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Review

Proton translocation in cytochrome *c* oxidase: Insights from proton exchange kinetics and vibrational spectroscopy☆

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ABSTRACT

Cytochrome *c* oxidase is the terminal enzyme in the electron transfer chain. It reduces oxygen to water and harnesses the released energy to translocate protons across the inner mitochondrial membrane. The mechanism by which the oxygen chemistry is coupled to proton translocation is not yet resolved owing to the difficulty of monitoring dynamic proton transfer events. Here we summarize several postulated mechanisms for proton translocation, which have been supported by a variety of vibrational spectroscopic studies. We recently proposed a proton translocation model involving proton accessibility to the regions near the propionate groups of the heme *a* and heme a_3 redox centers of the enzyme based by hydrogen/deuterium (H/D) exchange Raman scattering studies (Egawa et al., PLoS ONE 2013). To advance our understanding of this model and to refine the proton accessibility to the hemes, the H/D exchange dependence of the heme propionate group vibrational modes on temperature and pH was measured. The H/D exchange detected at the propionate groups of heme a_3 takes place within a few seconds under all conditions. In contrast, that detected at the heme *a* propionates occurs in the oxidized but not the reduced enzyme and the H/D exchange is pH-dependent with a pK_a of ~8.0 (faster at high pH). Analysis of the thermodynamic parameters revealed that, as the pH is varied, entropy/enthalpy compensation held the free energy of activation in a narrow range. The redox dependence of the possible proton pathways to the heme groups is discussed. This article is part of a Special Issue entitled: Vibrational spectroscopies and bioenergetic systems.

1. Introduction

Cytochrome *c* oxidase (C*c*O), the terminal enzyme of the respiratory chain in mitochondria, reduces dioxygen to water and harnesses the redox energy to translocate protons across the inner mitochondrial membrane. The generated electrochemical potential is utilized for the synthesis of ATP by F_oF_1 ATP synthase. C*c*O has four redox-active metal centers, Cu_A, heme *a*, heme a_3 and Cu_B [1]. Cu_A accepts electrons from cytochrome *c* and transfers them *via* heme *a* to the heme a_3–Cu_B binuclear center where the four-electron reduction of dioxygen to water takes place. Eight protons are taken up from the negative side (N-side) of the mitochondrial membrane; four of those are transferred to the binuclear center for the formation of water and four others are translocated across the membrane to the positive side (P-side). Three proton pathways (H, D and K, named after their entry residue) have been identified and heavily studied. In bacterial aa_3 enzymes the K-channel was shown to supply chemical protons to the binuclear center, while the D-channel was reported not only to supply chemical protons to the binuclear center, but also to serve as the pathway for all four of the translocated protons [2]. In contrast, in bovine C*c*O (bC*c*O), proton translocation has been attributed to the H-channel, which passes near heme *a* [3].

Although the proton conducting channels have been identified, the molecular mechanism for the proton translocation remains unresolved. Indeed, whether or not aa_3 enzymes from bacteria and bovine have different mechanisms of proton translocation remains an open question. This is a result of the difficulty of identifying protonation states of critical residues within the large membrane bound enzyme during its catalytic turnover, although some labile positions have been identified under equilibrium or quasi-equilibrium conditions by vibrational spectroscopy [4–17] and some have been postulated by electrostatic calculations [18–21]. Such studies have led to new insights into proposed mechanisms of proton translocation, some of which are discussed below.

Infrared absorption and resonance Raman spectroscopies have been extensively used with great success to study the properties of terminal

Abbreviations: bC*c*O, bovine Cytochrome *c* Oxidase; $[[CcO^{Ox}{}_D]_{H,t\ min}]^{Rd}$, treatment order of the enzyme, the oxidized (Ox) was treated in deuterated buffer (D) for at least 8 h, the buffer was exchanged for a protonated buffer (H) and incubated for t minutes before reduction (Rd); N-side and P-side, the negative and positive sides of the mitochondrial membrane, respectively

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oxidases. In general, the two techniques have yielded complementary information. The infrared spectrum reports on functional groups of the protein provided that there are changes in the dipole moment associated with their vibrational modes. As a result, the spectra tend to be complicated with overlapping features. In addition, the infrared spectrum can be obscured by the strong IR bands from water molecules, especially the water bending mode that occurs in the 1600 cm^{-1} region of the spectrum which overlaps with the Amide-I mode of the protein matrix as well as modes from sidechains from several types of amino acids. Various techniques have been used to address this problem. For instance, attenuated total reflection (ATR), in which a sample in contact with the ATR crystal is probed by the evanescent wave, makes the water pathlength short enough to reduce its contribution to the spectrum [6,22,23]. A new technique, reviewed in this issue by Nakashima, et al., uses a very intense IR laser beam to penetrate the water matrix surrounding the protein sample [17,24]. Difference spectra e.g. oxidized minus reduced [11], or CO-bound *versus* CO-photodissociated [13,14], are used to identify features in the infrared spectrum that may be functionally important.

In contrast to infrared absorption spectroscopy, resonance Raman scattering reports on those moieties with an optical transition which coincides with the excitation wavelength. Consequently, with visible excitation overlapping with the heme absorption bands, the resonance Raman spectrum probes the heme groups selectively, without spectral interference from the surrounding protein. The resonance Raman spectrum is sensitive to the electronic properties of the heme and perturbations of it induced by the protein environment. The contribution of water is relatively weak in the Raman spectrum, so it generally is not a factor in determining the quality of the spectrum. There is a great deal of information in the resonance Raman spectrum of the heme group, such as the redox and spin state of the iron atom, and the conformational properties of the heme macrocycle, as well as the peripheral groups attached to it. In addition, due to its strong scattering intensity, the resonance Raman spectrum of the heme can be obtained at relatively low sample concentrations; hence, it can be easily coupled with continuous-flow cells for time-resolved measurements, without excessive sample consumption, allowing determination of catalytic intermediates by time resolved techniques [25,26].

