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Review

Intermediates generated during the reaction of reduced *Rhodobacter sphaeroides* cytochrome *c* oxidase with dioxygen

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ABSTRACT

Cytochrome oxidase is one of the functionally most intriguing redox-driven proton pumps. During the last decade our increased understanding of the system has greatly benefited from theoretical calculations and modeling in the framework of three-dimensional structures of cytochrome *c* oxidases from different species. Because these studies are based on results from experiments, it is important that any ambiguities in the conclusions extracted from these experiments are discussed and elucidated. In a recent study Szundi et al. (Szundi et al. *Biochemistry* 2012, *51*, 9302) investigated the reaction of the reduced *Rhodobacter sphaeroides* cytochrome *c* oxidase with O_2 and arrived at conclusions different from those derived from earlier investigations. In this short communication we compare these very recent data to those obtained from earlier studies and discuss the origin of the differences.

1. Introduction

Cytochrome *c* oxidase (Cyt*c*O) is found in the mitochondrial inner membrane or the bacterial cytoplasmic membrane where it catalyzes reduction of O_2 to H_2O. The structure and function of Cyt*c*O have been reviewed, for example in [1–9]. During Cyt*c*O turnover, electrons are transferred from the one-electron donor, cytochrome *c*, to the primary electron acceptor copper A (Cu_A), located near the positive (*p*) side of the membrane. Upon reduction of Cu_A, electrons are transferred sequentially to heme *a* and to the catalytic site, which consists of heme a_3 and copper B (Cu_B). In its reduced state, the catalytic site binds O_2, which is reduced to H_2O. This reaction is also linked to proton uptake from the negative (*n*) side of the membrane. The transfer of a total of four electrons to the catalytic site is linked to the uptake of four protons and results in formation of two water molecules. Each coupled electron–proton transfer to the catalytic site is linked to proton pumping across the membrane. Thus, the proton is both the substrate of the reaction that drives proton pumping and the ion that is pumped across the membrane. In the bacterial oxidases, e.g. from *Rhodobacter* (*R.*) *sphaeroides*, both "types of protons" are transferred through one proton pathway (called D pathway, see Fig. 1), which complicates interpretation of the results from any mechanistic studies.

Three-dimensional structures of oxidases from a number of organisms have been determined at atomic resolution and a recent computational analysis of these structures implies the same characteristics relevant for function [10]. These structures have greatly contributed to understanding the system and have enabled application of theoretical tools to investigate the mechanisms of proton pumping [11–34]. Because theoretical calculations as well as application of computational tools build on and/or are evaluated based on results from experimental studies, it is important that any ambiguities in the conclusions or parameters extracted from these studies are elucidated. One such question that was raised recently concerns the relative timing of electron transfer from heme *a* to heme a_3 and proton uptake to the catalytic site in a specific time window of the reaction of reduced *R. sphaeroides* Cyt*c*O with O_2. These reactions and the problem are described in detail below.

To investigate the reaction of reduced Cyt*c*O with O_2, first the Cyt*c*O is reduced by four electrons, i.e. one at each of the redox sites Cu_A, heme *a*, heme a_3, and Cu_B (this state is called **R**), and a CO ligand is bound to heme a_3, i.e. the same site where O_2 normally binds. The Cyt*c*O–CO complex is mixed with O_2 and the CO ligand is dissociated using a short laser flash, which allows O_2 to bind and react (reviewed in [35]) (Fig. 2). After O_2 binding to heme a_3 forming a state that is called **A** with a time constant of approximately 8 μs at 1 mM O_2 (time constants are given for the *R. sphaeroides* Cyt*c*O and are taken from reference [36], see Table 1), an electron is transferred to the catalytic site with a time constant in the range of 45–70 μs forming state $\mathbf{P_R}$. In this state there is excess negative charge at the catalytic site

Abbreviations: Cyt*c*O, cytochrome *c* oxidase; **R**, the four-electron reduced Cyt*c*O; **A**, reduced Cyt*c*O with O_2 bound to heme a_3; $\mathbf{P_R}$, the "peroxy" state formed after transfer of a third electron to the catalytic site; **F**, the ferryl state formed at the catalytic site after protonation of $\mathbf{P_R}$; **O**, the oxidized Cyt*c*O

