetics components. Rate constants of the specific partial reactions observed at different wavelengths were averaged.

The concentration of cytochrome ba_3 was calculated from the difference absorption coefficient "reduced minus oxidized" for haem a_{s3} ($\varepsilon_{613-658}$=6.3 mM^{-1} cm^{-1} (Zimmermann et al. 1988). The concentration of enzyme that reacted with O_2 was estimated from the CO-dissociation absorbance change at 445 nm using $\Delta\varepsilon_{445}$=67 mM^{-1} cm^{-1} (Siletsky et al. 2007). Other data in the literature, based on cytochrome ba_3 static spectra, give a reduced-CO minus reduced difference absorption coefficient of $\Delta\varepsilon_{445}$=50 mM^{-1} cm^{-1} (Zimmermann et al. 1988). Using this value all absorbance changes in this paper would have to be multiplied by a factor of 1.3.

Proton uptake during oxidation of the fully reduced enzyme with oxygen was measured as described in (Ädelroth et al. 1997) using the pH indicator dye cresol red (pK_a=8.3) at the concentration of 40 μM. This indicator dye was selected because it enabled us to perform measurements at 580 nm where the contribution of absorbance changes of the haems is minimal. The measurements were carried out in a buffer-free solution containing 100 mM KCl and 0.1% DM with the pH adjusted to 8.0–8.2. The sample buffer was exchanged for this solution using a concentrating filter (Centriprep-30, BioRad). The traces obtained in the presence of buffer (20 mM Tris–HCl pH 8.0; 0.1% DM) were subtracted from those obtained in the absence of buffer to exclude possible contributions of the haems (20–25 traces were averaged). In order to estimate the amount of protons, the exhaust solution from the stopped-flow apparatus (in the absence of buffer) was collected, its pH was adjusted to 8.0 and absorbance changes corresponding to a given number of protons were determined by additions of known amounts of sulfuric acid and/or potassium hydroxide.

Results

The complex of the fully reduced cytochrome *c* oxidase from *T. thermophilus* with carbon monoxide bound to haem a_{s3} was mixed with an oxygen-containing solution in a stopped-flow apparatus. After approximately 20 ms the CO ligand was dissociation by a laser flash, which resulted in binding of O_2 to haem a_{s3}. The flash-induced dissociation of CO is accompanied by a rapid absorbance change (see e.g. Fig. 2A). The subsequent time-resolved absorbance changes reflect the reaction of the fully reduced cytochrome ba_3 with oxygen, where redox changes of the two haem groups and formation and decay of intermediate states at the catalytic site were detected at different wavelengths. These measurements, together with the data obtained by Siletsky et al. (2007), allowed us to identify the different kinetic phases with specific reactions in the enzyme.

The haem a_{s3}–CO-complex was formed in about 70% of the enzyme population, which corresponds to an equilibrium constant of about 2 for CO binding to Cu_B and haem a_{s3} with a larger fraction bound to the haem a_{s3}. This number is consistent with our studies of CO binding to the fully reduced cytochrome *c* oxidase under anaerobic conditions (unpublished data). Qualitatively, the same behaviour was observed by Giuffrè et al. (1999), but they obtained a different equilibrium constant.

At 445 nm (Fig. 2A) after the rapid increase in absorbance, associated with flash-photolysis of the CO-complex, the first phase displayed a rate constant of 6.8±0.5×10^4 s^{-1} (τ≅15 μs) and it is attributed to formation of the P_R state. The second phase, with a rate constant of 1.7±0.3×10^4 s^{-1} (τ≅60 μs) occurs over the same time

