Biochimica et Biophysica Acta 1787 (2009) 201–205

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Time-resolved $O_H \rightarrow E_H$ transition of the aberrant ba_3 oxidase from *Thermus thermophilus*

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ARTICLE INFO

Article history:
Received 20 November 2008
Received in revised form 23 December 2008
Accepted 30 December 2008
Available online 20 January 2009

Keywords:
Catalytic cycle
Cytochrome *c* oxidase
Electron transfer
Thermus thermophilus
Cytochrome ba_3

ABSTRACT

The kinetics of single-electron injection into the oxidized nonrelaxed state ($O_H \rightarrow E_H$ transition) of the aberrant ba_3 cytochrome oxidase from *Thermus thermophilus*, noted for its lowered efficiency of proton pumping, was investigated by time-resolved optical spectroscopy. Two main phases of intraprotein electron transfer were resolved. The first component ($\tau \sim 17$ μs) reflects oxidation of Cu_A and reduction of the heme groups (low-spin heme *b* and high-spin heme a_3 in a ratio close to 50:50). The subsequent component ($\tau \sim 420$ μs) includes reoxidation of both hemes by Cu_B. This is in significant contrast to the $O_H \rightarrow E_H$ transition of the aa_3-type cytochrome oxidase from *Paracoccus denitrificans*, where the fastest phase is exclusively due to transient reduction of the low-spin heme *a*, without electron equilibration with the binuclear center. On the other hand, the one-electron reduction of the relaxed O state in ba_3 oxidase was similar to that in aa_3 oxidase and only included rapid electron transfer from Cu_A to the low-spin heme *b*. This indicates a functional difference between the relaxed O and the pulsed O_H forms also in the ba_3 oxidase from *T. thermophilus*.

1. Introduction

The terminal oxidases are wide-spread membrane-bound enzymes that catalyze oxygen reduction by cytochrome *c* or ubiquinone, coupled to formation of protonmotive force across the membrane required for the synthesis of ATP [1–3]. 7 subgroups of terminal oxidases are combined into a superfamily of heme-copper oxidases [4]. The A-type oxidases form the major subgroup that includes the mitochondrial aa_3-type cytochrome *c* oxidases (CcO), and bacterial aa_3 oxidases, for example from *Paracoccus denitrificans* and *Rhodobacter sphaeroides*. The A-type oxidases couple 4-electron reduction of oxygen into water to translocation of four protons across the membrane [5,6]. Cytochrome ba_3 from *Thermus thermophilus* belongs to the heme-copper oxidases of type B, which have low sequence identity with the A-type enzymes, but with a clearly related three-dimensional structure [7]. Similar to aa_3 oxidases, cytochrome ba_3 has four redox centers: an electron-accepting bimetallic copper site (Cu_A), an electron-transferring low-spin heme *b*, and a binuclear center composed of a high spin heme a_3 with a copper ion nearby (Cu_B) (Fig. 1). Cytochrome ba_3 is expressed under limited oxygen supply; it catalyses oxidation of a highly specific electron donor, cytochrome c_{552} [8], and has been reported to have a reduced proton-pumping efficiency of 0.4–0.5 H^+/e^- [9].

Similar to the A-type CcOs, the catalytic cycle of cytochrome ba_3 may be described by distinguishing oxidative and reductive phases. The oxidative phase consists of four catalytic intermediates (see scheme in Fig. 1) [10]. The chemical nature and the spectral properties of three intermediates (compounds A, P and O_H) reproduce the general features of the aa_3-type oxidases, but the F intermediate in the ba_3 oxidase has a spectrum identical to that of the P state [10,11].

During catalytic turnover, the oxidative phase is followed by the reductive phase in which two electrons (and two protons) are delivered to the heme a_3–Cu_B site to reduce it back from O_H to the R state via a one-electron reduced intermediate E_H [12].