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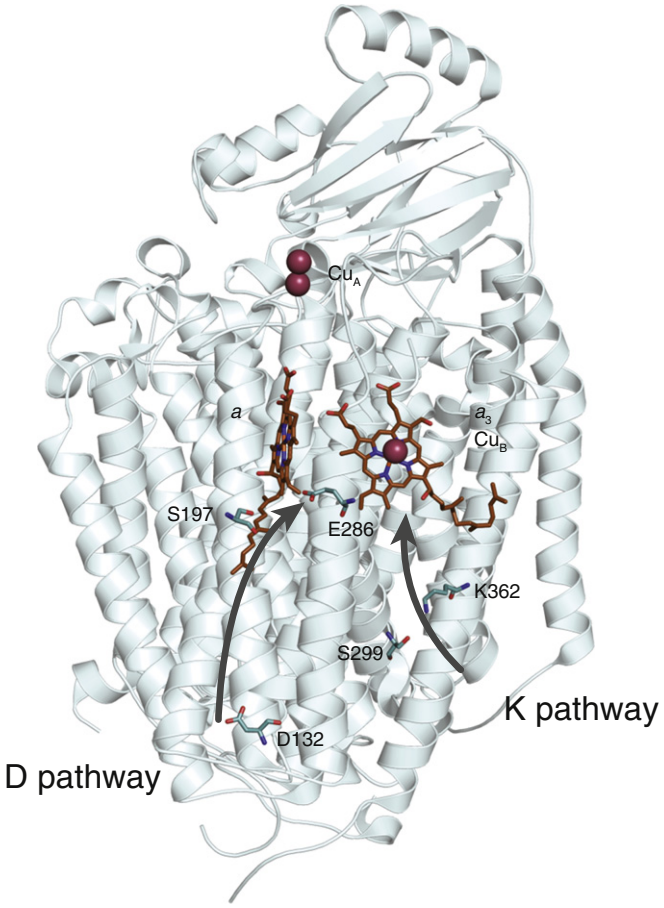

Fig. 1. Structure of the *R. sphaeroides* CytcO (PDB ID: 1M56 [58]) showing the redox-active sites and the D and K proton pathways. The side chains of the residues discussed in the text are shown explicitly.

(compared to all other states observed during this reaction) because the electron transfer is not accompanied by proton transfer to the catalytic site. Proton uptake is slower and is observed with a time constant in the range of 110–160 μs at neutral pH. This proton transfer results in formation of state **F** at the catalytic site. It is also accompanied by electron transfer from Cu_A to heme *a* with the same time constant as the proton uptake, as well as proton pumping across the membrane [37–39]. It should be noted that the functionality of the CytcO does not require a separation in time of the electron transfer to the catalytic site ($\mathbf{P_R}$ formation) and proton transfer to the catalytic site (**F** formation). Two distinct events, with different rate constants, are observed simply because the electron transfer is faster than proton transfer (see also more detailed discussion below). In the final step of the reaction, the electron in the Cu_A–heme *a* equilibrium is transferred to the catalytic site forming the oxidized CytcO (state **O**) with a time constant in the range of 1.2–1.4 ms at pH 7. For a recent review of the different intermediate states formed in CytcO, see [40].

In a recent paper, Einarsdóttir and colleagues report results from studies of the reaction of the reduced *R. sphaeroides* and bovine heart CytcO with O_2 [41]. As already mentioned above, the same reaction in these two systems was investigated previously, however, not at as many wavelengths as in the study by Einarsdóttir and colleagues. However, in this context it is important to note that in the studies prior to that of Einarsdóttir and colleagues the reaction was not investigated at a single wavelength, but at many wavelengths measured at a *single wavelength at a time*. Einarsdóttir and colleagues [41] performed a careful analysis of the optical absorption spectra of the intermediate states formed during the reaction of the CytcO with O_2 and concluded that the $\mathbf{P_R}$ state is not formed such that the reaction sequence is:

$$\mathbf{R \rightarrow A \rightarrow F \rightarrow O} \quad (1)$$

rather than

$$\mathbf{R \rightarrow A \rightarrow P_R \rightarrow F \rightarrow O} \quad (2)$$

as concluded earlier.