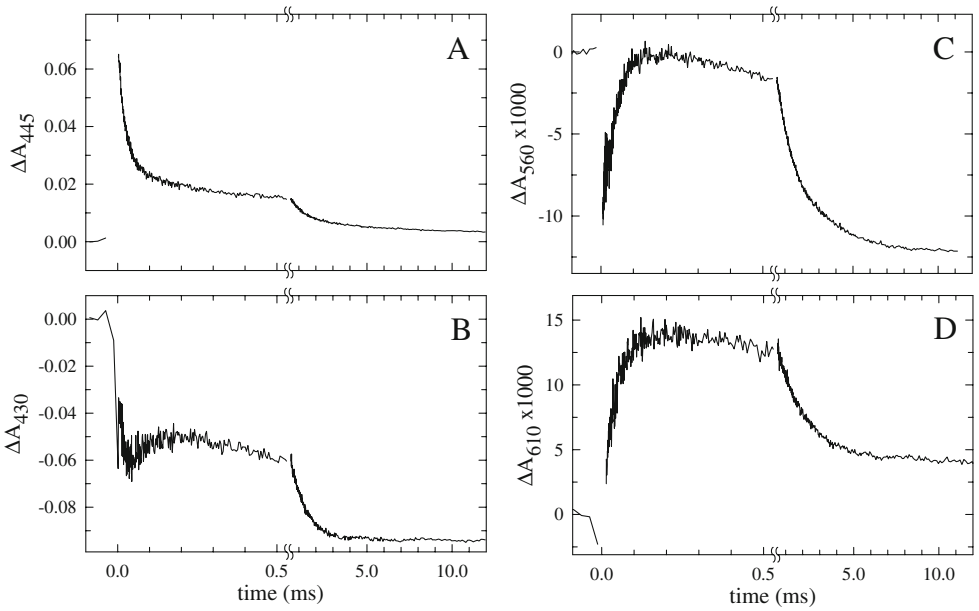

Fig. 2 Absorbance changes associated with reaction of the fully reduced ba_3 cytochrome *c* oxidase with oxygen. The absorbance changes were monitored at 445 nm (**A**), 430 nm (**B**), 560 nm (**C**) and 610 nm (**D**). Experimental conditions: 100 mM HEPES–KOH (pH 7.5); 0.1% dodecyl-β-D-maltoside; 22°C. Amplitudes are normalized to 1 μM reactive enzyme. The CO-ligand was dissociated by a laser flash at *t*=0

scale as formation of the F state (see "Discussion"). It is followed by two phases with rate constants of 1,100± 200 s^{-1} (τ≅0.9 ms) and ~200 s^{-1} (τ≅5 ms), associated with formation of the fully oxidized enzyme. A comparison of the total absorbance change at 445 nm immediately after the flash and at the end of the reaction shows that ~75% of the enzyme was oxidized.

At 430 nm the absorbance changes are dominated by redox changes of haem *b* with a minor contribution from the P_R and F intermediates formed at the catalytic site (haem a_{s3}). At this wavelength a rapid decrease in absorbance, associated with dissociation of CO, was followed by a further decrease in absorbance with a rate constant of $6.8\pm0.5\times10^4$ s^{-1}, associated with oxidation of haem *b*, and a subsequent increase in absorbance with a rate constant of $1.7\pm0.3\times10^4$ s^{-1}, associated with transient re-reduction of haem *b*. In the final step haem *b* was oxidized again, which is seen as a decrease in absorbance with a rate constant of 1,100±200 s^{-1} (Fig. 2B). Qualitatively, the same behaviour was observed at 422 nm (not shown) where the contribution of haem a_{s3} is close to zero and the absorbance changes originate primarily from redox changes at haem *b*.

In the α-region of the spectrum the reduced haem *b* displays a peak at about 560 nm. At this wavelength the $6.8\pm0.5\times10^4$ s^{-1} component, seen in the Soret region, was not resolved because it was partly masked by a laser artefact. After the initial decrease in absorbance we observed an increase in absorbance with a rate constant of $1.7\pm0.3\times10^4$ s^{-1} (Fig. 2C), consistent with reduction of haem *b*. Finally, haem *b* was oxidized with a rate constant of 1,100±200 s^{-1}. In addition, we observed a small component (13%) with a rate constant of ~200 s^{-1}.

Haem a_{s3} has a maximum at 612 nm in the reduced minus oxidized spectrum (Zimmermann et al. 1988), which is also close to a maximum for the P_R intermediate at 607 nm (on comparison with aa_3-type oxidases (Wikström and Morgan 1992). At 610 nm we observed an absorbance increase (80% contribution) with rate constants of $6.8\pm0.5\times10^4$ s^{-1}, associated with formation of the P_R intermediate, followed by a slower component with a rate constant of $1.7\pm0.3\times10^4$ s^{-1} (20% contribution; Fig. 2D), i.e. over the same time scale as that of F-intermediate formation. This observation suggests that the molar extinction of the F intermediate is even slightly larger than that of the P_R state. In case of the aa_3-type oxidases the F intermediate has a characteristic peak at 580 nm. For the ba_3 cytochrome *c* oxidase no absorbance changes were observed at 580 nm on this time scale, which is consisted with the results obtained by Siletsky et al. (2007) demonstrating a close similarity of the P_R and F-intermediate spectra. The absorbance decrease at 610 nm associated with the final oxidation of the enzyme was composed of two phases with rate constants of ~1,100 s^{-1} (90%) and ~200 s^{-1} (10%).

