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Review

Reaction mechanism of bovine heart cytochrome *c* oxidase

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Abstract

The 1.9 Å resolution X-ray structure of the O_2 reduction site of bovine heart cytochrome *c* oxidase in the fully reduced state indicates trigonal planar coordination of Cu_B by three histidine residues. One of the three histidine residues has a covalent link to a tyrosine residue to ensure retention of the tyrosine at the O_2 reduction site. These moieties facilitate a four electron reduction of O_2, and prevent formation of active oxygen species. The combination of a redox-coupled conformational change of an aspartate residue (Asp51) located near the intermembrane surface of the enzyme molecule and the existence of a hydrogen bond network connecting Asp51 to the matrix surface suggest that the proton-pumping process is mediated at Asp51. Mutation analyses using a gene expression system of the Asp51-containing enzyme subunit yield results in support of the proposal that Asp51 plays a critical role in the proton pumping process.
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1. Introduction

Cytochrome *c* oxidase reduces molecular oxygen (O_2) in a reaction coupled with a proton pumping process. Electrons are transferred from cytochrome *c* in the intermembrane space while protons are transferred from the matrix space in a process that increases the membrane potential across the mitochondrial membrane. Since 1995, when 2.8 Å resolution X-ray structures of the bovine and bacterial enzymes were obtained [1,2], the resolutions of X-ray structures of bovine enzyme have been improved to 1.8 Å for the fully oxidized state and 1.9 Å for the fully reduced state [3].

A mobile proton-donor functional group within a membrane protein can function as a redox-driven proton pumping site if it encounters a change in pK_a and alternates accessibility between two different aqueous phases. A change in the oxidation state of a protein redox site can induce this alternating accessibility. These pK_a and accessibility changes could be readily induced by conformational changes affecting the environment of the proton pumping site. Reliable methods other than X-ray structural analysis are not presently available for identification of these conformational changes.

In this article, recent progress in investigations of the mechanisms of the O_2 reduction and the proton-pumping processes using bovine heart cytochrome *c* oxidase will be reviewed to examine the applicability and limitations of X-ray structural and resonance Raman methods for studies of the reaction mechanism of cytochrome *c* oxidase.

2. Mechanism of O_2 reduction

One electron reduction of molecular oxygen in the ground state is energetically unfavorable while two electron reduction is favorable. This property contributes greatly to the stability of oxygenated hemoglobins and myoglobins, since heme is embedded within the interior of the protein to protect it from

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interacting with exogenous ferrous heme. In fact, ferrous protoheme in aqueous solution is readily auto-oxidized by formation of a μ-peroxo compound (Fe^{3+}–O–O–Fe^{3+}) as the second intermediate following the oxygenated compound (Fe^{2+}–O_2). One electron reduction of the two-electron reduced O_2 (O_2^{2-}) is energetically unfavorable since the process includes production of the highly reactive oxygen radical.

Thirty years before the 2.8 Å resolution X-ray structural determination of cytochrome *c* oxidase, a copper ion (Cu_B) was discovered to be present near the O_2 reduction site (heme a_3 iron) as a result of the observation of a magnetic coupling of Cu_B^{2+} to Fe_{a3}^{3+}[4]. This revelation combined with the knowledge of the intrinsic chemical properties of O_2 in the ground state led to the suggestion that the initial intermediate of the enzyme-catalyzed O_2 reduction process contains a peroxide bridged between Fe_{a3}^{3+} and Cu_B^{2+}, since the second electron for the two electron reduction of O_2 is readily available from Cu_B^{1+}. However, the initial intermediate was assigned as the oxygenated species (Fe^{2+}–O_2), as a result of experiments that monitored low-temperature absorption spectral changes. Additional experiments to support this assignment were not performed [5]. It should be noted that the fact that the absorption spectra of the initial intermediate and the CO-bound species used to model the O_2 species exhibit similar α-band positions does not provide conclusive evidence for the assignment since the absorption spectrum of heme a_3 does not provide unequivocal structural information about the ligand species.

Unexpectedly, the resonance Raman results indicate that Fe^{2+}–O_2 ,the first intermediate shows a band at 571 cm^{-1} while $Fe^{4+}=O^{2-}$, the second intermediate exhibits a band at 804 cm^{-1}. The $Fe^{4+}=O^{2-}$ species contains an oxidation equivalent near the O_2 reduction site [6,7]. The assignment is based on the $^{16}O=^{18}O$ isotopic shift effects [6,7]. The oxygenated form is stable enough to be detected while the putative peroxide intermediate is too labile to be identified as an intermediate species. The 1.9 Å resolution X-ray structure of the O_2 reduction site of the bovine heart cytochrome *c* oxidase in the fully reduced state [3] provides enough structural detail to suggest the following mechanism for O_2 reduction.