Einarsdóttir and colleagues conclude that the sequence as described in Eq. (2) still applies to the bovine heart mitochondrial CytcO and discuss the differences between these two systems in terms of structural differences. We found earlier that there are minor differences in the time constants measured with the *R. sphaeroides* and bovine heart mitochondrial CytcOs (Table 1). Also the electron equilibrium constants are different between the two systems such that the population of the reaction intermediates differ [36], but only slightly. There is also a difference in the Cu_A–heme *a* electron equilibrium in the two systems, which results in less heme *a* reduction during $\mathbf{P_R} \rightarrow \mathbf{F}$ in the *R. sphaeroides* than in the bovine CytcO [36]. All these differences were explained in terms of minor differences in the midpoint potentials of the redox sites in the two CytcOs, but the basic mechanism was concluded to be the same.

The indication that the $\mathbf{P_R}$ state is formed in the *R. sphaeroides* CytcO was originally based on the similarity in the absorbance changes (in the reaction of the four-electron reduced CytcO with O_2) obtained with the *R. sphaeroides* CytcO and the previously well characterized bovine CytcO

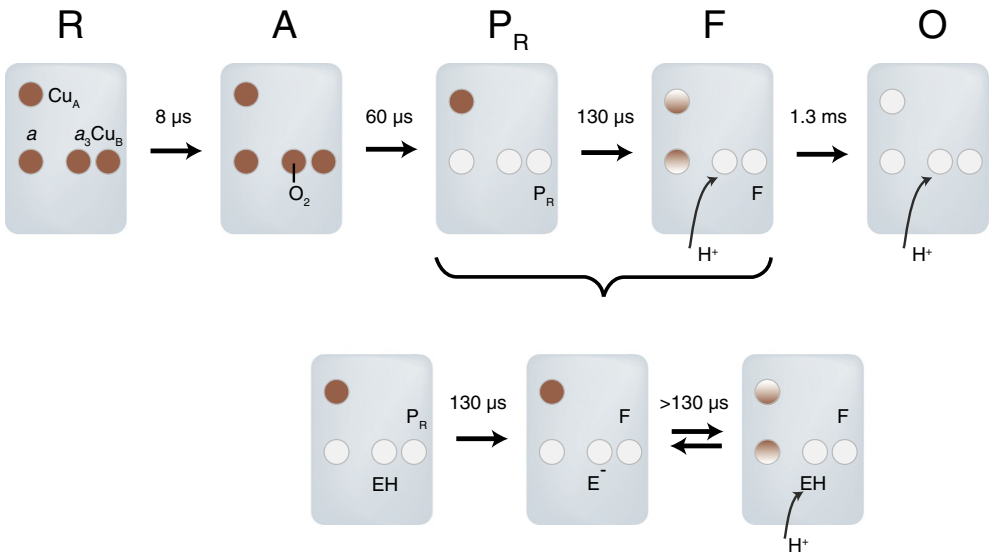

Fig. 2. The reaction sequence of the reduced CytcO with O_2. The filled, half-filled and empty circles represent reduced, partly reduced and oxidized states, respectively. Time constants are average values for the *R. sphaeroides* CytcO from Table 1. EH and E^- are the protonated and deprotonated forms, respectively, of Glu286. Proton pumping takes place in the reaction steps $\mathbf{P_R} \rightarrow \mathbf{F}$ and $\mathbf{F} \rightarrow \mathbf{O}$ (not shown in the figure).

[36]. Results from later measurements with the *R. sphaeroides* CytcO confirmed this assignment [4,38,42–47]. The main observation is that electron transfer from heme *a* to the catalytic site (to form the intermediate that is defined as $\mathbf{P_R}$) occurs *before* proton uptake to the catalytic site to form **F** (**F** is defined as $\mathbf{P_R} + H^+$). For example, we observed that after O_2 binding with a time constant of 8 µs at 1 mM O_2 (seen as an absorbance increase at 590 nm, see e.g. Fig. 4 in [48]), there is a decrease in absorbance with a time constant of 60 µs, which coincides in time with oxidation of heme *a* (absorbance decrease at 605 nm), i.e. formation of $\mathbf{P_R}$ at the catalytic site. An increase in absorbance at 580 nm, indicative of formation of **F** occurs only *after* oxidation of heme *a* over a slower time scale, with a time constant of 130 µs, which is the same as that observed for oxidation of Cu_A that is simultaneous with proton uptake from solution [49]. In other words, there is a clear separation in time between the electron transfer from heme *a* to the catalytic site (to form $\mathbf{P_R}$) and the proton uptake to form **F** (linked to oxidation of Cu_A). It should be noted that the $\mathbf{P_R} \rightarrow \mathbf{F}$ reaction is clearly seen as a separate phase in the *R. sphaeroides* CytcO only in a few regions of the spectrum.

