Kinetic design of the respiratory oxidases

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Energy conservation in all kingdoms of life involves electron transfer, through a number of membrane-bound proteins, associated with proton transfer across the membrane. In aerobic organisms, the last component of this electron-transfer chain is a respiratory heme-copper oxidase that catalyzes reduction of O₂ to H₂O, linking this process to transmembrane proton pumping. So far, the molecular mechanism of proton pumping is not known for any system that is driven by electron transfer. Here, we show that this problem can be addressed and elucidated in a unique cytochrome *c* oxidase (cytochrome *ba*₃) from a thermophilic bacterium, *Thermus thermophilus*. The results show that in this oxidase the electron- and proton-transfer reactions are orchestrated in time such that previously unresolved proton-transfer reactions could be directly observed. On the basis of these data we propose that loading of the proton pump occurs upon electron transfer, but before substrate proton transfer, to the catalytic site. Furthermore, the results suggest that the pump site alternates between a protonated and deprotonated state for every second electron transferred to the catalytic site, which would explain the noninteger pumping stoichiometry (0.5 H⁺/e⁻) of the *ba*₃ oxidase. Our studies of this variant of Nature's palette of mechanistic solutions to a basic problem offer a route toward understanding energy conservation in biological systems.

rapid kinetics | membrane protein | electrochemical gradient

Respiration in aerobic bacteria, archaea, and mitochondria is supported by membrane-bound enzymes, which belong to the so-called heme-copper oxidoreductase (oxidase) family. This group of enzymes is characterized by a catalytic site consisting of a heme group and a copper ion, where O₂ is bound and reduced to water. Electron donors are either cytochrome *c* (cytochrome *c* oxidases, Cyt_cO_s) or quinol (quinol oxidases). The chemical reaction catalyzed by the heme-copper oxidases is arranged topographically such that the electrons are donated from one side of the membrane (the more positive side, *p*) and protons to the catalytic site are taken up from the opposite, more negative (*n*) side, resulting in a charge separation across the membrane equivalent to moving a positive charge from the *n* to the *p* side:

where the subscripts refer to the two sides of the membrane. Furthermore, most heme-copper oxidases characterized to date are proton pumps, which couple the O₂ reduction to translocation of protons across the membrane:

where *n* is the number of protons pumped across the membrane per electron transferred to the catalytic site (*n* ≤ 1). The free energy stored in the electrochemical potential maintained by the oxidases, as well as other components of the respiratory chain, is used, e.g., for ATP production and transmembrane transport (for review of the above-described processes, see refs. 1–5).

The heme-copper oxidases have been classified into three main classes (A, B, and C) based on genomic and structural analyses (6, 7). The A-class Cyt_cO_s (A-Cyt_cO_s) includes, e.g.,

cytochromes *aa*₃ from mitochondria, *Rhodobacter sphaeroides*, and *Paracoccus denitrificans*, whereas the *Thermus thermophilus* (8–10) and *Rhodothermus marinus* (11) *ba*₃ oxidases belong to the B class of these enzymes, in which the distinction between these two classes is mainly based on the architecture of the proton pathways. In both the A-Cyt_cO_s and the *T. thermophilus* *ba*₃ oxidase, during turnover electrons are transferred one by one to a primary electron acceptor, copper A (Cu_A), located near the more positive (*p*) side of the membrane (Fig. 1). Then, electrons are transferred consecutively to heme *a* or *b* (in *aa*₃ and *ba*₃ oxidases, respectively) and then to the catalytic site, which is composed of heme *a*₃ and copper B (Cu_B). So far, the most detailed functional studies have been performed on the A-Cyt_cO_s. In these Cyt_cO_s, each electron transfer to the catalytic site is accompanied by proton uptake from the *n* side of the membrane and proton pumping, from the *n* to the *p* side, such that the average stoichiometry is one pumped proton per electron transferred to O₂ (*n* = 1 in formula 2 above) (2, 5, 12, 13). In the A-Cyt_cO_s, protons are transferred through two or three well-defined pathways denoted by letters D, K, and H (Fig. 1A) (14–17). The K pathway is used only for transfer of two of the substrate protons upon reduction of the Cyt_cO. In the bacterial A-type Cyt_cO_s the D proton pathway is used for transfer of the remaining two substrate protons as well as for all protons that are pumped across the membrane. The branching point from where protons are transferred either to the pump or catalytic sites is found at Glu286. The H pathway has been suggested to be used for transfer of protons that are pumped in the mitochondrial Cyt_cO_s (16).