Recent time-resolved measurements of intraprotein charge translocation with the A-type CcO indicated that the fully-oxidized O_H state that is formed during catalytic turnover is different from the resting fully oxidized (as isolated) O-state. It was found that these two states differ in the ability to pump protons across the membrane upon reduction. The O_H state is competent in fast electron transfer between the low-spin heme and the heme a_3–Cu_B site, and is fully capable in proton translocation [12,13]. In contrast, reduction of the relaxed O state is not competent in rapid electron transfer nor in proton pumping, and in this case single-electron injection from the photo-

Abbreviations: CcO, cytochrome *c* oxidase; DM, (dodecyl L-D-maltoside); E_m, midpoint redox potential; RubiPy, tris(2,2′-bipyridyl) ruthenium; TMPD, *N*,*N*,*N*,*N*-tetramethyl-*p*-phenylenediamine; Tris, tris(hydroxymethyl)aminomethane; τ, time constant

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doi:10.1016/j.bbabio.2008.12.020

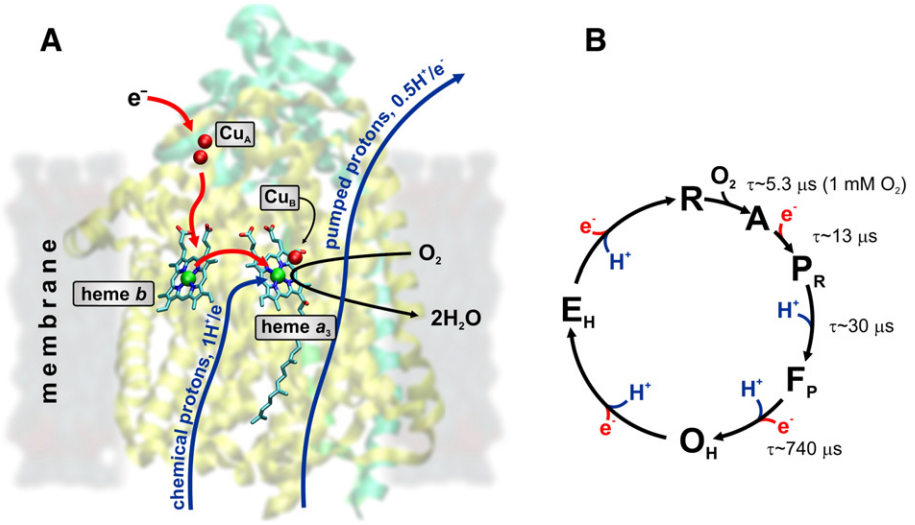

Fig. 1. (A) Structure and function of cytochrome ba_3 from *T. thermophilus* (PDB entry 1XME). Position of redox centers and direction of electron, proton, and oxygen pathways are shown. (B) Scheme of the catalytic cycle of cytochrome ba_3, time constants of certain transitions are indicated. Transitions from R to O_H form the oxidative part, while transitions from O_H to R form the reductive part of the cycle.

activated dye (RubiPy) results only in fast (10–50 μs) electron transfer from Cu_A to the low-spin heme [14–16].

The previous studies of the catalytic cycle of cytochrome ba_3 were mainly focused on the oxidative phase of the cycle, and a time-resolved study of the reductive phase is needed. In this work, the kinetics of single-electron injection into the oxidized nonrelaxed state (the $O_H \rightarrow E_H$ transition) of cytochrome ba_3 from *Thermus thermophilus* were investigated spectroscopically with adequate time resolution, and were compared with injection into the relaxed O state of the enzyme.

2. Materials and methods

2.1. Enzyme preparation

Cytochrome ba_3 was isolated from *T. thermophilus* HB8 cells as described in [17,18]. The enzyme concentration was measured using $\varepsilon^{red-ox}_{613-658} = 6.3 mM^{-1}cm^{-1}$ and $\varepsilon^{red}_{560-590} = 26 mM^{-1}cm^{-1}$. The values of oxidoreduction of the redox centers were calculated based on the following extinction coefficients: $\varepsilon^{red-ox}_{790-710} = 0.81 mM^{-1}cm^{-1}$ for Cu_A (deduced from the redox spectrum of the enzyme), for heme *b*, and $\varepsilon^{red-ox}_{613-658} = 6.3 mM^{-1}cm^{-1}$ for heme a_3 [19].

