RESONANCE RAMAN SPECTRA OF HEME *c* AND HEME d_1 IN *PSEUDOMONAS* CYTOCHROME OXIDASE

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1. Introduction

Pseudomonas cytochrome oxidase (ferrocytochrome c_{551}: oxygen oxidoreductase, EC 1.9.3.2), functions as a terminal electron carrier in *Pseudomonas aeruginosa* by accepting electrons from either *Pseudomonas* cytochrome c_{551} or *Pseudomonas* copper protein (azurin) and transferring these electrons to nitrite [1]. The enzyme is purified as a highly water soluble dimer (M_r 120 000) [2] with 2 apparently equivalent subunits, each of which contains 1 heme *c* and 1 heme d_1 [3]. In addition to the reduction of nitrite the enzyme is capable of the 4 e^- reduction of O_2 [4]. Thus, it serves as a model for mammalian cytochrome *c* oxidase. Although many of the properties of the oxidase have been determined from biochemical and biophysical studies, other questions concerning the basic structural and spectroscopic features of the enzyme remain unresolved. Here, we present resonance Raman spectra of *Pseudomonas* cytochrome oxidase in its oxidized and reduced forms and after adding cyanide to the reduced enzyme.

2. Materials and methods

All inorganic reagents were of analytical reagent grade. L-Ascorbic acid, certified grade, and sodium dithionite, purified grade, were from Fisher Scientific. *Pseudomonas* cytochrome oxidase was prepared as in [2]. The concentration of *Pseudomonas* cytochrome oxidase was determined from the absorbance of the ascorbate- or dithionite-reduced enzyme at 554 nm using an extinction coefficient of 30.2 $mM^{-1} \cdot cm^{-1}$ [5]. The enzyme was diluted in 50 mM KPO_4 (pH 7.0), to 3–5 mg/ml and incubated at room temperature for at least 30 min in a nitrogen-purged glove box prior to the addition of reductant. It was then reduced by the addition of a freshly prepared buffered solution of sodium ascorbate (final conc. ≈50 mM) or a few grains of solid sodium dithionite. After an additional 45 min incubation under a N_2 atmosphere to insure complete reduction, the absorption spectra of the samples were monitored with a Cary model 219 spectrophotometer. Freshly prepared, neutralized KCN was added to the reduced enzyme where indicated to final conc. 50 mM. The samples were then transferred under a N_2 atmosphere to cylindrical Raman cells and sealed. Raman spectra were obtained by the instrumentation in [6].

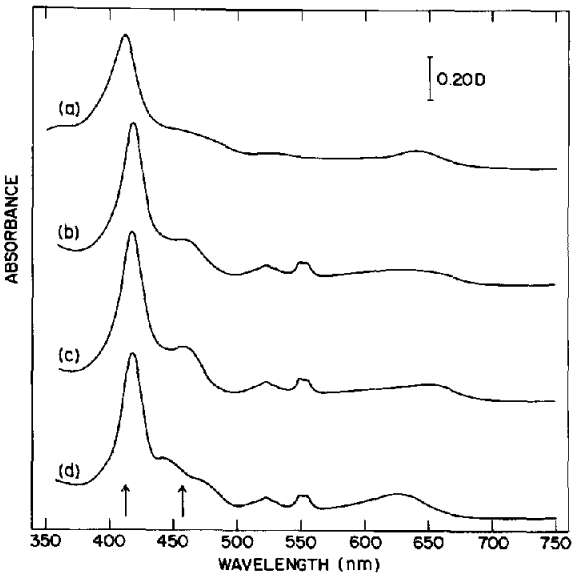

Fig.1. Optical absorption spectra of *Pseudomonas* cytochrome oxidase. Enzyme was 20 μM in 50 mM KPO_4 buffer (pH 7.0); optical cell pathlength, 0.1 cm: (a) oxidized preparation; (b) preparation reduced with $Na_2S_2O_4$; (c) preparation reduced with sodium ascorbate; (d) sample (c) plus 50 mM cyanide.