The situation is distinctly different in the *ba*₃ oxidase. Even though this oxidase harbors several putative proton pathways (8), only a K-pathway analogue is used for transfer of both pumped and substrate protons (Fig. 1B) (6, 10). In addition, in the *ba*₃ oxidases the pumping stoichiometry is lower than in the A-Cyt_cO_s such that on average 0.5 proton/electron is pumped (*n* ≈ 0.5 in formula 2 above) (18, 19).

One of the main current problems in Bioenergetics is to understand how oxygen reduction in Cyt_cO is energetically and mechanistically linked to proton pumping. When addressing this problem it is essential to investigate the individual electron- and proton-transfer reactions in the Cyt_cO_s (for review, see, e.g., ref. 20). In a representative experiment, all four electrons needed for reduction of O₂ to H₂O are preloaded into the four redox-active sites (Cu_A, heme *a/b*, heme *a*₃, and Cu_B) of these enzymes in the absence of O₂ but in the presence of carbon monoxide, which binds to the reduced heme *a*₃. This sample is mixed rapidly with an oxygen-containing solution, after which the blocking CO ligand is removed by a short laser flash. This allows O₂ to bind to the reduced heme *a*₃ and oxidation of the Cyt_cO can be investigated with a time resolution (microseconds) that allows monitoring individual internal electron- and proton-transfer reactions. This type of study has been performed mainly on the A-Cyt_cO_s (e.g., refs. 4, 5, and 21). Briefly, after O₂ binding to

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Fig. 1. Schematic structures and overall mechanism of the *R. sphaeroides* aa₃ (14) (A) and *T. thermophilus* ba₃ (8) (B) CytcOs. Electrons are transferred from a water-soluble cytochrome c to the primary electron acceptor Cu_A and then via heme a or b to the catalytic site (see red arrow), composed of heme a₃ and Cu_B, where O₂ binds and is reduced to H₂O. In the A-CytcO protons are taken up along two pathways, K and D. The former is used only for uptake of two substrate protons, whereas the latter is used for the uptake of all protons that are pumped as well as the two remaining substrate protons. In the ba₃ CytcO all protons are taken up through the K-pathway analogue. Some key residues of the different pathways are indicated. The A-class and ba₃ CytcOs pump four and two protons, respectively, per reduced O₂, as indicated in the overall reaction summarized below the pictures (subscripts *N* and *P* refer to the two sides of the membrane).

the reduced heme a₃ with a time constant (τ) of 10 μ s at 1 mM O₂, an electron is transferred from heme a to the catalytic site forming the peroxy state called **P³** (also **P_R**) ($\tau \approx 30$ μ s). Until this point there is no proton uptake from solution. Next, a proton is taken up to the catalytic site forming the ferryl state **F³**, linked to fractional electron transfer from Cu_A to heme a and proton pumping across the membrane ($\tau \approx 100$ μ s). Finally, the fourth electron from the Cu_A heme a equilibrium is transferred to the catalytic site, linked to proton uptake as well as pumping across the membrane ($\tau \approx 1.2$ ms). One main problem associated with understanding the results of these experiments is that in each step associated with proton uptake to the catalytic site (for O₂ reduction to H₂O), additional uptake and release of pumped protons occur over the same time scale, thereby complicating the dissection into individual reactions.

The above-described approach has also been used to investigate the sequence of reactions of the reduced ba₃ CytcO with O₂ (22–24). The results show that this reaction sequence is very similar to that found with the A-CytcOs. However, distinct differences were observed, which may imply that the timing of electron- and proton-transfer reactions is different in the ba₃ CytcO. To investigate the mechanism of O₂ reduction and proton pumping in the ba₃ CytcO, here we have studied the reaction of the three- and four-electron reduced enzyme with O₂ at different pH values.