The fully reduced species binds O_2 at the five-coordinate Fe_{a3}^{2+} site while Cu_B^{1+} is coordinated by three histidine imidazole groups in trigonal planar geometry [3,8]. In general, trigonal planar cuprous compounds are poor ligand acceptors and poor reductants due to their chemical stability. Therefore, the interaction between the O_2 molecule bound at Fe_{a3} and the cuprous Cu_B must be sufficiently weak to give rise to the resonance Raman band of the Fe_{a3}-O_2 stretching mode.

His240, one of the three histidines coordinated to Cu_B, is covalently linked to Tyr244 to constrain the phenol OH group to the O_2 reduction site [8]. However, a study which modeled the possible conformations of O_2 at Fe_{a3}^{2+} in the X-ray structure indicates that direct hydrogen bond formation between the bound O_2 and the Tyr244 OH group is not possible. Thus, a conformational change is required for hydrogen bond formation. The structure also stabilizes the O_2-bound form.

The covalent linkage between His240 and Tyr244 provides an effective electron transfer pathway from Cu_B to the OH group of Tyr244. Once the hydrogen bond is formed between Tyr244 and the bound O_2, Tyr244 functions as an effective hydrogen atom donor to the bound O_2 to form Fe_{a3}^{3+}–O–OH and the deprotonated tyrosine (Tyr244O^-) with Cu_B^{2+}. Cu_B^{2+} must react with any negatively charged ligand such as OH^-. The negatively charged phenol group of Tyr244, which is hydrogen-bonded to Fe_{a3}^{3+}–O–OH, is unstable within the hydrophobic environment of the protein interior and could function as an effective electron donor to the Fe_{a3}^{3+}-bound peroxide. These structures are expected to promote an effective two electron reduction of the O–O bond to provide $Fe_{a3}^{4+}=O^{2-}$ and OH^-. This process is mediated by one electron from Fe_{a3}^{3+}(which is converted to Fe_{a3}^{4+}) and one electron from Tyr244–O^-(which is converted to a Tyr244 radical).

Although support for the existence of the Tyr244 radical has been provided by a chemical analysis using radioactive iodide labeling followed by peptide mapping [9], spectroscopic evidence of the radical has not been obtained. It has been shown that, in the absence of reductant, the 804 cm^{-1} intermediate is quite stable ($t_{1/2}=70$ min at pH 8.0) [7]. On the other hand, the X-ray structure does not readily reveal a moiety near Tyr244 which could stabilize the radical [3,8]. These results appear to contradict the proposal that the 804 cm^{-1} intermediate contains a Tyr244 radical.

The oxidation equivalent of a Tyr244 radical could migrate to another redox active site to produce the 804 cm^{-1} intermediate. Possible candidates for the location of this oxidation equivalent are Cu_B^{3+}, the porphyrin π-cation radical of heme a_3, and Fe_{a3}^{5+}. The fact that a significant decrease is not observed in the Soret absorption intensity of the intermediate state relative to those of the other enzyme species argues against assignment of the porphyrin π–cation radical as the candidate species. Although the high-oxidation state metal sites such as Fe_{a3}^{5+} and Cu_B^{3+} have not yet been detected in a metalloprotein system, the large electron density surrounding Fe_{a3} would be expected to stabilize the high oxidation state of the iron atom.

The third intermediate exhibits an oxygen isotope sensitive band at 785 cm^{-1} and is assigned as $Fe_{a3}^{4+}=O^{2-}$. The fourth intermediate species exhibits a band at 450 cm^{-1} and is assigned as Fe_{a3}^{3+}–OH^-. The lower oxidation state of the third (785 cm^{-1}) intermediate compared to the second (804 cm^{-1}) intermediate is obvious since the third intermediate does not appear in the reaction between the mixed valence enzyme (the two electron reduced enzyme) and O_2[6,7]. The first, second and third intermediate species have absorption maxima at 590 nm (A), 607 nm (P) and 580 nm (F), respectively [6].

The 607 nm species (or the P species) can be prepared by oxygenation of either the fully reduced enzyme or the mixed valence enzyme [10–13]. The P species resulting from oxygenation of the fully reduced enzyme is named the P_r species while the P species obtained from the mixed valence enzyme and O_2 is called the P_m species. It has been proposed that the oxidation state of P_r is one equivalent lower, relative to P_m by four observations [12,13]. Firstly, the appearance of the 607 nm species (P_r) in the reaction of the fully reduced enzyme with O_2 at low temperature is associated with absorption spectral changes of heme *a* indicative of oxidation [12]. Secondly, the 607 nm species appears to be associated with an unusual EPR signal which

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