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RESONANCE ENHANCEMENT OF THE VIBRATIONS OF CYTOCHROME a_3 AND ITS CONFORMATION IN OXIDIZED CYTOCHROME OXIDASE

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Summary: Resonance enhancement of high and low frequency vibrational modes of oxidized cytochrome a_3 in several cytochrome oxidase species have been observed when flowing samples and excitation frequencies of 413.1 nm and 406.7 nm were used. The data are interpreted by excitation profile arguments which incorporate optical parameters from the spectra of cytochromes a and a_3 deduced by Vanneste (Biochemistry 5, 838-848 (1966)). The carbonyl of oxidized cytochrome a_3 is conjugated with the heme a π-electron system independent of both its iron spin state and the redox state of cytochrome a. This contrasts with the behavior of cytochrome a and of reduced cytochrome a_3. Mechanistic implications of these conformational differences are briefly considered.

INTRODUCTION

Cytochrome a_3 catalyzes the efficient and rapid reduction of dioxygen to water in the respiratory protein, cytochrome oxidase. Strong evidence exists to indicate that both iron, incorporated in the formyl-containing porphyrin heme a, and copper are involved in the cytochrome a_3 active site (1-4). The structural details are uncertain, however, and much recent discussion concerns the identity of the bridging ligand which mediates the strong (> 200 cm^{-1}) antiferromagnetic exchange coupling between the two metals (2,5-8). The presence of the formyl at the heme a periphery and its role in enzyme catalysis is also perplexing, although recent Raman and optical results implicate conformational modulation of the electron-withdrawing properties of the formyl as a means of regulating heme a iron reactivity in situ (9).

Two phenomena complicate the application of resonance Raman spectroscopy to the oxidized enzyme: a) the protein is susceptible to photoreduction (10,11)

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and b) considerable uncertainty exists regarding both the visible and Soret band absorption properties of the oxidase chromophores, cytochromes a and a_3 (12-15).

To overcome the photoreduction problem we developed a flowing sample system and used it, along with 441.6 nm laser excitation, to obtain Raman data for the oxidized protein (16). At this exciting frequency, the spectrum is essentially that of oxidized, low-spin cytochrome a. This result is predicted by combining excitation profile arguments with the oxidase Soret spectral assignments of Vanneste (17) and suggests that deeper blue exciting lines are necessary for resonance enhancement of oxidized, high-spin cytochrome a_3. In agreement with this, we report here that with 413.1 nm and 406.7 nm excitation of oxidized cytochrome oxidase, strong enhancement of cytochrome a_3^{3+} vibrational modes in both high and low frequency regions occurs. In contrast to cytochrome a, the formyl stretching vibration is observed for oxidized cytochrome a_3 independent of both its spin state and the redox state of cytochrome a. The latter observation shows that the conformation of heme a in the cytochrome a_3 site is distinct from its conformation in the cytochrome a site, while the former provides convincing support for Vanneste's spectral assignments.

MATERIALS AND METHODS

Beef heart cytochrome oxidase and its inhibitor complexes were prepared as described previously (3). Enzyme concentrations were typically 20 μM in a buffer consisting of 50 mM Hepes, 0.5% Brij 35, pH 7.4. Raman spectra for flowing samples were recorded with the Spex 1401 double monochromator and associated Ramalog electronics used in earlier studies (13). Excitation at 413.1 nm and 406.7 nm was obtained from a Spectra Physics 164-11 laser head equipped with a high field magnet. A prism, external to the laser cavity, was used to separate the two frequencies. Power incident on the sample was typically 15 mW and never exceeded 25 mW. Beam polarization and Raman depolarization ratios were determined as before (13).