Results

A sample containing reduced cytochrome ba₃ incubated with CO was mixed with O₂ after which the CO ligand was dissociated by means of a laser flash at time = 0. Fig. 2A shows absorbance changes at 560 nm where the initial decrease in absorbance after flash photolysis of CO at pH 7.5 ($\tau \approx 15$ μ s) is associated with electron transfer from heme b to the catalytic site forming state **P³**. This process was also seen as an increase in absorbance at 610 nm (Fig. 2B), which is mainly due to formation of **P³**. It was followed in time by an absorbance increase at 560 nm (Fig. 2A) and an increase at 830 nm (Fig. 2D) with $\tau \approx 60$ μ s, associated with electron transfer from Cu_A to heme b as well as proton uptake (Fig. 2C). Finally, the absorbance at both 560 nm (oxidation of heme b) and 610 nm (decay of state **P³**) decreased with $\tau \approx 0.9$ ms,

also linked to proton uptake from solution, resulting in formation of the oxidized CytcO (**O⁴**).

In an attempt to separate in time the electron-transfer and proton-transfer reactions, we repeated the same experiment at pH 10. Also at this pH heme b was oxidized and state **P³** was formed with $\tau \approx 15$ μ s (Fig. 2A and B). Re-reduction of heme b (by Cu_A) was seen with $\tau \approx 80$ μ s, linked to proton uptake from solution (Fig. 2A, C, and D). Thus, these reactions displayed time constants similar to those at pH 7.5. However, the subsequent reaction at pH 10 followed a markedly different route. Here, both the **P³** state decay (Fig. 2B) and proton uptake (Fig. 2C) occurred at $\tau \approx 1.5$ ms, whereas the final oxidation of the enzyme occurred at $\tau \approx 7$ ms, as evidenced from the absorbance changes at 560 nm (Fig. 2A). The net amount of proton uptake upon oxidation was lower at pH 10 than at pH 7.5, which is consistent with the data obtained with the mitochondrial CytcO (25).

The processes observed with the ba₃ CytcO at pH 7.5 could at first be correlated to the corresponding reactions in the A-CytcO: formation of **P³** (15 μ s), **P³** $\rightarrow$ **F³** (60 μ s), and **F³** $\rightarrow$ **O⁴** (0.9 ms), where the two latter reactions are linked in time to proton uptake. However, as noted previously (22, 23), in the ba₃ enzyme there is no absorbance change that would be linked to decay of the **P³** state on the 60- μ s time scale even though a proton is taken up, which in the A-CytcOs is indicative of the **P³** $\rightarrow$ **F³** reaction. Furthermore, the data at pH 10 indicate that the **P³** $\rightarrow$ **F³** reaction occurs *after* uptake of the first proton, over a time scale of 1.5 ms, linked to uptake of a *second* proton. Thus, this scenario suggests that electron transfer from heme b to the catalytic site, forming **P³**, triggers uptake of two protons, one being transferred to an unknown site (see Discussion) and one to the catalytic site to form state **F³**. This scenario is remarkably different from the situation observed with the A-CytcOs where only a net of one proton is taken up from solution upon forming **P³** (26). To further elucidate the differences between the A-type and ba₃ oxidases, we studied the reaction of the three-electron reduced ba₃ CytcO with O₂ (Fig. 2E–H). Here, oxidation of heme b (Fig. 2E) and formation of **P³** (Fig. 2F) were clearly seen, although slower than with the four-electron reduced CytcO ($\tau \approx 30$ μ s). However, re-reduction of heme b was not observed, as also evidenced from the lack of Cu_A absorbance changes at 830 nm (Fig. 2H).