2.2. Time-resolved spectrophotometric measurements

Time-resolved multiwavelength absorption changes were followed by a home-constructed CCD-based instrument, which allows recording of absorption change surfaces with a time resolution of 1–16 μs between the spectra. This setup has been described in detail in [13]. The reaction was initiated by a laser flash (BrilliantB; Quantel, Les Ulis, France; frequency-doubled YAG, 532 nm, pulse energy −120 mJ).

2.3. Electron injection

To obtain the activated oxidized state (O_H) for absorption measurements, a solution of 90 μM CcO in 2 mM Tris (pH 8); 0.05% DM; 20 mM aniline; 15 μM TMPD was first made anaerobic on a vacuum line, and then fully reduced by 1 mM potassium ascorbate. Then, anaerobic fully reduced CcO 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 RubiPy) resulting in complete oxidation of CcO and formation of the pulsed O_H state. Immediately after the mixing, a laser flash initiated the electron injection. For a detailed description of the electron injection approach, see [1,13,14].

2.4. Data analysis and software

The surfaces of optical changes were fitted by a sequential reactions model [20]. MATLAB (the Mathworks, South Natick, MA) was used for data analysis and presentation. Computer software for experimental setups was written by Dr. N. Belevich (Helsinki, Finland).

3. Results

The single-electron injection into the O_H state (the $O_H \rightarrow E_H$ transition) was carried out in the presence of the photo-activatable dye RubiPy and its sacrificial donor, aniline. The small quantum efficiency of the electron injection assures that either one or no electron is injected. The trajectory of the injected electron through the heme centers of the enzyme was monitored by time-resolved optical spectroscopy.

3.1. Electron transfer kinetics of the $O_H \rightarrow E_H$ transition

The O_H state was formed by direct mixing of the fully reduced enzyme with oxygen- and RubiPy-containing buffer. Immediately after mixing ($\Delta t = 5$ ms) a laser flash initiates electron injection. The estimated quantum yield of electron injection into O_H was approximately 2.5%, which is several times smaller than for aa_3 CcO from *P. denitrificans* [13], and which can probably be explained by a lower affinity of RubiPy to the cytochrome c_{552} binding-site in cytochrome ba_3.

Fig. 2A shows two kinetic traces characteristic for the hemes *b* and a_3 selected from the complete surface of absorbance changes, and reflecting the electron transfer during the $O_H \rightarrow E_H$ transition. The trace at 560 nm is characteristic for heme *b*, while the trace at 611 nm is specific for heme a_3 oxidoreduction. The traces show reduction and subsequent oxidation of both hemes during the course of the reaction. The surface of optical absorbance changes during the $O_H \rightarrow E_H$ transition was globally fitted with a 2-step sequential model. The resulting kinetic spectra of the two phases are shown in Fig. 2B. The spectrum of the first phase ($\tau_1 \sim 17$ μs) has sharp peaks at 560 nm and 611 nm, and a wide peak centered at 790 nm. The peak at 790 nm can

