Communication

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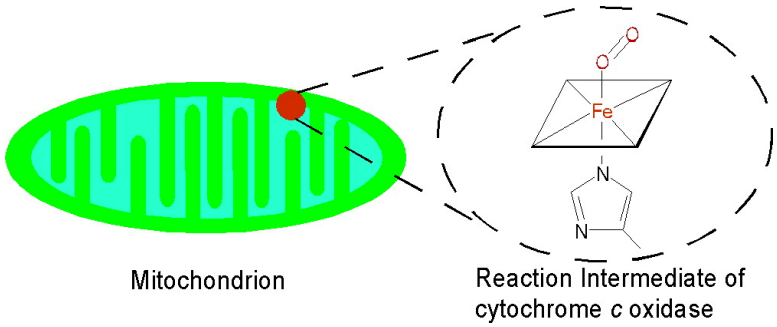

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Probing the Oxygen Activation Reaction in Intact Whole Mitochondria through Analysis of Molecular Vibrations

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The mitochondrial inner membrane contains high concentrations of various functional membrane protein complexes and includes the proteins of the respiratory chain. It has been estimated that three-quarters of the weight of the mitochondrial inner membrane is occupied by membrane proteins,[1] some of which are as yet unidentified. The phospholipid bilayer region also includes various classes of lipids and other hydrophobic compounds. All membrane proteins within the mitochondrial inner membrane interact strongly with other membrane proteins and components of the phospholipid bilayer, and these interactions are removed when the membrane proteins are purified from cellular components. It is desirable to examine the function of these membrane proteins within the mitochondrial membrane—in an environment as close as possible to their natural biological state. Unfortunately, most analytical techniques used for structure/function investigations of proteins are typically amenable only for analysis of pure samples. Purification of the protein from cellular components is usually the most challenging and time-consuming step in the pursuit of structural and functional investigations of complex proteins. The purification procedures for most membrane proteins include solubilization of the membrane proteins with harsh detergents which often contribute to degradation of the protein of interest. Thus, it is desirable to establish new methods for analysis of the structural properties of membrane proteins within functional membrane systems in the cell for the sake of assessment of the integrity of a purified membrane protein sample. In this communication, we present the identification of the resonance Raman bands which we assign to the first three intermediates in the reaction of cytochrome *c* oxidase with dioxygen in intact mitochondria.

Resonance Raman spectroscopy is a powerful tool for this purpose since, in principle and in practice, this technique is generally not influenced by contaminants in the protein sample. Vibrational spectra of cytochromes in whole mitochondria have been reported.[2,3] Recently, the CO-binding function of heme a_3 of cytochrome *c* oxidase has been examined in a sample of intact bovine heart mitochondria, after significant improvements were made that contributed to sensitivity and precision of the Raman measurement for the highly turbid and viscous mitochondrial suspensions.[4] In the present work, the resonance Raman technique is also applied for measurement of the cytochrome *c* oxidase-catalyzed O_2 reduction process in an intact porcine mitochondrial preparation. It should be noted that the time-resolved resonance Raman method described here is not amenable for investigations of the native proteoliposome system due to the low overall enzyme concentration in this system. Furthermore, in highly concentrated proteoliposome suspensions, the vesicles interact to induce aggregation and fusion of the vesicles which disrupt the native positioning of the enzyme within the liposomes. The highest overall concentration of cytochrome *c* oxidase obtained in a stable proteoliposome preparation[5] is on the order of 0.1 μM, which is far below the lower limit of about 10 μM required for a resonance Raman measurement. Fortunately, intact porcine or bovine mitochondrial preparations are stable even at overall concentrations of cytochrome *c* oxidase as high as 50 μM. The resonance Raman technique is expected to be the only direct method capable of revealing the influence of membrane potential on the structure and binding mode of the heme a_3 oxygen adduct, although this effect is currently too small to detect at present levels of experimental accuracy. Absorption spectroscopy is also useful for kinetic and thermodynamic investigations of intermediate species, such as the P and F forms of cytochrome *c* oxidase.[6] However, these results do not provide high quality structural information for these intermediates.