Fig. 2. Absorbance changes associated with reaction of ba_3 Cyt c O with O_2 . Data in A–D were obtained with the 4-electron reduced Cyt c O at pH 7.5 (black) and pH 10 (green), respectively. Data in E–H were obtained with the 3 (red), approx. 3.5 (blue), and 4 (black)-electron reduced Cyt c O at pH 7.5. The main contributions at the different wavelengths follow: 560 nm—mainly redox reactions of heme b ; 610 nm—mainly formation and decay of the P^3 state (absorbance increase and decrease, respectively); 570/575 nm—pH dye phenol red at pH 7; m -cresol purple at pH 10, where an increase in absorbance is equivalent to proton uptake from solution; 830 nm—redox reaction of Cu_A . Where applicable, a slow pH drift has been subtracted from traces C and G. The vertical bars in these traces indicate the proton-uptake stoichiometry. Experimental conditions after mixing: (A), (B), (D–F), and (H): 100 mM Hepes-KOH/100 mM Ches [pH 7.5/pH 10, 0.05% dodecyl- β -D-maltoside (DDM), 22 °C]. The CO ligand was dissociated by a laser flash at time zero. Amplitudes are normalized to 1 μ M reacting ba_3 . (C and G): 150 mM KCl, pH 7.5/pH 10, 0.05% DDM, 40 mM phenol red/ m -cresol purple.

Remarkably, proton uptake occurred in two kinetic phases with $\tau \approx 70$ μ s and 1 ms (Fig. 2G), respectively; i.e., the first proton uptake did not correlate with any redox events within the oxidase, whereas the second proton was taken up over the same time scale as the absorbance decrease at 610 nm, i.e., decay of state P^3 (Fig. 2F).

Discussion

In the A-type oxidases the sequence of reactions upon binding of O_2 to the four-electron reduced Cyt c O is relatively well understood (Fig. 3A) (reviewed in ref. 27). As outlined at the beginning of the paper, after binding of O_2 to heme a_3 an electron is transferred from heme a to the catalytic site forming state P^3 with a time constant of approximately 30 μ s without any proton uptake from solution (step I). The reaction displays a very weak pH dependence such that the time constant increases to approximately 50 μ s at pH 10 (Fig. 3C, I). In the next step, one proton is transferred to a hydroxide group bound to Cu_B , which results in a spectral shift that defines formation of state F^3 (decrease in absorbance around 610 nm and appearance of a peak at 580 nm). In addition, one proton is pumped across the membrane and there is also fractional electron transfer from Cu_A to heme a . All these reactions display a time constant of approximately 100 μ s at pH 7.5 (Fig. 3A, II). At pH 10 the $P^3 \rightarrow F^3$ reaction is slowed to approximately 400 μ s and at this pH there is no proton pumping, so the reaction step is linked only to proton uptake to the catalytic site (Fig. 3C, II) (28). In addition, at high pH the Cu_A heme a equilibrium is shifted toward Cu_A in the F^3 state. Finally, the fourth electron and a proton are transferred to the catalytic site reforming the oxidized Cyt c O (O^4) (Fig. 3A, III).

The reaction sequence at the catalytic site of the reduced ba_3 Cyt c O with O_2 is the same as that of the A-Cyt c O (see below) (22–24). However, the sequence of specific proton-uptake reactions appears to be distinctly different. Although with the A-type oxidases uptake of the first proton from solution ($\tau \approx 100$ μ s) displays the same time constant as the $P^3 \rightarrow F^3$ reaction (Fig. 3A, II), with the ba_3 Cyt c O the first proton uptake ($\tau \approx 60$ μ s) is not linked to the absorbance decay at 610 nm where state P^3 contributes. This observation was previously explained in terms of identical optical absorption spectra of the P^3 and F^3 states; i.e., the $P^3 \rightarrow F^3$ reaction was assumed to take place during the first proton uptake, but being optically invisible (22–24). However, the data from the present study suggest that this is not the case. The results with the three-electron reduced ba_3 Cyt c O show that upon transfer of three electrons to the catalytic site, there was first a proton uptake with a time constant of 70 μ s, which was followed in time by additional proton uptake with a time constant of approximately 1 ms (Fig. 3B, IIa and IIb). This second proton uptake was linked in time to the absorbance decrease at 610 nm, characteristic of the decay of the P^3 state (see Fig. 2F and G). In other words, it appears that the second proton, but not the first, is transferred to the catalytic site to form F^3 . In contrast, with the A-type oxidases, during reaction of the three-electron reduced Cyt c O with O_2 , only a net of one proton is taken up, which occurs during the $P^3 \rightarrow F^3$ reaction with a time constant of approximately 100 μ s (Fig. 3B, II) (in addition, a proton is pumped across the membrane) (13). Also the data for the ba_3 Cyt c O at pH 10 suggest that there is indeed an absorbance change associated with decay of the P^3 state ($P^3 \rightarrow F^3$ reaction),