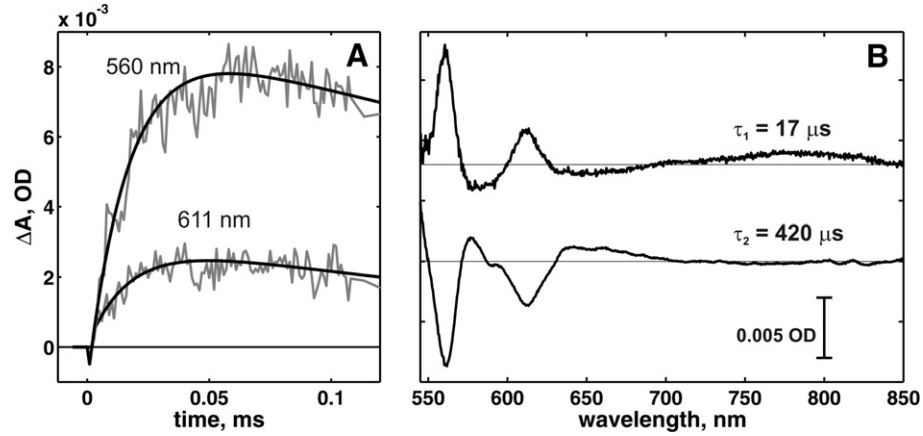

Fig. 2. Electron injection into the pulsed form of cytochrome ba_3 (the $O_H \rightarrow E_H$ transition). (A) The redox kinetics of heme *b* (560 nm) and heme a_3 (611 nm) picked from the surface of absorption spectra. (B) Kinetic absorption spectra of the phases obtained by the global fit of the experimental data. Conditions: see Materials and methods.

be assigned to oxidation of Cu_A [21], while the other peaks are due to reduction of heme *b* and heme a_3, respectively. The Cu_A center was photoreduced by RubiPy on a submicrosecond time scale, and is not resolved here.

From the amplitudes of the peaks it is possible to estimate the extent of oxidoreduction of the redox centers. The 17 μs phase consists of oxidation of ~1.15 μM of Cu_A, and reduction of ~0.59 μM and ~0.55 μM of hemes *b* and a_3, respectively, based on the extinction coefficients of these centers (see Materials and methods). The absence of optical changes around 790 nm during the later stage of the reaction indicates that Cu_A is completely oxidized by the two hemes already during the 17 μs phase; after this phase, the electron is thus almost equally shared between hemes *b* and a_3. The Cu_B center is most likely still in the oxidized state since the extent of reduction of the hemes is equal to the extent of oxidation of Cu_A.

The spectrum of the slow phase (τ_2 ~420 μs) includes troughs at 560 and 613 nm that can be assigned to reoxidation of both hemes by the Cu_B site with parallel kinetics. Because the hemes in cytochrome ba_3 have good spectral separation, it is much easier (compared to aa_3) to distinguish the spectral contributions of the low and high spin hemes. Formation of the high-spin oxidized state of heme a_3 is corroborated by the appearance of the characteristic charge-transfer band at ~660 nm (Fig. 2B, lower spectrum).

3.2. Electron Transfer kinetics of the O→E transition

Electron injection into the O_H state of cytochrome ba_3 produced interesting results that differ profoundly from those observed previously with the aa_3-type enzymes: already during the fast phase we detect simultaneous reduction of both low and high spin hemes, which was not the case for the aa_3-type oxidases [13]. To test whether reduction of both hemes during the fast phase might be a specific property of the O_H state of cytochrome ba_3, we injected electrons into the fully oxidized resting state O (as isolated), as shown in Fig. 3A. The kinetic traces at 560 and 611 nm were extracted from the surface of absorbance changes, again representing oxidoreduction of hemes *b* and a_3 respectively. A global fit of the complete surface resulted in a two exponential process with time constants of about 10.5 μs and 230 μs. The kinetic spectrum of the 10.5-μs component (Fig. 3B) has a peak at 560 nm and can be assigned to pure reduction of heme *b*, without any electron transfer to heme a_3. Thus, the fast phase of electron injection into the relaxed O state of cytochrome ba_3 is similar to that found for the O state of cytochrome aa_3, but clearly different from the above results for the O_H state. The fast phase consists in both cases of electron transfer from Cu_A to the low-spin heme, and also occurs with approximately the same time constant [15,16,22]. The fast phase in the experiments using the O state is followed by a slower 230 μs component, which consists of partial reoxidation of the low-

