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Electron and Proton Transfer in the *ba*₃ oxidase from *Thermus thermophilus*

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Abstract

The *ba*₃-type cytochrome *c* oxidase from *T. 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 O₂ 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, *R. sphaeroides* and *P. 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* ($k \cong 6.8 \cdot 10^4 \text{ s}^{-1}$) and formation of the peroxy (P_R) intermediate. In the next step a proton was taken up from solution with a rate constant of $\sim 1.7 \cdot 10^4 \text{ s}^{-1}$, 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 $\sim 1100 \text{ s}^{-1}$, 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.* (2007) *Biochim Biophys Acta* 1767, 138) 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

Introduction

The *ba*₃-cytochrome *c* oxidase from the extremely thermophilic eu-bacterium *Thermus* (*T.*) *thermophilus* is expressed at low oxygen concentrations. The enzyme is an integral 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

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proton gradient (for review on oxidases in general, see (1–8)). Presumably, the enzyme pumps protons with a stoichiometry $\sim 0.5 \text{ H}^+/\text{e}^-$ (9) (see also (10)), i.e. lower than that of the aa_3 -type oxidases ($\sim 1 \text{ H}^+/\text{e}^-$). The three-dimensional structure of the ba_3 -cytochrome *c* oxidase from *T. thermophilus*, determined to a resolution of 2.4 Å (11) 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_{53} and Cu_B . Haem *b* has a low-spin iron and two axial histidine residue ligands. Haem a_{53} 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 (R.) sphaeroides*, *Paracoccus (P.) denitrificans* and mitochondria from bovine heart (12–16). Furthermore, haem a_{53} 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 (11).

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 (8). 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. 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 Tyr294, 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) and Ser261 provide the proton connection to the surface.

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, 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 (1,17–19). In the *aa*₃-type oxidases, e.g. from *R. sphaeroides*, initially oxygen binds to the reduced haem *a*₃ forming state A ($k \cong 1 \cdot 10^5 \text{ s}^{-1}$). 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 ($k \cong 2 \cdot 10^4 \text{ s}^{-1}$). 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* ($k \cong 1 \cdot 10^4 \text{ s}^{-1}$). Finally, the fully-oxidized enzyme (O) is formed concomitantly with proton uptake ($k \cong 0.8 \cdot 10^3 \text{ s}^{-1}$). During the reaction, protons are pumped in the P_R to F and F to O transitions (20–22).

Very recently, results of a study of the reaction of the reduced *ba*₃-cytochrome *c* oxidase with oxygen were published (10). 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 → 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 → 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.* (10) 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 *Thermus thermophilus* HB8 and purification of the *ba*₃-cytochrome *c* oxidase were performed as described in (23).

Electron transfer and proton uptake measurement

The cytochrome *ba*₃ samples were prepared in anaerobic cuvettes as described in (24,25). Completely reduced (i.e. with 4 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 (24). The enzyme and O₂-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*₃ ($\epsilon_{613-658} = 6.3 \text{ mM}^{-1}\text{cm}^{-1}$ (26)). The concentration of enzyme that reacted with O₂ was estimated from the CO-dissociation absorbance change at 445 nm using $\Delta\epsilon_{445} = 67 \text{ mM}^{-1}\text{cm}^{-1}$ (10). Other data in the literature, based on cytochrome *ba*₃ static spectra, give a reduced-CO minus reduced difference absorption coefficient of $\Delta\epsilon_{445} = 50 \text{ mM}^{-1}\text{cm}^{-1}$ (26). 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 (24) using the pH indicator dye cresol red ($\text{pK}_a=8.3$) 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 7.5–7.7. The sample buffer was exchanged for this solution using a concentrating filter (Centriprep-30, BioRad). The traces obtained in the presence of buffer (100 mM HEPES-KOH pH 7.5; 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 7.5 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*₃ 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 O₂ to haem *a*₃. The flash-induced dissociation of CO is accompanied by a rapid absorbance change (see e.g. Fig. 1A). 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.* (10), allowed us to identify the different kinetic phases with specific reactions in the enzyme.

The haem *a*₃-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*₃ with a larger fraction bound to the haem *a*₃. 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.*, but they obtained a different equilibrium constant (27).

At 445 nm (Fig. 1A) after the rapid increase in absorbance, associated with flash-photolysis of the CO-complex, the first phase displayed a rate constant of $6.8 \pm 0.5 \cdot 10^4 \text{ s}^{-1}$ ($\tau \cong 15 \mu\text{s}$) and it is attributed to formation of the **P_R** state. The second phase, with a rate constant of $1.7 \pm 0.3 \cdot 10^4 \text{ s}^{-1}$ ($\tau \cong 60 \mu\text{s}$) occurs over the same time scale as formation of the **F** state (see Discussion). It is followed by two phases with rate constants of $1100 \pm 200 \text{ s}^{-1}$ ($\tau \cong 0.9 \text{ ms}$) and $\sim 200 \text{ s}^{-1}$ ($\tau \cong 5 \text{ ms}$), 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 $\sim 75\%$ 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*₃). 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 $6.8 \pm 0.5 \cdot 10^4 \text{ s}^{-1}$, associated with oxidation of haem *b*, and a subsequent increase in absorbance with a rate constant of $1.7 \pm 0.3 \cdot 10^4 \text{ s}^{-1}$, 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 $1100 \pm 200 \text{ s}^{-1}$ (Fig. 1B). Qualitatively, the same behaviour was observed at 422 nm (not shown) where the contribution of haem *a*₃ 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 $6.8 \pm 0.5 \cdot 10^4 \text{ s}^{-1}$ 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 $1.7 \pm 0.3 \cdot 10^4 \text{ s}^{-1}$ (Fig. 1C), consistent with reduction of haem *b*. Finally, haem *b* was oxidized with a rate constant of $1100 \pm 200 \text{ s}^{-1}$. In addition, we observed a small component (13%) with a rate constant of $\sim 200 \text{ s}^{-1}$.