Proton uptake associated with oxygen reduction was investigated using the pH-indicator dye cresol red and monitored at 580 nm (this wavelength was selected in order to minimize the contribution of the *b*-haem; Fig. 3). Two major phases were resolved with rate constants of $1.7\pm0.3\times10^4$ s^{-1} and 1,100±200 s^{-1}, and approximately equal contributions (44% and 50%, respectively). A much slower phase with rate constant of ~200 s^{-1} contributed with 6% of the amplitude. The net proton uptake stoichiometry during the two fast phases was estimated to be 1.5±0.1 H^+/enzyme.

Discussion

The reaction sequence of the four-electron reduced cytochrome ba_3 with O_2 was similar to that of the aa_3-type oxidases (Siletsky et al. 2007; Fig. 4). After dissociation of CO from reduced haem a_{s3} binding of O_2 was previously observed to occur with a second-order rate constant of ~1.7×10^8 M^{-1} s^{-1} (Siletsky et al. 2007), which at 1 mM O_2 corresponds to a time constant of ~6 μs. This initial reaction was not resolved in our measurements.

The first resolved reaction after binding of O_2 was oxidation of both haems *b* and a_{s3} and formation of the P_R intermediate with a rate constant of $6.8\pm0.5\times10^4$ s^{-1} (τ≅15 μs), which is a factor of 2–3 faster than in the *R. sphaeroides* and mitochondrial aa_3 oxidases (Einarsdóttir 1995; Ferguson-Miller and Babcock 1996; Namslauer and Brzezinski 2004; Ädelroth et al. 1998). This reaction was seen at 610 nm (Fig. 2D) as an increase in absorbance and also as a decrease in absorbance at 445 nm (Fig. 2A).

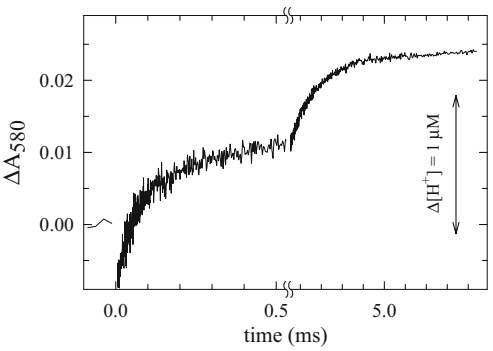

Fig. 3 Proton uptake upon reaction of the fully reduced ba_3 cytochrome *c* oxidase with oxygen. The trace is the difference between the averaged trace obtained without buffer and in the presence of buffer (see "Materials and methods"). Experimental conditions: 0.1% dodecyl-β-D-maltoside; 40 μM cresol red; 22°C and, 100 mM KCl (without buffer, pH adjusted to 8.0–8.2 with KOH) or 20 mM Tris–HCl (pH 8.0) (with buffer). The amplitude is normalized to 1 μM reactive enzyme. The CO-ligand was dissociated by a laser flash at *t*=0

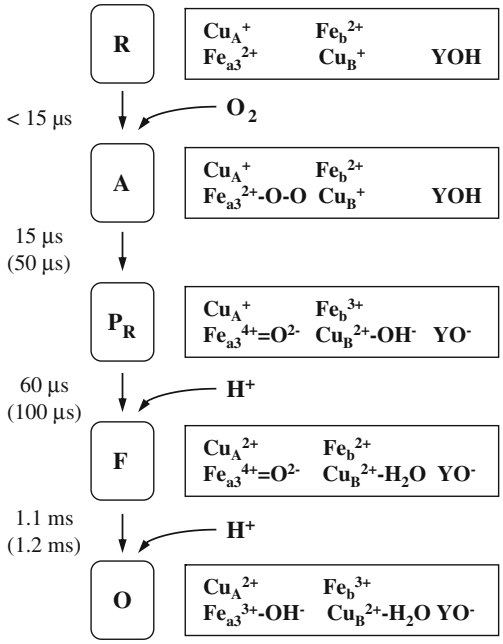

Fig. 4 A reaction scheme illustrating the oxidative part of the reaction cycle of the ba_3 cytochrome *c* oxidase. The suggested structures of the intermediates are shown in *rectangles* to the *right* (Cu_A: copper A; Fe_b: the iron ion of haem *b*; Cu_B: copper B; Fe_{a3}: the iron ion of haem a_{s3} and *Y*: Tyr 237 proton-donor group in a vicinity of the binuclear centre). Time constants for the transitions are shown to the *left* (time constants for analogous reactions in the aa_3 oxidase from *R. sphaeroides* (Namslauer and Brzezinski 2004) are given in the *parentheses*)

Oxidation of haem *b* was seen as a decrease in absorbance at 422 nm (not shown) and 430 nm (Fig. 2B), where mainly haem *b* contributes.