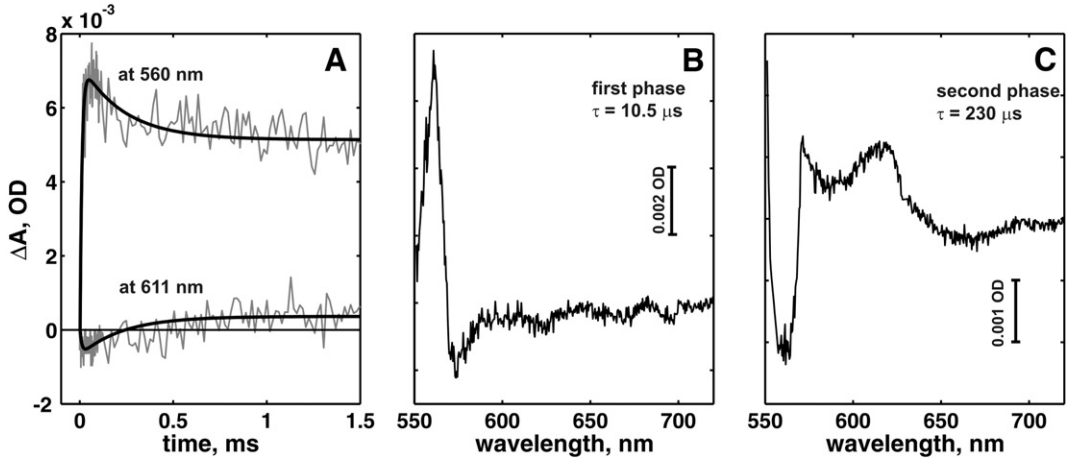

Fig. 3. Electron injection into the relaxed form of cytochrome ba_3 (the O→E transition). (A) The redox kinetics of heme *b* (560 nm) and heme a_3 (611 nm) picked from the surface of absorption spectra. (B) Kinetic absorption spectrum of the fast phase. (C) Kinetic absorption spectrum of the slow phase. Conditions: cytochrome ba_3, 50 μM; Tris (pH 8), 2 mM; DM, 0.05%; aniline, 20 mM; RubiPy, 200 μM.

spin heme *b* by the binuclear center. A spectrum of the 230 μs phase (Fig. 3C) has a sharp trough at 560 nm together with a broad peak around 615 nm, and a broad trough near 660 nm, consistent with this interpretation. These optical features indicate that by the end of the reaction the injected electron resides on hemes *b* and a_3 in the proportion 70/30. The extent of the absorption changes due to heme *b* oxidation and heme a_3 reduction indicate that there is no significant electron transfer to the Cu_B site. It is clear, therefore, that electron injection into the relaxed O state of ba_3 oxidase results in a fundamentally different response as compared to electron transfer into the metastable O_H state.

4. Discussion

The cytochrome ba_3 from *T. thermophilus* is so far the only aberrant member of the heme-copper oxidases for which a high-resolution X-ray structure is available [7]. Whereas there is a clear structural homology to the proton-pumping A-type oxidases of mitochondria, there are also crucial differences, such as the absence of a glutamic acid at the end of the D-pathway of proton transfer [7]. Several functional differences have also been reported [23–25], many of which concern the binuclear heme a_3–Cu_B site of O_2 activation and reduction, and its vicinity. A study of the details of ba_3 function is of particular interest because the enzyme has been reported to pump protons with an efficiency of only about one half of that in the A-type enzymes [9]. Hence, a comparison to the aa_3 enzymes might reveal important aspects of the proton pump mechanism.

The data on equilibrium redox titrations of ba_3 oxidase, presented up to now [26,27] are of interest to compare with the transient data observed in the present study. The equilibrium data of [27] predicts 50:50 electron distribution between the hemes under general conditions similar to those employed here. This finding agrees with ours in the transient conditions of electron injection into state O_H, but not for the case of the O state. In contrast, Hellwig et al. [26] showed that the midpoint redox potential of heme a_3 is at least 60 mV more positive than of heme *b*, which predicts a practically complete electron shift to the former. Here it is important to realize that in the fast kinetic mode the system passes through transient states, which may not be accessible in equilibrium redox titrations. Therefore, redox potentials measured at equilibrium may not be applicable for these conditions. This is clearly seen from the large difference in electron distribution between the redox centers after electron injection into O and O_H states in our experiments.