2. The catalytic reaction of C*c*O

To translocate protons against an electrochemical gradient, energy has to be supplied to the system. For terminal oxidases, energy is supplied by the reduction of oxygen to water at the binuclear center, a process that involves several intermediates, which have been well-characterized by resonance Raman studies [27–32]. The catalytic cycle, illustrated in Fig. 1, shows the stretching modes of the oxygen intermediates that were determined by time-resolved experiments carried out primarily by three different groups using either continuous-flow or pulsed laser spectroscopic techniques [29,31,32]. In the continuous-flow procedure, the CO-inhibited reduced enzyme is mixed with oxygen and flowed into an observation cell, where one laser beam photodissociates the CO, allowing the O_2 to bind to the enzyme, and a second laser, downstream from the photolysis beam, excites the Raman spectrum [25]. The reaction time at a given probe position is determined by the flow rate and the separation between the two laser beams. The signal to noise ratio of the Raman spectrum is determined by the spectral integration time at a given position. In the pulsed experiments, the CO-bound enzyme, mixed with O_2, is loaded into the observation cell, where a laser pulse photodissociates the CO and a second laser pulse, time delayed from the first, excites Raman spectrum [33,34]. Subsequently, a new sample is loaded into the observation cell and the process is repeated a number of times until a spectrum with a satisfactory signal to noise ratio is obtained. The reaction time is given by the time delay between the two laser pulses.

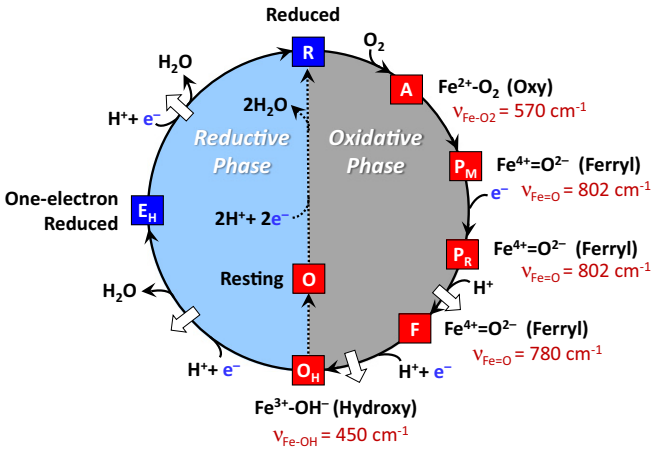

Fig. 1. The redox cycle of cytochrome *c* oxidase. The white arrows designate the proposed proton translocation events. If, after the $\mathbf{O_H}$ species is formed, the enzyme is rapidly reduced, it passes through $\mathbf{E_H}$ on its way to the reduced state, **R**, and two protons are translocated. However, if the reduction does not occur immediately, the resting oxidized species (**O**) is formed, and no proton translocation is associated with its reduction to the **R** species. The characteristic vibrational mode associated with each oxygen intermediate is indicated in red.

Raman measurements led to the firm determination of the identity of the catalytic intermediates. In the initial O_2-bound species (the **A** intermediate), the Fe – O_2 stretching mode was identified at 570 cm^{-1}, the same as that found in myoglobin, indicating that the nearby Cu_B does not significantly perturb the Fe-O_2 moiety [35,36]. The identification [37] of the Fe═O mode of the **P** intermediates at 802 cm^{-1} is particularly interesting as initially the **P** intermediate was thought to be a peroxy species. However, resonance Raman studies of a **P** analog derived from the H_2O_2 reaction with C*c*O, carried out by Kitagawa and coworkers, demonstrated that the species had only one oxygen atom, proving that it was a ferryl rather than a peroxy species [37]. This assignment was subsequently confirmed by biochemical [38] and other spectroscopic measurements [39]. In addition, the structure of the intermediate was confirmed by additional resonance Raman measurements of the catalytic reaction [31,40,41]. In the **F** intermediate, another ferryl species was detected, in which the Fe═O stretching mode was identified at 780 cm^{-1} by several groups [28–30,42]. Although both **P** and **F** are ferryl species, they differ by one redox equivalent with **P** having an additional oxidation equivalent, the location of which is under constant debate. Presumably, they also differ by the ligand coordinated to Cu_B, likely a hydroxide in **P** and a water molecule in **F** [2]. Finally, the $\mathbf{O_H}$ species was shown to be a hydroxide-bound high-spin intermediate with a Fe – OH stretching mode at 450 cm^{-1} [28]. The Fe – OH stretching frequency is very low with respect to other hydroxide bound heme species; we attribute this very low frequency to the presence of a strong H-bond to the oxygen atom, thereby weakening the Fe – O bond and generating a high-spin configuration.

3. Postulated proton pumping mechanisms

Although the properties of the catalytic intermediates are reasonably well-understood, how the oxygen reduction reaction leads to the concerted conformational changes and electrostatic processes that drive protons across a membrane against a proton gradient remains to be determined. Maintaining charge neutrality at the metal centers as electrons and protons flow through the protein during the redox processes is a critical component in coupling oxygen reduction to proton translocation [43]. Schematically, for proton translocation to occur efficiently, a proton loading site with two gates, where protons can be stored and released in a controllable fashion, is needed. In principle, the loading site can have a variable pK_a, which is regulated by the redox and ligand binding states of the heme group. Under conditions