Haem *a*₃ has a maximum at 612 nm in the reduced minus oxidized spectrum (26), which is also close to a maximum for the P_R intermediate at 607 nm (on comparison with *aa*₃-type oxidases (28)). At 610 nm we observed an absorbance increase (80% contribution) with rate constants of $6.8 \pm 0.5 \cdot 10^4 \text{ s}^{-1}$, associated with formation of the P_R intermediate, followed by a slower component with a rate constant of $1.7 \pm 0.3 \cdot 10^4 \text{ s}^{-1}$ (20% contribution) (Fig. 1D), 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 consistent with the results obtained by Siletsky *et al.* (10) 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 $\sim 1100 \text{ s}^{-1}$ (90%) and $\sim 200 \text{ s}^{-1}$ (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. 2). Two major phases were resolved with rate constants of $1.7 \pm 0.3 \cdot 10^4 \text{ s}^{-1}$ and $1100 \pm 200 \text{ s}^{-1}$, and approximately equal contributions (44% and 50%, respectively). A much slower phase with rate constant of $\sim 200 \text{ s}^{-1}$ contributed with 6% of the amplitude. The net proton uptake stoichiometry during the two fast phases was estimated to be $1.5 \pm 0.1 \text{ H}^+/\text{enzyme}$.

Discussion

The reaction sequence of the four-electron reduced cytochrome *ba*₃ with O₂ was similar to that of the *aa*₃-type oxidases (10) (Figure 3). After dissociation of CO from reduced haem *a*₃ binding of O₂ was previously observed to occur with a second-order rate constant of $\sim 1.7 \cdot 10^8 \text{ M}^{-1} \text{ s}^{-1}$ (10), which at 1 mM O₂ corresponds to a time constant of $\sim 6 \text{ } \mu\text{s}$. This initial reaction was not resolved in our measurements.

The first resolved reaction after binding of O₂ was oxidation of both haems *b* and *a*₃ and formation of the P_R intermediate with a rate constant of $6.8 \pm 0.5 \cdot 10^4 \text{ s}^{-1}$ ($\tau \cong 15 \text{ } \mu\text{s}$), which is a factor of 2–3 faster than in the *R. sphaeroides* and mitochondrial *aa*₃ oxidases (17,19,25, 29). This reaction was seen at 610 nm (Figure 2d) as an increase in absorbance and also as a decrease in absorbance at 445 nm (Figure 2a). Oxidation of haem *b* was seen as a decrease in absorbance at 422 nm (not shown) and 430 nm (Figure 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 $1.7 \pm 0.3 \cdot 10^4 \text{ s}^{-1}$ ($\tau \cong 60 \text{ }\mu\text{s}$), 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 → F** transition itself could not be seen (this study and (10)), 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 $\sim 0.75 \text{ H}^+$ per enzyme molecule, which is similar to that observed with the *R. sphaeroides aa*₃-type oxidase (25). Because proton uptake is required for formation of state F, our results indicate that the **P_R → 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 ($\sim 1.4 \cdot 10^4 \text{ s}^{-1}$) and *R. sphaeroides* ($\sim 1.1 \cdot 10^4 \text{ s}^{-1}$) (17,19,25,29). 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.* (10), 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 nm 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.* (10) 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 Figure 3, 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*₃ displayed a rate constant of $1100 \pm 200 \text{ s}^{-1}$. In addition there was a minor component with a rate constant of $\sim 200 \text{ s}^{-1}$, which has also been observed for the **F → O** transition in the *aa*₃-oxidases, primarily in electrometric studies (21). These two reactions were accompanied by the uptake of 0.66 and 0.09 H^+ per enzyme molecule, respectively. In the **F → 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 → F** and **F → 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 → O** transition (10) and the **P_R → F** transition only involves uptake of substrate protons. The absence of proton pumping in the **P_R → 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 p*K*_a of the proton acceptor is too low, the free energy for protonation of the acceptor may not be sufficient for proton pumping (30).

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)

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Figure 1.

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 μ M reactive enzyme. The CO-ligand was dissociated by a laser flash at $t = 0$.

Figure 2.

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 μ M cresol red; 22°C and, 100 mM KCl (without buffer, pH adjusted to 7.5–7.7 with KOH) or 100 mM HEPES-KOH (pH 7.5). The amplitude is normalized to 1 μ M reactive enzyme. The CO-ligand was dissociated by a laser flash at $t = 0$.

Figure 3.

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*₃ 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* (19) are given in the parentheses).