The "resting" fully oxidized state (O) of the aa_3-type enzyme is not the same as the oxidized state during catalytic turnover ("pulsed" form), as was originally shown by Antonini et al. [28]. The O state can be activated into a metastable pulsed form (O_H) by reduction of the enzyme followed by reoxidation with O_2. One key difference between the two states is the much slower electron transfer from heme *a* to the binuclear center in the O state. Discrete electron injection into the O state of the aa_3 enzyme results in fast electron transfer to heme *a* via Cu_A, but not further, at least on a millisecond time scale [15,16], whereas the O_H state responds by further electron transfer to heme a_3 and Cu_B within about a millisecond [13,29].

Most importantly, electron injection into the O state yields no proton translocation whereas the O_H state is fully competent in that respect [12,30]. Despite these crucial differences in reactivity, no significant spectroscopic difference between the two states have been detected [31], and the structural difference between the two states is hence still unknown. In this respect the present results with the aberrant ba_3 enzyme are of considerable significance. First of all, this enzyme also exhibits the O/O_H difference in the sense that electron transfer beyond the low spin heme appears to be slowed down in the O state, but is very fast in O_H. However, there is nevertheless also a clear difference in the reactivity of the relaxed O states between the two types of enzyme. In cytochrome ba_3, electron equilibration between heme *b* and heme a_3, although slowed down by a factor of at least 20 compared to the O_H state, still takes place on a submillisecond time scale ($\tau \sim 230$ μs), whilst reduction of Cu_B is blocked on this time scale. In contrast, in the aa_3-type enzyme no significant electron transfer beyond heme *a* is observed on a 100 ms time scale for the O state. On the other hand, in the metastable O_H states of both types of enzyme there is complete electron transfer into the Cu_B site within about a millisecond.

These observations give strong support to the notion that the relaxed O state is not an artifact of enzyme purification, but a true low-energy state of an oxidized CcO when it is not quickly replenished with an electron donor [30]. This is an important conclusion from this work, because it supports and emphasizes the existence of two functionally different forms of oxidized CcO [21–23] that have been largely neglected in the past.

Secondly, in the O_H state of the ba_3 enzyme the rate of electron transfer to heme a_3 is as fast ($\tau \sim 17$ μs), or even faster than that from Cu_A to the low spin heme (cf. [25]). This is very different from the aa_3 enzyme in which electron transfer to heme a_3 is delayed to $\tau \sim 150$ μs [13].

The data reported in this work directly show that, in contrast to the relaxed state O, the activated O_H state of cytochrome ba_3 is fully competent in rapid electron transfer from the input redox-centers into the catalytic heme-copper site. However it is still unknown whether the $O_H \rightarrow E_H$ transition in the ba_3 enzyme is capable of proton-pumping across the membrane, as was found earlier for *P. denitrificans* aa_3 oxidase.

The proposed mechanism of proton pumping for aa_3 oxidase [13] includes loading of a "pump site" that take place during the 150 μs phase, and associated with a shift of the electron distribution from heme *a* to heme a_3. The subsequent 800 μs proton-transfer step is coupled to electron redistribution from heme a_3 (and heme *a*) to Cu_B. In contrast, in cytochrome ba_3 the electron transfer to heme a_3 proceeds together with reduction of heme *b* already in the 17 μs phase, which is then followed by simultaneous oxidation of both hemes by Cu_B with a time constant of about 420 μs. Most probably, in the O_H state of cytochrome ba_3, the E_m value of heme a_3 is much higher than in the O_H state of aa_3 oxidase. The higher E_m may be caused by the presence of an extra proton close to the binuclear center, or by fast proton transfer to the binuclear center from a nearby amino acid donor residue. The transfer of the electron to heme a_3 already during first electron transfer step may indicate possible decoupling of the $O_H \rightarrow E_H$ transition from proton pumping. A time-resolved study of charge translocation during the $O_H \rightarrow E_H$ transition in cytochrome ba_3 is required to answer this crucial question.

We conclude that 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*. It is interesting that whilst the O state show similar (although not identical) properties in both oxidases, the O_H states behave very differently in the two types of enzymes.

Acknowledgments

This work was supported by Biocentrum Helsinki, the Sigrid Juselius Foundation, the Academy of Finland, Russian Fund for Basic Research Grant (06-04-48608 and 09-04-00140 to S.A.S.), the Science Foundation Ireland (BICF685).

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