RESONANCE RAMAN SPECTROSCOPY OF CYTOCHROME c OXIDASE AND ELECTRON TRANSPORT PARTICLES WITH EXCITATION NEAR THE SORET BAND

I. Salmeen, L. Rimai, D. Gill
Scientific Research Staff, Ford Motor Co., Dearborn, MI 48121

T. Yamamoto and G. Palmer
Biophysics Research Division, Institute of Science and Technology, University of Michigan, Ann Arbor, MI 48104

C. R. Hartzell
Department of Biochemistry, Pennsylvania State University, University Park, PA 16802

H. Beinert
Institute for Enzyme Research, University of Wisconsin, Madison, WI 53706

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SUMMARY We report the resonance Raman spectra of cytochrome c oxidase, both solubilized and in electron transport particles using laser excitation near the Soret band. As in the spectra of other hemoproteins, such as cytochrome c, the shape and intensity of a number of bands change when the oxidation state is varied. However, one of the hemes of solubilized cytochrome c oxidase shows redox behavior which is anomalous. Spectra of electron transport particles are dominated by cytochrome c oxidase. There are, however, definite differences between spectra of solubilized cytochrome c oxidase and electron transport particles in the oxidized states.

INTRODUCTION The mechanisms of oxidation and reduction of cytochrome c oxidase (c oxidase) remain obscure, in part because spectroscopic studies of the a and a_3 components have been difficult to interpret (1, 2). Vibrational spectra can be sensitive to small structural differences and might provide useful information. Accordingly we have investigated the heme groups of c oxidase by resonance Raman (RR) spectroscopy. This investigation was prompted by recent reports of the RR spectra of hemoglobin (3-5) and cytochrome c (4, 6), using excitation near the visible α and β bands. Our own observations on these and other hemoproteins show that RR spectra excited near the Soret band have improved sensitivity over that obtained with excitation near the α and β bands (3-6). With 441.6 nm excitation we have obtained good quality spectra of both oxidized and reduced c oxidase at concentrations as low as 5 μM. This sensitivity is comparable to that of optical absorption (1, 2) and low temperature EPR spectroscopy (7, 8). Our preliminary results are: (1) The RR spectrum of oxidized c oxidase has contributions from two hemes; one is similar in

redox behavior to that of other hemoproteins, the other is anomalous. (2) Various lines can be used for the unique characterization of fully reduced c oxidase. (3) With 441.6 nm excitation, RR spectra of electron transport particles (ETP) are strongly dominated by c oxidase. Under the conditions of these experiments, the redox behavior of solubilized c oxidase and c oxidase in ETP is different.

METHODS AND MATERIALS Beef heart mitochondrial c oxidase, suspended in 0.1 M potassium phosphate (pH 7.2) and 0.5% cholate, was prepared by the method of Fowler et al. (9) as modified by MacLennan and Tzagoloff (10). Lipid free c oxidase, suspended in 0.1 M potassium phosphate (pH 7.2) and 0.5% Tween 20, was prepared according to an unpublished method of C. R. Hartzell. Electron transport particles (ETP) suspended in tris-sucrose were prepared essentially according to the method of Linnane and Ziegler (11). Cytochrome c Type VI was purchased from Sigma and dissolved without further purification in 0.1 M phosphate (pH 7.2). Sample concentrations are noted in figure legends. Both cytochrome c and c oxidase (in the presence of catalytic amounts of cytochrome c) were reduced aerobically by adding a minimal amount of sodium dithionite; the c oxidase was reoxidized by gently blowing a stream of oxygen gas over the sample and allowing at least 20 minutes for the conversion to an oxidized form. The redox state was checked optically before and after the Raman measurements by transferring a portion of the sample into a suitable cuvette. The oxidized and reduced c oxidase had Soret bands at 420 nm and 445 nm respectively. The Raman spectra of the dense suspension of reduced ETP were obtained by adding sodium succinate or sodium dithionite until no further changes occurred in the spectra.

The spectra were recorded with a system described previously (12) using the 441.6 nm line of a Spectra Physics Model 185 HeCd laser. The power incident on the sample did not exceed 25 mW and spectra taken with 3 mW showed only the decrease in signal to noise ratio expected from the lower power. The depolarization ratios $\rho = I_{\perp}/II_{\parallel}$ are indicated for the principal bands. The spectra were taken at 5°C, cooling being achieved by blowing cold nitrogen gas through a jacketed cuvette holder.

