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Time-resolved single-turnover of ba_3 oxidase from *Thermus thermophilus*

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Abstract

The kinetics of the oxidation of fully-reduced ba_3 cytochrome *c* oxidase from *Thermus thermophilus* by oxygen were followed by time-resolved optical spectroscopy and electrometry. Four catalytic intermediates were resolved during this reaction. The chemical nature and the spectral properties of three intermediates (compounds **A**, **P** and **O**) reproduce the general features of aa_3-type oxidases. However the **F** intermediate in ba_3 oxidase has a spectrum identical to the **P** state. This indicates that the proton taken up during the **P**→**F** transition does not reside in the binuclear site but is rather transferred to the covalently cross-linked tyrosine near that site. The total charge translocation associated with the **F**→**O** transition in ba_3 oxidase is close to that observed during the **F**→**O** transition in the aa_3 oxidases. However, the $\mathbf{P_R}$→**F** transition is characterized by significantly lower charge translocation, which probably reflects the overall lower measured pumping efficiency during multiple turnovers.

Keywords: Catalytic cycle; Cytochrome *c* oxidase; Electron transfer; Electric potential generation; *Thermus thermophilus*

1. Introduction

The four-electron reduction of oxygen to water in living organisms is catalyzed by terminal oxidases [1,2], which convert redox energy into a transmembrane difference of proton electrochemical potential ($\Delta\mu_H^+$) [3]. Cytochrome ba_3 from *Thermus thermophilus* belongs to the large family of structurally related heme-copper oxidases that can be divided into at least 3 large groups denoted as A-, B- and C-type differing in the structure of proton channels and some other characteristics [4]. Whereas the most thoroughly explored canonical cytochrome *c* oxidases like the aa_3 enzymes from bovine heart, *Paracoccus denitrificans* or *Rhodobacter sphaeroides* belong to type A, the ba_3 cytochrome c oxidase from *T. thermophilus* exemplifies the B-type enzyme. This three-subunit protein is expressed under limited oxygen supply and catalyses oxidation of a highly specific electron donor, cytochrome c_{552}, by molecular oxygen [5]. Like the canonical cytochrome oxidases, ba_3 contains four redox-centers: low-spin heme *b,* high-spin heme a_3 and two copper centers (binuclear Cu_A and mononuclear Cu_B) [6,7]. Cu_A and heme *b* are the input redox-centers, through which electrons are transferred from cytochrome c_{552} into the oxygen-reducing binuclear site [8,9].

The O_2 reduction site of ba_3 oxidase is formed by heme a_3 and Cu_B as in the canonical aa_3 oxidases but nevertheless it reveals a

Abbreviations: **A**, ferrous oxy species; CcO, cytochrome *c* oxidase; DM, dodecyl-β-D-maltoside; **F**, ferryl species; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; N-side, electrically negative side of the membrane (bacterial cytoplasm); **O**, fully oxidized species; P-side, electrically positive side of the membrane (periplasmic space in bacteria); **P**, "peroxy" species; **R**, fully reduced species; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine; τ, time constant, reciprocal of rate constant

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number of odd features, including an unusual pattern of reactivity towards exogenous ligands such as CN^-, N_3^-, SH^-, CO, NO, and H_2O_2 [8,10,11]. Thus, in cytochrome ba_3 cyanide binds preferentially to the ferrous heme a_3 [11], while in the A-type oxidases its ligation to the ferric heme a_3 is very much stronger. In contrast to A-type oxidases, the ferric ba_3-enzyme does not react readily with H_2O_2 or any other heme a_3 ligands tested (cyanide, azide, sulfide) and the binuclear center was postulated to be "closed" in the oxidized enzyme, opening up after one electron reduction [8]. The affinity of Cu_B to CO in the reduced ba_3 oxidase is about 100 times higher than that in cytochrome *c* oxidase from bovine heart [12]. Some of the unusual ligand-binding features of the ba_3 oxidase from *T. thermophilus* may be shared by other B-type oxidases, such as an aa_3-type quinol oxidase from *Acidianus ambivalens* [13,14].

All known members of the heme-copper oxidase superfamily are capable of pumping protons from the N- to the P-side of the membrane against the transmembrane electrochemical gradient of protons [4]. Three proton-conducting pathways were resolved from the atomic crystal structure of cytochrome ba_3 [6]. Two of these pathways are reminiscent of the previously identified D- and K-pathways of cytochrome aa_3 [6,15–17]; however the principal amino acid residues are not conserved between cytochromes aa_3 and ba_3 [6]. For instance, the highly conserved Glu-278 (*P. denitrificans* numbering or Glu-286 from *R. sphaeroides* numbering) in the D-pathway of cytochrome aa_3 is exchanged to Ile-235 (*T. thermophilus* numbering), and has been assumed to optimize the oxygen input channel [6] crucial for survival at low oxygen concentrations.