According to our interpretation, the above-described observations clearly exclude that **F** would be formed directly from **A**. In this context it is also relevant to mention that our previously described reaction sequence obtained with the *R. sphaeroides* CytcO is in agreement with that obtained for the *Paracoccus* (*P.*) *denitrificans* CytcO, where the sequence $\mathbf{A} \rightarrow \mathbf{P_R} \rightarrow \mathbf{F}$ is observed at neutral pH [8,35,50]. Further support for formation of state $\mathbf{P_R}$ before **F** is obtained from studies of the pH dependence of the reaction of the reduced CytcO with O_2—while the $\mathbf{A} \rightarrow \mathbf{P_R}$ reaction displays essentially pH-independent kinetics, the $\mathbf{P_R} \rightarrow \mathbf{F}$ reaction is strongly pH dependent above pH ~ 9, i.e. the pK_a of 9.4 associated with this reaction. Consequently, at pH values above this pK_a, the separation between the two reactions is even more obvious [45]. A clear time separation between the $\mathbf{A} \rightarrow \mathbf{P_R}$ and $\mathbf{P_R} \rightarrow \mathbf{F}$ reaction steps was also seen in the presence of Zn^{2+} or in D_2O where the latter reaction is slowed more than the former [51]. The same time difference was also seen with the Glu286Ala/Ile112Glu double mutant CytcO [52]. Taken together, these data show that the $\mathbf{P_R}$ state is populated to a significant level during the reaction of the reduced CytcO with O_2.

To summarize this far, Einarsdóttir and colleagues [41] concluded that the **F** state is formed directly from **A** and they only report one event with a time constant of 53 µs, i.e. they do not observe the 130-µs reaction, which we attribute to the $\mathbf{P_R} \rightarrow \mathbf{F}$ reaction and electron transfer from Cu_A to heme *a*. As described above, in our studies the 130 µs reaction is not only seen in the optical changes in the alpha region (at 580 nm), but also at 830 nm (redox reactions of Cu_A), from studies of proton uptake from solution and it is also clearly seen as a lag in traces at 605 nm (see Fig. 4 in [49]). In other words, independently of the interpretation of the data reported by Einarsdóttir and colleagues [41], there is an obvious difference between the new data [41] and the older results—after O_2 binding we observe two clearly separable events ($\mathbf{A} \rightarrow \mathbf{P_R} \rightarrow \mathbf{F}$, Table 1, Eq. (2)), while Einarsdóttir and colleagues observe one event ($\mathbf{A} \rightarrow \mathbf{F}$). Different conclusions are reached as a result of differences in the data, not from interpretation of the same data.

The altered reaction route $\mathbf{A} \rightarrow \mathbf{F}$ (instead of $\mathbf{A} \rightarrow \mathbf{P_R} \rightarrow \mathbf{F}$), as reported by Einarsdóttir and colleagues may in principle be due to slowed $\mathbf{A} \rightarrow \mathbf{P_R}$ or an accelerated $\mathbf{P_R} \rightarrow \mathbf{F}$. As pointed out by the authors of the new study, the most likely explanation is an accelerated $\mathbf{P_R} \rightarrow \mathbf{F}$ reaction that coincides in time with the previous reaction step ($\mathbf{A} \rightarrow \mathbf{P_R}$) [41]. In other words, in the CytcO preparation used in the recent study [41], proton transfer to the catalytic site would be accelerated compared to the earlier investigated *R. sphaeroides* preparations of CytcO. It is difficult to speculate on the molecular origin of this difference. A change in the rate of proton transfer from Glu286 to the catalytic site is likely to be caused by structural changes around this residue, which is buried within the membrane-spanning part of the protein. On the other hand, a very small change in the proton-transfer rate constant would be required to yield the observed effect. In his context it would also be interesting to measure the rate of proton uptake from solution to determine whether only the internal proton transfer from Glu286 is accelerated or also reprotonation from solution displays the same effect. Additionally, monitoring absorbance changes at 830 nm from the Cu_A–heme *a* equilibration would also offer information whether Glu286 is reprotonated immediately or if it is transiently left in the unprotonated state after the

Table 1
Time constants associated with specific steps of the reaction of reduced CytcO with O_2. The data in the first two columns are from [36,43,48].