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3. Results and discussion

The optical absorption spectrum of reduced *Pseudomonas* cytochrome oxidase (fig.1) has two bands in the Soret region. One, with a maximum at ≈417 nm, has been proposed to originate from the heme *c* moiety of the enzyme [7]. The other which has a peak at ≈460 nm, has been ascribed to the heme d_1. As may be seen from fig.1 the krypton ion-laser excitation frequency at 413.1 nm and the argon ion-laser excitation at 457.9 nm, are nearly coincident with these 2 maxima.

The resonance Raman spectrum of dithionite-reduced *Pseudomonas* cytochrome oxidase, obtained using an excitation of 413.1 nm and shown in fig.2a, is very similar to the spectrum of reduced *Pseudonomas* cytochrome c_{551} illustrated in fig.2c. The frequencies of the major lines are labeled on the spectra. Only in

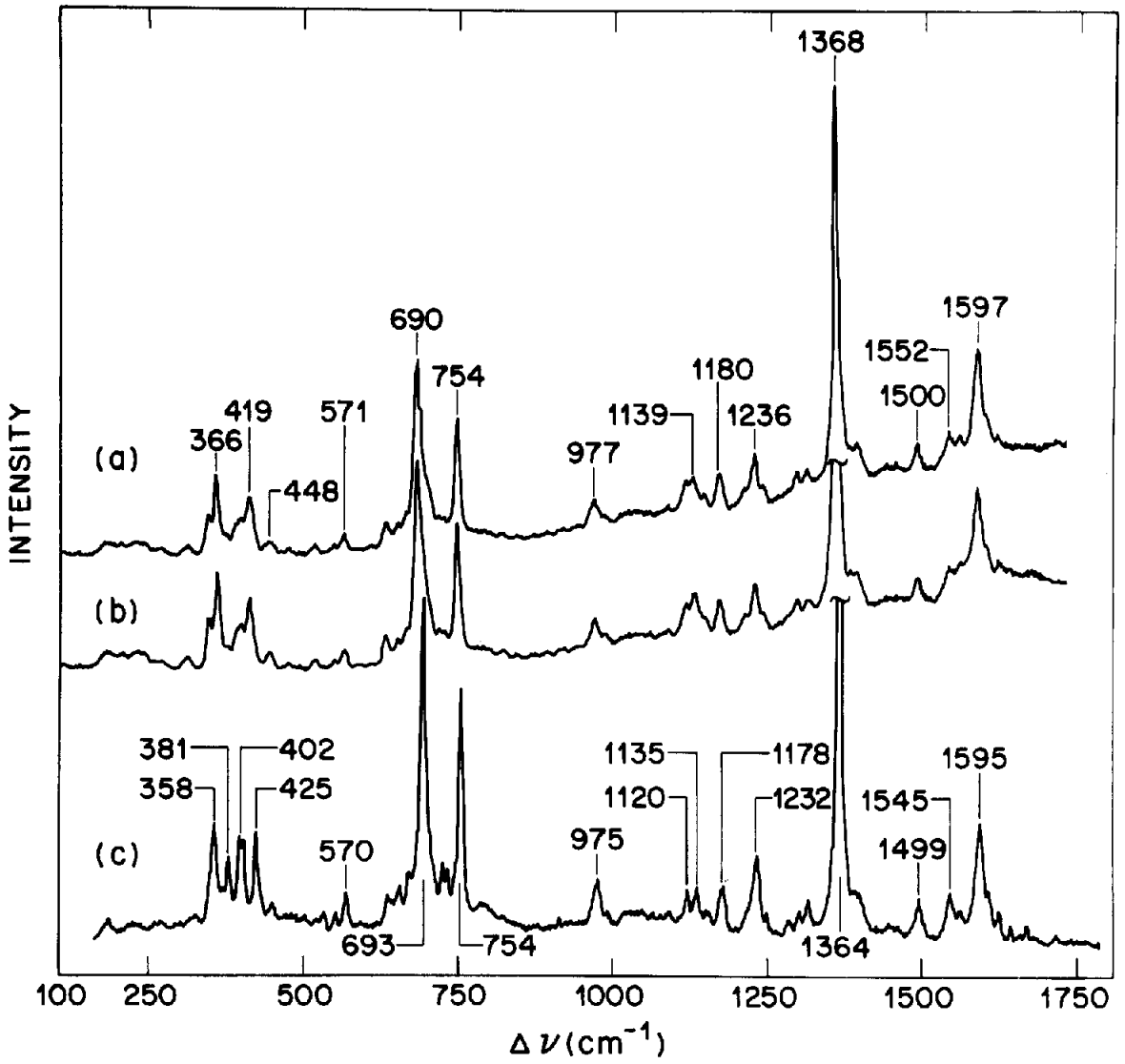

Fig.2. Resonance Raman spectra of reduced *Pseudomonas* cytochrome oxidase obtained with excitation in the Soret region (413.1 nm) of the heme *c* prosthetic group. The sharp line at 1345 cm^{-1} marked with an asterisk is a laser fluorescence line. Enzyme was 20 μM; laser power 40–50 mW; spectral slit width, ~4 cm^{-1}: (a) *Pseudomonas* cytochrome oxidase preparation reduced with $Na_2S_2O_4$; (b) sample (a) plus 50 mM cyanide; (c) dithionite-reduced *Pseudomonas* cytochrome c_{551} (40 μM).

the 300–400 cm^{-1} regions do significant qualitative differences appear. It is in this region that band splitting in cytochrome *c* has been reported to occur, presumably due to removal of axial degeneracy [8]. Differences among other cytochromes *c* are also evident in this region [9].

In fig.3a,b the resonance Raman spectra of dithionite- and ascorbate-reduced *Pseudomonas* cytochrome oxidase, obtained with 457.9 nm excitation, are presented. There are very few similarities in the peak positions or relative intensities between these spectra and those obtained with 413.1 nm. Cytochrome *c*