RESULTS AND DISCUSSION The spectra are shown in Figures 1 and 2; cytochrome c is included here for purposes of comparison. Figure 2 also included c oxidase in some intermediate oxidation state which was obtained fortuitously in the course of successive additions of dithionite to the aerobic sample. (The corresponding optical absorption spectrum showed a peak at 445 nm and a prominent shoulder at about 420 nm, consistent with partially reduced protein.) Two cholate solubilized c oxidase preparations and the lipid-free Tween 20 sample showed identical spectra. Two independent preparations of ETP were spectroscopically identical to each other. There are no detectable contributions to any of these spectra from the buffer.

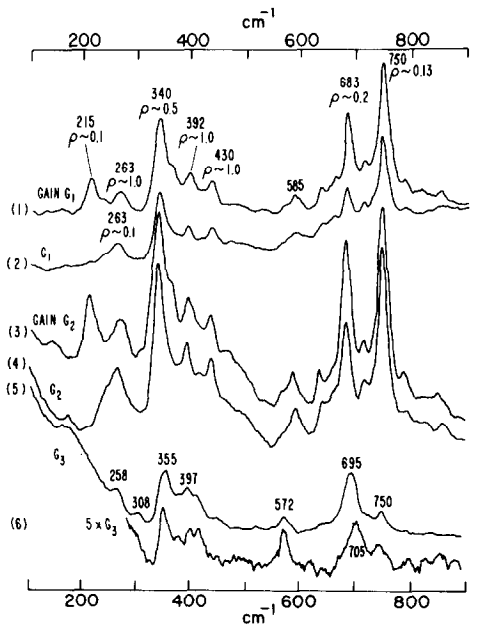

Figure 1 Raman spectra in the 100-900 cm^{-1} region. Relative system gains indicated adjacent to each spectrum. Spectra are unpolarized, reflection mode, single scans using standard 5 mm x 10 mm rectangular cuvettes, slit width ca 5 cm^{-1}; scan rate of 1 cm^{-1}/sec.; sample volumes ca 0.3 ml. (1) Cytochrome c oxidase ca 70 μM reduced with 0.5 μM cytochrome c and a few crystals of sodium dithionite. (2) Oxidized cytochrome c oxidase, same sample as in (1) prior to reduction. (3) ETP, ca 70 mg/ml protein, reduced with a few crystals of sodium dithionite. (4) ETP sample as in (3) oxidized by addition of ferricyanide. (5) Cytochrome c ca 40 μM reduced with dithionite. (6) Oxidized cytochrome c same sample as in "5" prior to reduction.

The spectra of cytochrome c at 441.6 nm excitation are markedly different from those of Strekas and Spiro (4, 6) which were obtained with laser excitation (514.5 nm) near the α and β bands. These differences are related to the differences in the electronic bands in resonance with the incident laser frequencies; spectra of oxyhemoglobin and deoxyhemoglobin excited at 488.0 nm (5) resemble more closely the hemoprotein spectra that we have observed with 441.6 nm excitation.

As expected from the general structural similarities in the heme chromophore, the gross features of the spectra of c oxidase, cytochrome c, and other hemoproteins are similar. Nevertheless there are differences between c oxidase

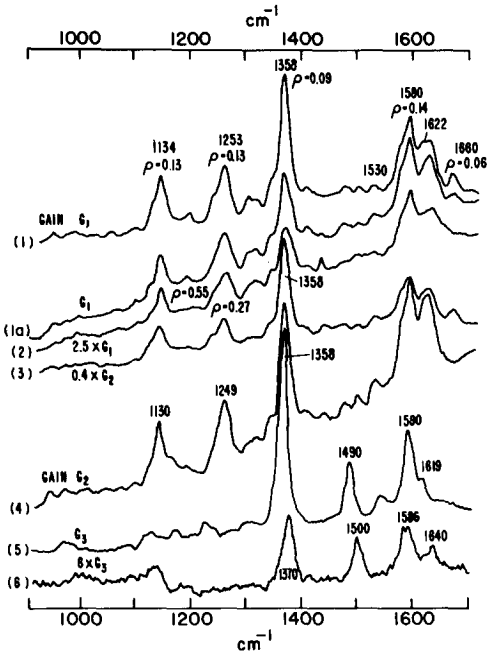

Figure 2 Raman spectra (unpolarized) in the 900-1700 cm^{-1} region, instrumental conditions as in Figure 1. (1) Reduced cytochrome c oxidase as in Fig. 1-1. (1a) Partially reduced cytochrome c oxidase obtained fortuitously in the course of dithionite reduction. Intensity of 1660 cm^{-1} about one-half that in fully reduced. Traces 2 to 6 are from same samples as corresponding traces in Figure 1.