The porcine heart mitochondrial preparation used in this study exhibits an acceptor control ratio and an absorption spectrum essentially identical to those of the purified bovine cytochrome *c* oxidase. Resonance Raman spectra of the CO-bound heme a_3 in the fully reduced and CO-bound enzyme in porcine heart mitochondria are very similar to those of the purified bovine cytochrome *c* oxidase and bovine whole mitochondria, indicating that the Fe−C−O environments of these species are essentially identical, although the assignment of the Fe−C−O bending mode remains the subject of some controversy.[7−9]

Figure 1 shows resonance Raman difference spectra for the reaction of porcine heart mitochondria with dioxygen expressed as $^{16}O_2$-spectrum minus $^{18}O_2$-spectrum at three delay time points (Δt) after initiation of the reaction. In the 400−950 cm^{-1} frequency region, vibrational modes associated with the heme-bound oxygen are expected.[10−12] These spectra were recorded using an apparatus[13,14] that includes an "artificial cardiovascular system" for circulating the enzyme sample which was originally developed to maximize the accumulation time of Raman scattering using a limited amount of purified CcO. The following improvements of the system depicted in Figure 1 of ref 14 are necessary for measurements of the viscous and highly concentrated mitochondrial suspensions: (1) all of the connecting points were improved to withstand high pressure; (2) the "oxygen-remover" was replaced with a glass tube-type, and the inner diameter of the "sample reservoir" was increased from 10 to 45 mm; and (3) the "kidney/liver" component, which supplies reductant, was removed since the levels of naturally occurring reductants contained in the mitochondrial preparation were sufficient for complete reduction of the mitochondrial preparation before exposure to CO in the circulating system.

Figure 1A shows that the band at 571 cm^{-1} is down-shifted to 544 cm^{-1} upon $^{18}O_2$-substitution, indicating that the band at 571

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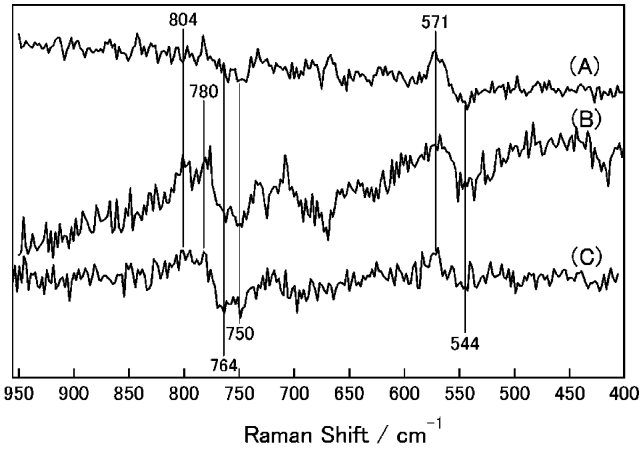

Figure 1. Time-resolved resonance Raman difference spectra of CcO in whole mitochondria measured during a reaction with dioxygen at pH 7.4. Delay time after initiation of the reaction is 0.4 (A), 0.6 (B), and 1.4 ms (C). Accumulation time of Raman scattering was 16 (A), 8 (B), and 32 min (C) for each of $^{16}O_2$ and $^{18}O_2$ experiments. The excitation wavelength of resonance Raman scattering was 423.0 nm. The temperature of the sample was 11.6 °C at the flow cell (cross section, 0.6 mm × 0.6 mm), and the flow rate was 40 mL/min. Other conditions of the artificial cardiovascular system were the same as those described in ref 14. Mitochondria were suspended in 10 mM Tris-HCl, pH 7.4, supplemented with 70 mM sucrose and 210 mM mannitol. The volume of the mitochondrial suspension was 80 mL and contained 10^9 mitochondria per mL, which is equivalent to 20 μM CcO. Sodium ascorbate was added as a reductant, to a final concentration of 100 mM, for a total of 7 h per experiment. An acceptor control ratio of 3 was observed throughout the Raman measurements. Reproducibility of the intensity of the 571 cm^{-1} band was within 20% in several different measurements.