In the next step, haem *b* was re-reduced in nearly 100% of the enzyme population with a rate constant of $1.7\pm0.3\times10^4$ s^{-1} ($\tau\cong60$ µs), as seen at 430 nm and 560 nm. On the same time scale, in the aa_3 oxidases, the F state is formed, which is also linked to electron transfer from Cu_A to haem *a*. In the ba_3 oxidase the $P_R \rightarrow F$ transition itself could not be seen (this study and Siletsky et al. 2007), and based on the observation of the Cu_A to haem *b* electron transfer Siletsky et al. proposed that the P_R and F states have the same optical absorption spectra. In the present study we found that the 60-µs electron transfer to haem *b* was accompanied by proton uptake from solution with a stoichiometry of ~0.75 H^+ per enzyme molecule, which is similar to that observed with the *R. sphaeroides* aa_3-type oxidase (Ädelroth et al. 1998). Because proton uptake is required for formation of state F, our results indicate that the $P_R \rightarrow F$ transition occurred with a time constant of 60 µs. In other words, our data support the conclusion that state F is formed from P_R without any significant absorbance changes associated with the transition itself.

The rate constant of the P_R to F transition and the accompanying proton uptake in the ba_3 oxidase is about the same as that observed with the aa_3-type oxidases from bovine heart mitochondria (~1.4×10^4 s^{-1}) and *R. sphaeroides* (~1.1×10^4 s^{-1}; Einarsdóttir 1995; Ferguson-Miller and Babcock 1996; Namslauer and Brzezinski 2004; Ädelroth et al. 1998). This results shows that the proton pathway in the ba_3 oxidase is capable of delivering protons with the same rate as in the aa_3 oxidases despite the significantly different architecture (see "Introduction").

According to our data, as well as those presented recently by Siletsky et al. (2007), the P_R to F transition in the cytochrome ba_3 differs from the analogous step in cytochrome aa_3 from e.g. *R. sphaeroides* in that the shift in the absorbance maximum from 610 to 580 nm does not occur (see above). Such a shift of the maximum to 580 nm upon formation of F intermediate for the aa_3 type oxidases is assumed to be related to protonation of a group at the catalytic site. As discussed by Siletsky et al. (2007) the difference in the absorbance changes at e.g. 580 nm for the aa_3 and ba_3 oxidases may be due to different proton acceptors in the two enzymes, i.e., $Tyr288^-$ or a hydroxide bound to Cu_B. Independently of the origin of this proton acceptor in the aa_3-oxidases, it would be the other one in the ba_3 enzyme (In Fig. 4, we have chosen to indicate the OH^- as the proton acceptor in the ba_3 oxidase).

The last step of the oxidative part of the catalytic cycle is the formation of the oxidized enzyme. Oxidation of the haems *b* and a_{s3} displayed a rate constant of $1{,}100\pm200$ s^{-1}. In addition there was a minor component with a rate constant f ~200 s^{-1}, which has also been observed for the $F \rightarrow O$ transition in the aa_3-oxidases, primarily in electrometric studies (Jasaitis et al. 1999). These two reactions were accompanied by the uptake of 0.66 and 0.09 H^+ per enzyme molecule, respectively. In the $F \rightarrow O$ transition, the proton is transferred to the oxo-ferryl (at haem a_3) and therefore the proton acceptor is the same in both the aa_3 and ba_3 oxidases.

As discussed above, the proton-uptake rates and extents during the $P_R \rightarrow F$ and $F \rightarrow O$ transitions during the oxidative part of the reaction cycle are similar to those observed for e.g. the *R. sphaeroides* cytochrome aa_3. Yet, in the ba_3 oxidase, during this part of the reaction cycle proton pumping occurs exclusively in the $F \rightarrow O$ transition (Siletsky et al. 2007) and the $P_R \rightarrow F$ transition only involves uptake of substrate protons. The absence of proton pumping in the $P_R \rightarrow F$ transition may be explained by the different nature of the proton acceptor in the ba_3 oxidase as compared to the aa_3 enzymes (see discussion above), specifically during this transition; if the pK_a of the proton acceptor is too low, the free energy for protonation of the acceptor may not be sufficient for proton pumping (Blomberg et al. 2006).

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