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and for example, cytochrome c; these occur in the ranges 1500-1670 cm^{-1}, 1100-1250 cm^{-1}, 1350-1370 cm^{-1} and below 900 cm^{-1}. The bands in the 1500-1670 cm^{-1} region correspond to normal modes with major contributions from C=C and C=N double bond stretching vibrations of the conjugated ring (13-16), while those below 900 cm^{-1} are mainly skeletal vibrations of the heme (13-16). The differences in these regions presumably are due to the detailed structural differences between heme a and heme c and to the different modes of binding to the protein (1, 2). The lines at 1660 cm^{-1} and 215 cm^{-1} in the spectrum of reduced c oxidase are absent in the oxidized protein and thus appear to be markers for the fully reduced protein. The line at 1660 cm^{-1} could be due to the formyl group on the heme a since this frequency is usually characteristic of a C=O

stretching (12); its appearance upon reduction could be due to structural changes accompanying electron transfer which enhance the coupling of the C=O to the conjugated system, thus causing the C=O frequency to become resonance enhanced.

The behavior of the strong, polarized line in the 1350-1370 cm^{-1} region of c oxidase is consistent with the presence of the two chemically nonequivalent heme components. Figure 3 shows expanded scans of this region. Every dithionite reduced c oxidase sample exhibits a single symmetrical line at 1358 cm^{-1} (Figure 3A), while c oxidase, as isolated, shows a band at ca 1358 cm^{-1}

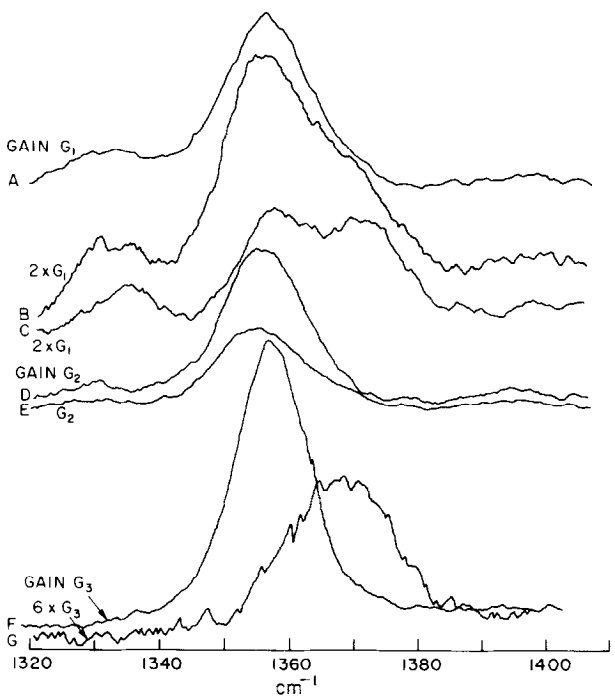

Figure 3 Raman spectra in the 1300-1400 cm^{-1} region. Relative system gains indicated adjacent to each spectrum. Single scans, slit width ca 5 cm^{-1}, scan rate 0.25 cm^{-1}/sec. (A) Reduced cytochrome c oxidase as in Figure 1-1. (B) Sample as in (A) prior to reduction. This is not the same intermediate as in Fig. 2-1a; it is characterized by the absence of 215 cm^{-1} and 1660 cm^{-1} lines as in Figure 1-2 and Figure 2-2 (see text). (C) Ferricyanide oxidized cytochrome c oxidase. Traces D to G correspond to the same samples as traces 3 to 6 of Figure 1.

which varies between the two spectra exemplified by Figures 3B and 3C. Samples which only showed the single asymmetric line (3B) could be converted to samples showing spectrum 3C either by exposure to oxygen or by addition of ferricyanide. Samples which show the doublet (Figure 3C) are presumed to be oxidized because: (1) the spectra show the same relative intensity distribution as in other oxidized hemoproteins, such as cytochrome c, over the entire spectral range and (2) the lines at 1660 and 215 cm^{-1} characteristic of fully reduced c oxidase are absent. In contrast, the spectra of cytochrome c, hemoglobin, myoglobin, horseradish peroxidase, cytochrome b_5, and the cytochrome b - c_1 combination of mitochondrial complex III, show only a single line at ca 1358 cm^{-1} in the reduced state which is shifted to ca 1370 cm^{-1} upon oxidation (unpublished results). Thus oxidized c oxidase appears to have a "normal" heme which contributes the 1370 cm^{-1} band and an anomalous component whose frequency is low even in the oxidized state. It is tempting to ascribe these two lines to the two moieties a and a_3.