of the membrane (cf pumped proton) (Fig. 4). This site is most likely located around the A and/or D propionates of heme a_3 , where in particular a cluster consisting of His376 and Asp372, together with surrounding water molecules have been proposed (29). The proton bound to this site would then be released to the p side during the next reaction step, i.e., upon formation of the oxidized Cyt c O (O^4). This scenario also explains the lower pumping stoichiometry of the ba_3 oxidases because here a proton would be loaded to a pump site in one reaction step and then released in a later reaction step. In other words, at each step of the reaction the pump site would be either loaded or unloaded, which is in contrast to the situation observed with the A-Cyt c O s where at each step the loading and unloading of the pump site are synchronized. We note that this mechanism does not contradict general models for proton pumping by heme-copper oxidases where proton transfer to the pump site is proposed to precede proton uptake to the catalytic site (see, e.g., ref. 30). The only difference is that the two processes occur at distinct times rather than being apparently synchronized as in the A-type oxidases. The scenario is also consistent with the observed electrogenic reactions with the ba_3 Cyt c O (22), which show that the extent of proton transfer for the $P^3 \rightarrow F^3$ reaction is about 1/2 of that associated with the $F^3 \rightarrow O^4$. According to our interpretation these results would translate into 50% electrogenicity of step *Ila* compared to *Iib* + *III* in Fig. 3A, i.e., no proton pumping in step *Ila*.

We note that a number of site-directed mutants of the aa_3 oxidases have been studied in which the proton-pumping stoichiometry is lower than one (5, 31–33). In these variants the lower stoichiometry could be explained in terms of a competition of proton transfers from a protonatable site (Glu286) within the D proton pathway to a pump site and the catalytic site, respectively (20). In this case the proton-pumping stoichiometry can adopt any value 0–1 depending on the relative rates of internal proton transfers to these two sites. The situation is distinctly different in the ba_3 oxidase where we propose that the lower pumping stoichiometry is a consequence of the pump site alternating between a protonated and deprotonated state for every second electron transfer to the catalytic site (cf. Fig. 3 where the pump site is loaded in *Ila* and then unloaded in *III*).

As discussed above, the ba_3 Cyt c O uses only the one-proton pathway to transfer all substrate and pumped protons. This is most likely a consequence of adaptation to the low-oxygen habitat of *T. thermophilus* (19). In the A-type oxidases the D proton pathway overlaps near Glu286 with a channel through which O_2 is transferred to the catalytic site. The presence of a specific gas channel has been proposed to be a consequence of a generally rigid protein structure in a segment around the catalytic site, which is necessary to control proton access to the two sides of the membrane, but at the same time prevents free gas diffusion through the protein matrix (34, 35). In the ba_3 oxidases a rapid O_2 delivery at low O_2 concentrations may require a more hydrophobic channel environment, which would be incompatible with proton transfer through the same protein segment. Nevertheless, it is generally assumed that the pumping mechanism is the same in all oxidases, which suggests that the mechanistic variant represented by the ba_3 oxidase, leading to a lower pumping stoichiometry, is linked to the one-proton pathway structural design. In general, a separation in time of the proton loading and unloading events would reduce the probability of proton leaks because the two events do not have to be synchronized within one specific reaction step. At the same time this mechanistic variant allows observation of previously inaccessible reactions that are at the core of the pumping machinery of all heme-copper oxidases.

Materials and Methods

Purification of Cyt c O. His-tagged wild-type Cyt c O was expressed and purified as described (24) with the following modifications: The membrane solubilization was done in the presence of 2% Triton X-100 overnight at 4°C.

The membrane extract was then bound to a 5-mL precast Ni-NTA column (His Prep HP, Amersham Biosciences) in the presence of 10 mM imidazole. Washing and elution of the ba_3 oxidase bound to the column were performed in the presence of 50 mM and 200 mM imidazole, respectively. Purified protein (100 μ M) was kept at 4°C in 5 mM Hepes, pH 7.5, 0.05% dodecyl- β -D-maltoside (DDM).