RESULTS

Raman spectra recorded for several cytochrome oxidase species are shown in Figure 1. The spectrum we report for the reduced enzyme is identical to that recorded previously with 413.1 nm excitation (10). For the oxidized enzyme and excitation at either 406.7 or 413.1 nm, the oxidation state marker

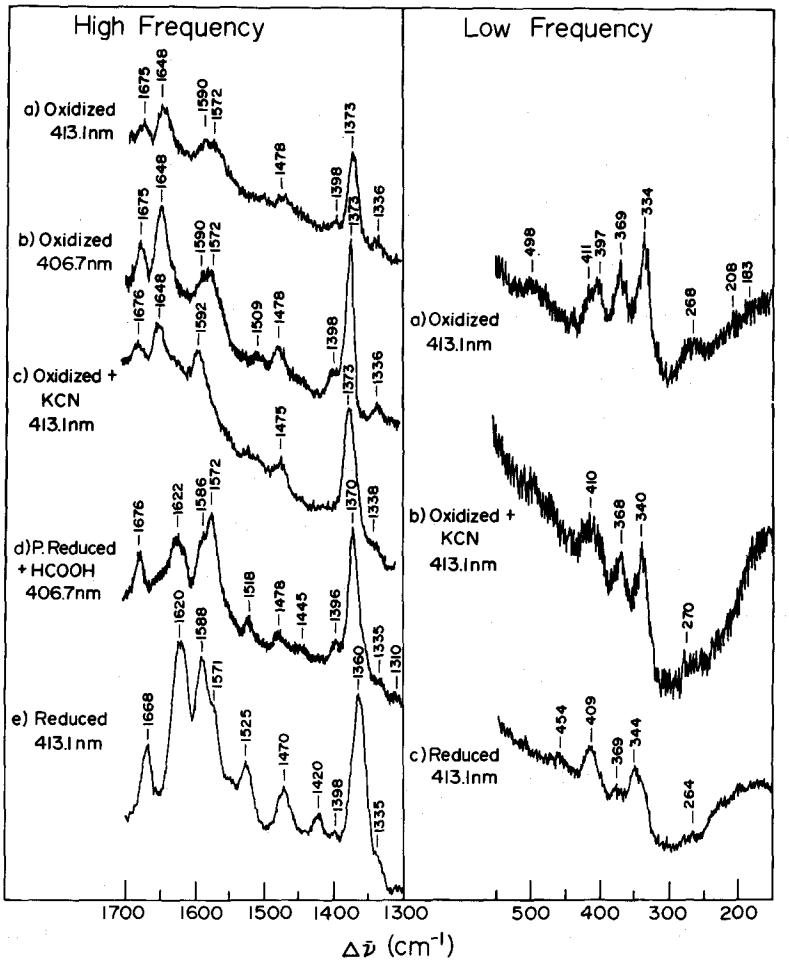

Figure 1. Resonance Raman spectra of cytochrome oxidase species with Soret excitation. Left Panel: the high frequency region for a) and b) the oxidized enzyme, c) the oxidized enzyme plus 6 mM neutralized potassium cyanide, d) the enzyme in the presence of 60 mM sodium formate, 10 mM sodium ascorbate and 0.1 mM tetramethylphenylenediamine and e) the enzyme following dithionite addition. The instrument gain and resolution (8 cm^{-1}) were the same for all five spectra, in b) and d) the instrument time constant was 1.0 sec, for a), c) and e) the time constant was increased to 2.5 sec. Right panel: the low frequency region for a) the oxidized enzyme, b) the oxidized enzyme plus 6 mM neutralized potassium cyanide and c) the enzyme following dithionite addition. The instrument resolution, 8 cm^{-1}, and time constant, 2.5 sec, were the same for all spectra; instrument gain in a) and b) was 10 times higher than in the high frequency region, in c) the gain was 3.3 times that of the high frequency spectrum. All vibrational lines in both frequency regions are polarized. Laser frequencies are indicated for all spectra.

occurs at 1373 cm^{-1}, indicating that the flowing sample remains oxidized, the formyl carbonyl vibration occurs at 1675 cm^{-1}, and a doublet is observed in the 1570, 1590 cm^{-1} region. These spectra are remarkably different from that recorded for oxidized oxidase with 441.6 nm excitation where neither the 1570