	R. sphaeroides	Bovine	*R. sphaeroides* from [41]
$\mathbf{R} \rightarrow \mathbf{A}$	8 µs (at 1 mM O_2)	~10 µs (at 1 mM O_2)	18 µs (at 0.5 mM O_2)
$\mathbf{A} \rightarrow \mathbf{P_R}$	45–70 µs	25–30 µs	$\mathbf{A} \rightarrow \mathbf{F}$
$\mathbf{P_R} \rightarrow \mathbf{F}$	110–160 µs	65–80 µs	53 µs
$\mathbf{F} \rightarrow \mathbf{O}$	1.2–1.4 ms	0.9–1.1 ms	1.3 ms

accelerated **F** formation because this electron equilibration takes place only when a proton is taken up from solution to reprotonate Glu286 [42,49].

When discussing the differences in the data it may be relevant to mention that there are conditions under which the $\mathbf{P_R} \rightarrow \mathbf{F}$ reaction is for different reasons not seen with the *R. sphaeroides* CytcO. For example, if electron transfer from heme *a* to the catalytic site (to form $\mathbf{P_R}$) is slowed by at least a factor of ~3 without slowing the proton transfer, then the electron and proton-transfer reactions occur simultaneously and appear as one coupled electron–proton transfer to the catalytic site to form state **F** without any significant population of $\mathbf{P_R}$. This situation is observed, for example, upon injecting an electron, via Cu_A and heme *a* to the $\mathbf{P_M}$ state (where the catalytic site is reduced by two electrons in the presence of O_2) [53,54]. A similar scenario is also observed upon replacement of specific amino-acid residues in the K proton pathway (Lys362, Ser299) (Fig. 1) [48,55], where formation of state $\mathbf{P_R}$ is bypassed and state **A** is converted directly to **F** (presumably because charge compensation, via the K pathway, upon electron transfer to the catalytic site is impaired [48,55]). On the other hand, at high pH (>9.4) when Glu286 is deprotonated and proton uptake to the D pathway is rate limiting, the **F** state is not populated (due to too slow proton uptake) and $\mathbf{P_R}$ is converted directly to state **O**, $\mathbf{P_R} \rightarrow \mathbf{O}$ [45]. A direct conversion of $\mathbf{P_R}$ to **O** is also observed upon replacement of Glu286 by Gln, for example in the *P. denitrificans* CytcO [56]. Thus, it is evident that there are ways to slow specific reaction steps during oxidation of the reduced CytcO thereby modulating the relative rates of the transitions such that a specific reaction step is not seen. A situation exactly mimicking that reported by Einarsdóttir and colleagues was observed earlier in experiments with the Ser197Asp mutant CytcO where formation of state **F** was accelerated, compared to the wild-type CytcO such that $\mathbf{P_R}$ was not observed (the time constant of the reaction $\mathbf{A} \rightarrow \mathbf{F}$ was ~65 μs) [57].

2. Summary

In conclusion, in our earlier studies the $\mathbf{P_R}$ state is clearly observed in the reaction of the *Rhodobacter sphaeroides* CytcO with O_2. Thus, in contrast to the conclusions reached by Szundi et al. [41], we do not believe that there are any fundamental differences in the reaction sequences of the bovine mitochondrial and *R. sphaeroides* CytcOs. As also pointed out by Szundi et al. the explanation for the different conclusions reached in the different studies is a change in the relative rates of $\mathbf{P_R}$ formation and proton uptake to form state **F**. This conclusion means that the $\mathbf{P_R}$ state is not populated to a significant level in the *R. sphaeroides* CytcO preparation used in the recent study [41], but it does not imply a different mechanism.

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