Sample Preparation for Flow-Flash Measurements. A sample containing ba_3 oxidase (approximately 10 μ M enzyme in 2 mM Hepes, pH 7.5, 0.05% DDM, 1 μ M phenazine methosulfate) was placed in the bottom of a Thunberg cuvette and 2 mM Na ascorbate was placed in the sidearm of the cuvette. The atmosphere was exchanged for N_2 on a vacuum line and reduction was initiated by mixing the ascorbate with the enzyme. Full reduction of the enzyme was awaited before the atmosphere was exchanged for CO and the sample was allowed to incubate for at least 30 min. Reduction and CO binding were monitored spectrophotometrically.

In order to reduce the ba_3 oxidase only partially, two different methods were utilized. (i) The enzyme solution was placed in the bottom of the Thunberg cuvette together with 50 μ M EDTA, acting as electron mediator, whereas 50–100 μ M Fe_2SO_4 was placed in the sidearm as the electron source. The atmosphere was exchanged for N_2 and reduction was initiated by addition of the Fe_2SO_4 . The sample was incubated until the increase of the 560-nm absorption ceased and the atmosphere was then exchanged for CO. Again, the sample was allowed to incubate until the absorption increase at 430 nm had ceased. This procedure yielded approximately 100% reduction of heme b and 0–30% reduction of Cu_A . (ii) The enzyme solution was transferred in a Thunberg cuvette and made anaerobic by the exchange of the atmosphere for N_2 . Subsequently, the atmosphere was exchanged for CO and the sample was allowed to incubate for >12 h, yielding 60–75% reduction of heme b (no reduction of Cu_A) at pH 7.5.

Reduction Level of Partially Reduced Enzymes. The reduction level in partially reduced enzymes varied slightly in our preparations. When using Fe_2SO_4 /EDTA, a slight excess of electrons was used in order to compensate for enzyme oxidation during sample transfer from the cuvette to the stopped-flow apparatus. The reduction level of Cu_A was estimated from the absorption at 830 nm after mixing with O_2 . In most samples (denoted $3e^-$) including the ones reduced with CO only, no trace of Cu_A reduction was observed. In a few samples (denoted $3.5e^-$), a partial reduction of Cu_A was found. In order to reach the fully reduced state (denoted $4e^-$), ascorbate and PMS were directly added to the partially reduced enzyme and incubated for 5 min prior to measurement. These experiments were repeated several times (>7 times) with different enzyme preparations yielding similar results.

Flow-Flash Measurements. Measurements of the reaction of the reduced enzyme with oxygen were performed in a locally modified stopped-flow apparatus (Applied Biophysics) as described in ref. 36. The enzyme solution (2 mM Hepes, pH 7.5, 0.05% DDM) was rapidly mixed 1:5 with an oxygen-saturated solution (100 mM Hepes, pH 7.5/100 mM 2-(N -cyclohexylamino)ethanesulfonic acid (Ches), pH 10, 0.05% DDM), and the reaction was initiated after 30 ms by flash photolysis (10 ns, 200 mJ, 532 nm, Nd:YAG laser from Quantel) of the enzyme-CO complex. The kinetic traces were recorded at different wavelengths on an oscilloscope.

Proton-Uptake Measurements. Proton uptake from solution during oxidation was performed as described (24): Briefly, the purified enzyme solution was run over a PD-10 column (GE Healthcare) and the buffer was exchanged for 150 mM KCl, pH 7.5, 0.05% DDM. The protein was then diluted with the same solution to the appropriate concentration (approximately 10 μ M) and placed in a Thunberg cuvette. For full or partial reduction of the ba_3 oxidase, the samples were treated as described above. In the stopped-flow apparatus, the enzyme was mixed 1:5 with an unbuffered solution containing 150 mM KCl, 0.05% DDM, 1 mM O_2 , and 50 μ M pH sensitive dye (pH 7.5, phenol red; pH 10, m -cresol purple) and the absorption changes at 570 nm (phenol red) and 575 nm (m -cresol purple) were followed. Quantification of proton uptake was performed with spectroscopic titration of the exhaust with defined additions of NaOH and HCl (23).

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