cm^{-1} component of the doublet nor the 1675 cm^{-1} vibration is observed (16). Using inhibitor complexes, model compounds and excitation profile arguments, we assigned the 441.6 nm spectrum to be essentially that of cytochrome $\underline{a}^{3+}$. The appearance of new bands with 413.1 nm excitation indicates that at this wavelength we are enhancing vibrations of both oxidase iron chromophores. In agreement with our earlier heme $\underline{a}$ model compound results (16) and with results on hemoglobin and myoglobin derivatives (18,19), we assign the 1572 cm^{-1} vibration to high-spin cytochrome $\underline{a}_3^{3+}$, and note that its carbonyl, but not that of cytochrome $\underline{a}^{3+}$, is resonance enhanced by Soret excitation. Additional support for these assignments and further information on the oxidase comes from Raman spectra of its inhibitor complexes. In the oxidized enzyme-cyanide complex (Fig. 1c) both $\underline{a}^{3+}$ and $\underline{a}_3^{3+}$ are low-spin and the 1572 cm^{-1} band, indicative of the high-spin state, is absent. The carbonyl vibration remains enhanced and, based on a comparison with our earlier 441.6 nm spectra (16), can be assigned to the cytochrome $\underline{a}_3^{3+}\cdot CN^-$ complex. Reduction of only cytochrome $\underline{a}$ is achieved by addition of a mild reducing system in the presence of formate to form the partially reduced complex, $\underline{a}^{2+}$ $\underline{a}_3^{3+}\cdot$HCOOH (20). The Raman spectrum, Figure 1d, retains the 1572 cm^{-1} feature, as well as the 1676 cm^{-1} formyl vibration, which we assign to the high-spin $\underline{a}_3^{3+}\cdot$HCOOH complex. The broadening on the low frequency side of the 1370 cm^{-1} band and the bands at 1586 cm^{-1} and 1622 cm^{-1} are attributed to reduced cytochrome $\underline{a}$. The right panel of Figure 1 shows low frequency Raman spectra for three oxidase species; the strong resonance enhancement of these modes indicates that they may be useful in providing insight into the identity of the bridging ligand in the $\underline{a}_3^{3+}$ active site.

DISCUSSION

The pattern of resonance enhancement for Soret excitation of cytochrome oxidase can be rationalized using Vanneste's optical assignments (17) and simple excitation profile arguments. In the Soret band of heme proteins Franck-Condon scattering dominates and the scattered intensity should be

TABLE I: SPECTRAL AND RAMAN PARAMETERS

- CYTOCHROME $\underline{a}$ and $\underline{a}_3$ SPECIES[a]

Species	λ_{max} nm	Γ cm^{-1}	ε $mM^{-1}cm^{-1}$	R x 10^3 441.6 nm	413.1 nm	406.7 nm
$\underline{a}_3^{3+}$	414	800	81	2.2	10.2	7.9
$\underline{a}_3^{3+}\cdot CN^-$	427	550	65	4.7	4.6	2.5
$\underline{a}_3^{3+}\cdot HCOOH$	414	750	98	3.3	17.0	12.8
$\underline{a}^{3+}$	427	800	120	12.3	11.4	7.2
$\underline{a}^{2+}$	444	550	113	40.3	4.1	2.8

[a]Spectral parameters from Reference 17.

proportional to the quantity R defined by

$$R = \frac{\varepsilon^2}{(\nu - \nu_o)^2 + \Gamma^2}$$

where ε is the molar extinction coefficient at the frequency, ν, of the Soret band maximum, ν_o is the laser frequency and Γ is the Soret bandwidth at half maximum (16,21,22). Table I summarizes R values for cytochrome $\underline{a}$ and $\underline{a}_3$ at three different excitation frequencies. The dominance of cytochrome $\underline{a}^{3+}$ modes in the oxidized enzyme under 441.6 nm excitation and the appearance of cytochrome $\underline{a}_3^{3+}$ modes in the spectra obtained with 413.1 nm excitation is apparent when one ratios the R values for the two species at the different laser frequencies.

A comparison of Figures 1(a) and (c) shows that the formyl of oxidized cytochrome $\underline{a}_3$ is conjugated with the porphyrin π system independent of the ferric iron spin state. This contrasts with its behavior in reduced cytochrome $\underline{a}_3$, where formyl resonance enhancement is lost upon the high-spin →

low-spin transition (11). The mechanistic implications of this shift in geometry during dioxygen reduction have been discussed recently (9). The behavior of the heme a carbonyl of cytochrome a_3 also contrasts with that of cytochrome a where the formyl carbonyl vibration is not enhanced in either iron valence state. This suggests that the carbonyl of cytochrome a may carry out a specialized function and makes it a potential site for the proton pumping activity of cytochrome oxidase (23) in analogy with the aldehyde/ Schiff's base mechanism in the bacteriorhodopsin system (24).

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