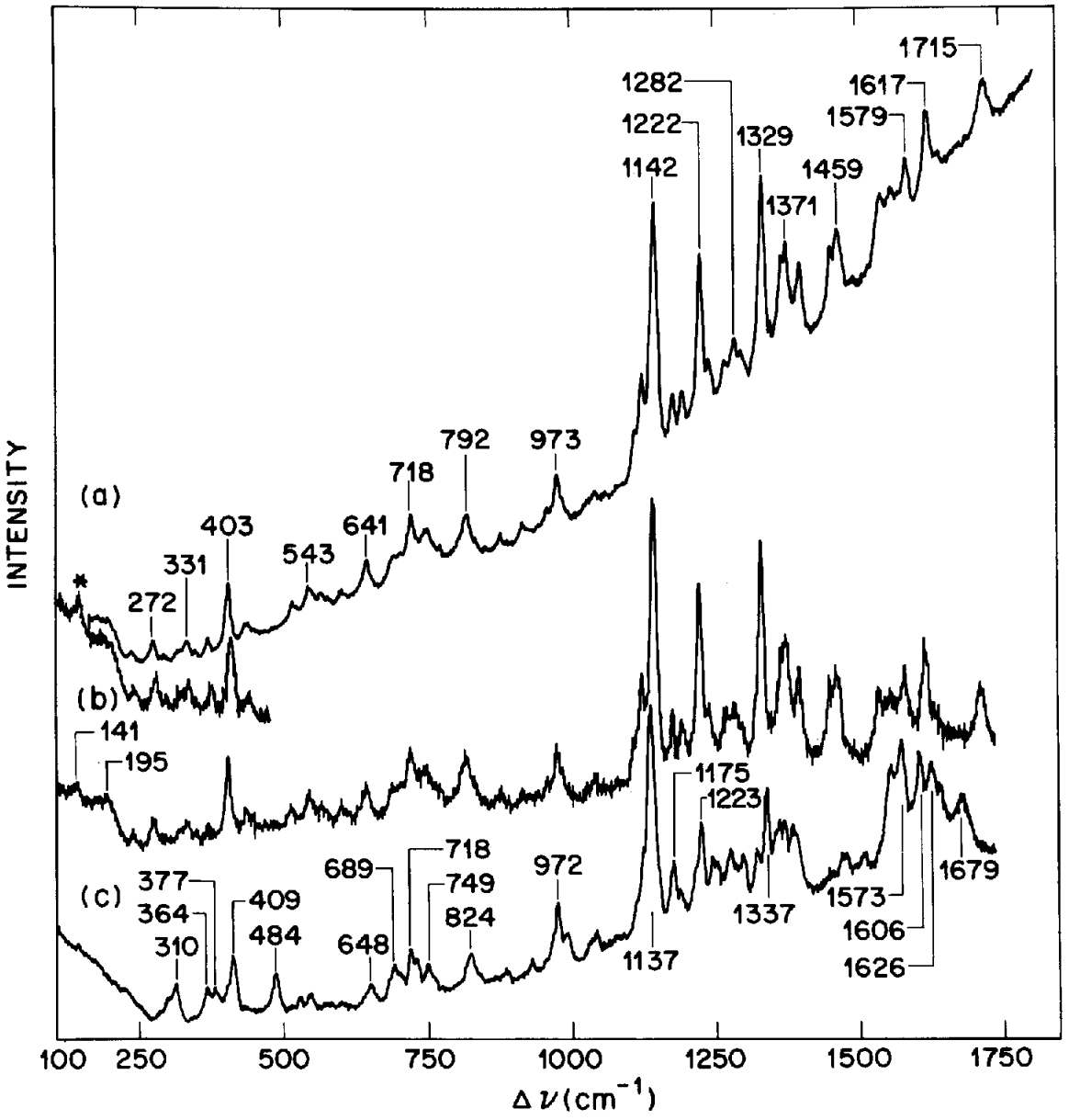

Fig.3. Resonance Raman spectra obtained with excitation in the Soret region (457.9 nm) of the heme d_1 prosthetic group of reduced *Pseudomonas* cytochrome oxidase. Enzyme was 30 μM; laser power ~300 mW; spectral slit width, ~4 cm^{-1}: (a) preparation reduced with $Na_2S_2O_4$. The insert was obtained from a second dithionite reduced sample. The sharp feature denoted by an asterisk is a laser fluorescence line; (b) preparation reduced with sodium ascorbate; (c) sample (b) plus 50 mM cyanide.

excited at 457.9 nm does not have spectra which resemble the spectrum of the *Pseudomonas* cytochrome oxidase excited at this wavelength. Thus, we attribute the spectrum obtained with 413.1 nm to the heme *c* entity and that excited with 457.9 nm to the heme d_1 entity. Spectra of cytochromes *cd* were reported [10] with 457.9 nm and 514 nm excitation. However, in those data complete separation was not obtained. Some of the heme d_1 lines were detected in the heme *c* spectrum excited at 514.5 nm. It was also concluded that some heme *c* lines detected in these spectra were also present in the heme d_1 spectrum obtained with excitation at 457.9 nm. We are puzzled by these results since, in addition to showing very few coincidences with the Soret band-excited heme *c* spectrum, we find that all the lines in the heme d_1 spectrum are fully polarized, indicating the absence of depolarized and anomalously polarized bands of heme *c* excited by resonances near its β-transition.

The spectra of heme d_1 are particularly noteworthy. The number of resonance Raman active vibrations of the heme d_1 moiety far exceeds those apparent in Soret-excited scattering from other hemes. This is presumably due to the reduction in symmetry of the chromophore from D_{4h} to C_{2v} or lower, allowing the Raman-forbidden E_u vibrational modes of the higher symmetry structure to become Raman-active modes in C_{2v} symmetry [11]. The number and position of the high frequency modes (>1000 cm^{-1}) of heme d_1 compare well with those of Cu^{2+} chlorin and isochlorin model compounds [12]. The dominance of A-term scattering, as evidenced by the polarized nature of all the bands obtained with 457.9 nm excitation, indicates resonance with a strongly allowed electronic state in the chlorin similar to the resonance Raman scattering behavior from other hemes with Soret band excitation.