cm^{-1} can be assigned to the Fe−O_2 stretching mode of the oxygenated intermediate with an end-on geometry[14] with an Fe−O−O bond angle of 120°. The band is reduced in intensity as a function of time, as indicated in Figure 1B,C. Similarly, the bands at 804 and 780 cm^{-1} in Figure 1B are down-shifted to 764 and 750 cm^{-1}, respectively, upon $^{18}O_2$-substitution in Figure 1B and are reduced in intensity in Figure 1C. The frequencies of these bands at 804 and 780 cm^{-1} are very similar to those observed for the solubilized enzymes (804 and 785 cm^{-1}), which have been assigned to the Fe═O stretching vibrations of the P and the F intermediates, respectively.[12,15] The frequencies of the three oxygen-isotope-sensitive bands at 571, 804, and 780 cm^{-1} of the oxygenated, P, and F intermediates are identical to those of the solubilized enzyme within limits of experimental accuracy. This indicates that the oxygen activation process historically observed in the solubilized enzyme also occurs in mitochondria. We note that the lifetime of the oxygenated intermediate (571 cm^{-1} band in Figure 1A) is significantly longer than that observed for solubilized enzyme; the band is detectable at $\Delta t = 1.4$ ms in mitochondria (11.6 °C), but no trace of the band has been detected at Δt longer than 1 ms for the solubilized state[12] at 3 °C. Results obtained at room temperature[10,11] for solubilized enzyme are essentially the same, and the band at 571 cm^{-1} is no longer observed even at $\Delta t = 0.5$ ms. This phenomenon is unlikely to be due to modification of the heme a_3 site in the solubilized enzyme since the resonance Raman spectra of the CO-bound forms provide good evidence that the integrity of heme a_3 site has been maintained. It has been shown that the transition from the oxygenated form to the P intermediate is several times slower at pH 8 than that at pH 6.8 in the solubilized enzyme system. The slower oxygenated → P transition suggests that the pH control site is located on the enzyme surface facing the matrix phase, where pH is expected to be at an approximate value of 8. Alternatively, the oxygenated form could be stabilized by the membrane potential created by the P → F transition in the other enzyme molecules within the same mitochondrial membrane. At $\Delta t = 0.6$ ms (Figure 1B), the 571 cm^{-1} band coexists with 804 and 780 cm^{-1} bands, showing the coexistence of the P → F transition during the process of degradation of the oxygenated form. Furthermore, the other components of the mitochondrial inner membrane could be involved in interactions with the enzymes that cause a decrease in the transition rate. The influence of the higher effective O_2 concentration relative to that of the solubilized enzyme system is less likely to be the cause of this effect since the maximum intensity of the 571 cm^{-1} band of the enzyme given in Figure 1 is essentially the same as that observed for the solubilized enzyme system. Further improvements of the experimental accuracy, particularly for the spectral band positions, would provide important insights with regard to these issues. These studies are now underway in our laboratory.

In summary, this study demonstrates that the time-resolved resonance Raman method described herein is able to dissect and monitor the dioxygen reduction process driven by cytochrome *c* oxidase in the mitochondrial inner membrane in the midst of a complex matrix that contains many other membrane proteins with strongly absorbing chromophores, with a resolution comparable to that obtained in reaction systems comprised of solubilized and isolated cytochrome *c* oxidase. This method is applicable for investigations of other mitochondrial physiological processes that occur at similar time scales.

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Supporting Information Available: Absorption and resonance Raman spectra of the fully reduced form of and fully reduced and CO-bound form of porcine heart mitochondria. This material is available free of charge via the Internet at http://pubs.acs.org.

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