This conclusion is also supported by the behavior of the relative intensities of various lines as the oxidation state of the protein is changed. Figure 4 summarizes these results for some of the prominent lines. In the intermediate state corresponding to Figure 2-1a the lines at 1660 and 1358 cm^{-1} have intensities that are roughly halfway between their values in the fully oxidized and reduced states whereas other lines (1130, 1620 and 1580 cm^{-1}) have intensities much closer to those in the reduced state. Although this intermediate state is not well defined because dithionite was added aerobically, such behavior would be expected of two nonequivalent heme units which, under these conditions, accept electrons at different potentials and which are in different environments so that they contribute differently to the Raman spectra.

We find a correspondence between the Raman spectra of reduced ETP and reduced c oxidase. However the spectra of ETP, both as isolated and further oxidized by either ferricyanide or oxygen, do not develop the doublet at 1358-

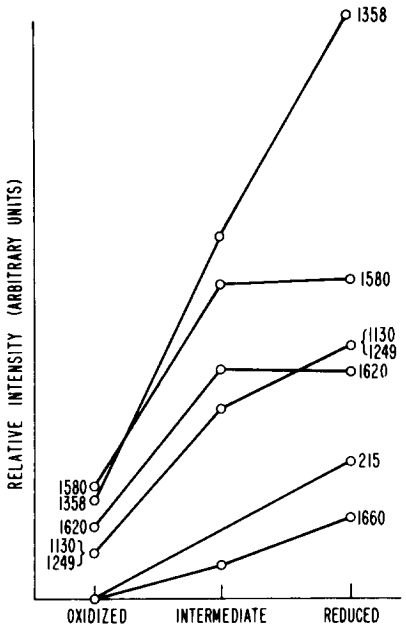

Figure 4 Comparison of relative intensities of selected lines from spectra of cytochrome c oxidase. Changes in oxidation state were achieved without significant change in concentration and the instrument stability was such that for different runs on the same sample the intensities were reproducible to better than 15%, making most of the observed intensity variations significant. The low frequency data on the intermediate state were not taken.

1370 cm^{-1}, but rather retain a sharp singlet at 1358 cm^{-1} even though the two marker lines at 215 cm^{-1} and 1660 cm^{-1}, characteristic of reduced c oxidase, are absent. In addition, upon oxidation the intensity of the 1358 cm^{-1} line (relative to other lines in the same spectrum) decreases less in ETP than in c oxidase. On the basis of other preliminary data of mitochondrial complex III it is unlikely that these differences are due to contributions to the ETP spectra from cytochromes b and c_1, since for the same heme concentration we find that the intensities of the spectra for oxidized and reduced solubilized c oxidase are respectively ca 28 and 3 times larger than those of oxidized and reduced complex III estimated from the combined concentration of cytochromes b and c_1 (17) presumably because the Soret band of c oxidase is closer to

441.6 nm. From the estimated concentrations of cytochromes $\underline{a}+\underline{a}_3$, $\underline{b}$ and $\underline{c}_1$ in ETP (18) we conclude that the contribution of $\underline{c}$ oxidase to the ETP spectra in the oxidized and reduced states must be respectively 40 and 4 times larger than that of the combined contributions of cytochromes $\underline{b}$ and $\underline{c}_1$.

We believe we have observed $\underline{c}$ oxidase in three of its many possible states: (1) State A of Figure 3 which is fully reduced and nonequivalent hemes are not observable; (2) State B of Figure 3 which is often observed for the resting protein and can be reached during progressive oxidation from aerobically reduced protein; and (3) State C of Figure 3, which is fully oxidized and suggests nonequivalent heme groups. The correlation of these observations with existing optical and EPR studies requires complete anaerobic Raman redox titrations. The preliminary results presented here suggest that these titrations will be instructive.

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