The spectra of heme d_1 reported here differ greatly from those in [10] with surface-enhanced excitation on an electrode and quite surprisingly also differ from their solution spectra. For example, the moderately strong lines at 1459 and 1715 cm^{-1} in our reduced *Pseudomonas* cytochrome oxidase spectrum (of heme d_1) are absent from their surface-enhanced spectrum and present only weakly in their solution spectrum. Also the line they report at 1674–1686 cm^{-1} is not present in our spectrum of the heme d_1.

To determine the origin of the differences between our data and that in [10] we first examined the spectrum of heme d_1 for *Pseudomonas* cytochrome oxidase in the presence of cyanide. Cyanide binds preferentially to the heme d_1 [15] in the reduced enzyme and, as expected, alters the resonance Raman spectrum of that moiety substantially. Of particular significance are the changes which occur in the very low frequency region of the Raman spectrum where, in the absence of cyanide, there are lines at ~140 and 195 cm^{-1}. (The sharp line at 141 cm^{-1} is a laser fluorescence line superimposed on the broader Raman line.) Raman lines are also seen in 5-coordinate deoxyhemoglobin [16]. The 220 cm^{-1} line in deoxyhemoglobin has been shown to result from an imidazole–iron stretching mode [17]. Its presence in the *Pseudomonas* cytochrome oxidase spectrum is thus consistent with 5-coordination with an imidazole ligand. Upon cyanide binding and conversion to a 6-coordinate, low-spin configuration [18], these low frequency modes disappear in a manner analogous to the spectral behavior observed upon O_2 binding to hemoglobin.

Of additional interest in the cyanide-bound spectrum are the changes that occur in the high frequency region. In particular, note the absence of the line at 1459 cm^{-1} in the 6-coordinate spectrum and the shift of the line at 1715 to 1679 cm^{-1}. Comparison of these data to those in [10] indicates that their solution spectrum is apparently a mixture of 5- and 6-coordinate states and that their surface-enhanced spectrum is primarily 6-coordinate. The optical absorption spectra obtained from our dithionite- and ascorbate-reduced samples confirm that our samples are 5-coordinate. The origin of the 6-coordinate species evident in the spectra of [10] is uncertain. However, it appears that interactions with the electrode favor a 6-coordinate species since bands diagnostic of 5-coordination which are weakly present in their solution spectrum are absent in the spectrum of the surface-adsorbed protein. At present, it is not clear if the surface-enhanced spectra in [10] result from sample denaturation, selective enhancement of the low-spin component also present in their solution sample, or conformational changes due to the surface interactions. However, the observation in [19] that denaturation of ferric myoglobin may occur on an electrode surface, suggests that denaturation may be occurring in the *Pseudomonas* cytochrome oxidase case as well. Clearly, caution must be exercised in evaluating surface-enhanced Raman data from biological molecules.

The optical absorption spectrum (fig.1) in the 600–700 nm region is sensitive to whether the reductant is dithionite or ascorbate. This difference has been attributed to the binding of a sulfoxy anion to the

heme d_1 region [13]. To test this proposal we have obtained the resonance Raman spectra of samples in the presence of each reductant and found no detectable differences in the heme d_1 spectra (fig.3). This indicates that it is unlikely that the sulfoxy ion binds directly to the heme d_1 and some other explanation of the absorption differences must be considered [14]. Raman excitation in the 600–700 nm, currently in progress, should help clarify the origin of the optical absorption changes.