Although cytochrome ba_3 lacks most of the highly conserved amino acid residues that form input proton channels in the A-type oxidases, it nevertheless generates an electric transmembrane potential difference under steady-state conditions, and pumps protons [18,19]. While 1 H^+/e^- is taken up from the N-side of the membrane for water formation, cytochrome ba_3 shows a reduced proton-pumping efficiency of 0.4–0.5 H^+/e^- instead of $1H^+/e^-$ found in the canonical heme-copper oxidases [4,18].

Despite of the known three-dimensional structure of cytochrome ba_3 [6,7,20], the mechanisms of proton pumping and oxygen reduction in this enzyme are still poorly understood, because there have been few time-resolved studies on electron and proton transfer events during the catalytic cycle [8,9,12]. The aim of this work was to resolve the sequence of intermediates of the catalytic cycle and to follow the transitions in this cycle where proton translocation occurs. We used the flow-flash method [21], which allows for a time-resolved study of the reaction of the fully reduced enzyme with dioxygen.

When the oxygen reaction is initiated from the so-called mixed valence (2-electron reduced) state of bovine enzyme, formation of compound **A** is followed by reduction of dioxygen yielding the $\mathbf{P_M}$ state with ferryl heme iron and cupric Cu_B. The three out of four electrons required for oxygen reduction are taken from the binuclear center, while the fourth electron is delivered from a local tyrosine residue [22] that is covalently bonded to one of the histidine ligands of Cu_B [23–25]. It is believed that the delivery of the fourth electron to the dioxygen molecule is accompanied by a proton that is provided by the cross-linked tyrosine as well, resulting in a neutral tyrosyl radical [26,27].

In the fully-reduced bovine cytochrome oxidase, binding of O_2 to heme a_3 is followed by electron transfer from heme *a* to the binuclear center with a time constant of ~30–40 μs, forming the $\mathbf{P_R}$ state [28,29]. Both $\mathbf{P_M}$ and $\mathbf{P_R}$ are characterized by a broken O–O bond, an oxo-ferryl state of heme a_3, and the same visible absorption spectrum (peak with maximum at 607 nm) with the only variation due to heme *a* oxidation in the latter case [30,31]. Thus, the $\mathbf{P_R}$ state is characterized by the presence of an extra electron in the binuclear site that is assumed to be transferred from heme *a* and to results in transient formation of a tyrosinate [22,26,27,32]. As with $\mathbf{P_M}$, formation of $\mathbf{P_R}$ is not associated with proton uptake from the outside of the protein [33].

Later, protonation of the binuclear center from the N-side of the membrane with a time constant of ~50–140 μs [28,34,35], gives rise to the next compound, the **F** state with absorption maximum at 580 nm. Formation of the **F** state is accompanied by electron equilibration between Cu_A and heme *a* [28,36], and is rate-limited by internal proton transfer from the conserved Glu-286 (numbering from *R. sphaeroides*), followed by re-protonation of Glu-286 from the N-side of the membrane [37]. During the next stage, electron transfer from the equilibrating heme a/Cu_A pair to the binuclear center is accompanied by proton uptake from the N-side to produce a water molecule in the binuclear center and to complete formation of the fully oxidized state (**O**). In addition, the $\mathbf{P_R \rightarrow F}$ and $\mathbf{F \rightarrow O}$ transitions are both linked to proton pumping across the membrane in the canonical oxidases [38]. Charge translocation across the membrane can be directly monitored by the time-resolved electrometric technique [39–43].

In this work, the kinetics of single-turnover oxidation of fully-reduced ba_3 cytochrome oxidase from *T. thermophilus* by molecular oxygen were resolved spectroscopically for the first time. Additionally, parallel kinetics of membrane potential generation was followed and partial charge transfer events determined which are coupled to the conversion of the corresponding intermediates of the binuclear center. The data obtained directly identify the transition $\mathbf{A \rightarrow P_R \rightarrow F}$ in ba_3 oxidase to be responsible for the lowered proton-pumping efficiency of this enzyme.

2. Materials and methods

2.1. Enzyme preparation and reconstitution into phospholipid vesicles

Cytochrome ba_3 was isolated from *T. thermophilus* HB8 cells as described in [12,44]. The enzyme was reconstituted into vesicles by the Bio-Beads method (SM-2 adsorbent; Bio-Rad, Hercules, CA) as described [45], except that the concentration of CcO during reconstitution was increased to 5–8 μM.

2.2. Time-resolved measurement of electric potential generation

The development of electric potential across the vesicle membrane was monitored by an electrometric technique [46], as adapted for time-resolved experiments with CcO [41,43]. Details of the sample preparation and the methodology can be found in [45].