In [20], it was reported on the basis of MCD measurements that when reduced, the electronic states of the heme *c* of *Pseudomonas* cytochrome oxidase are strongly affected by the electronic states of the heme d_1. However, this heme *c*/heme d_1 electronic interaction was called into question by a reappraisal of the MCD spectroscopic results [21]. Due to the clear separation between the 2 types of heme by selective laser excitation, we have been able to examine the heme *c* spectra in the absence or presence of cyanide bound to the heme d_1, conditions under which the heme d_1 undergoes a high–low-spin transition [18]. In contrast to the pronounced spectral changes detected in the heme d_1 upon binding cyanide, the Raman spectrum of the heme *c* is not significantly affected. The lack of effect of cyanide on the heme *c* spectra indicates that there is no large electronic interaction between the heme *c* and the heme d_1 and confirms the conclusions in [21].

Also of interest is the mode at 1715 cm^{-1} in the reduced enzyme which shifts to 1679 cm^{-1} in the presence of cyanide. No Raman modes have been reported in this region for porphyrins or chlorins. However, bands at 1706–1720 cm^{-1} have been detected in Cu^{2+} di-ketoisochlorins [12]. This is also the region where keto- and aldo-group frequencies appear in aliphatic and aromatic hydrocarbons. In mammalian cytochrome oxidase and *Spirographis*-substituted myoglobins, carbonyl groups on the periphery of the hemes result in a pronounced red-shift of the electronic spectra. Carbonyl modes are evident in the spectra of these proteins appearing at 1645–1670 cm^{-1} [22,23] and are quite conformationally sensitive. Since the carbonyl groups on the propionic acids should not be resonantly enhanced, the occurrence of a mode in the 1700 cm^{-1} region suggests [10] the presence of an α-keto group on a heme substituent or on the chlorin macrocycle. This would also account for the large red shift of the heme d_1 absorption peaks relative to those of octaethychlorins. If this 1680–1720 cm^{-1} line does

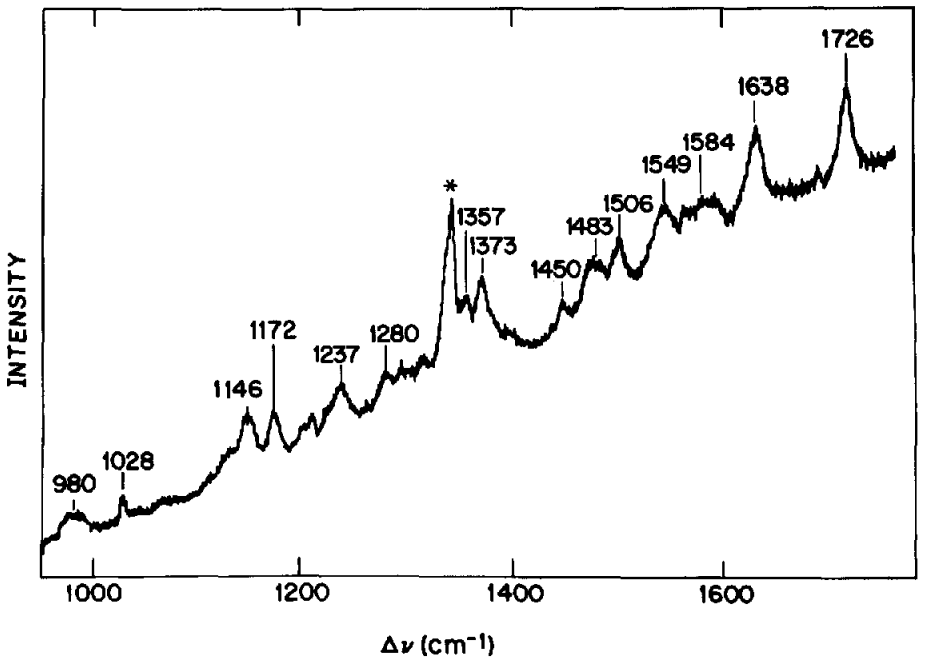

Fig.4. Resonance Raman spectra of oxidized *Pseudomonas* cytochrome oxidase with excitation (457.9 nm) in the Soret region of the heme d_1. Enzyme was 40 μM; laser power ~300 mW; spectral slit width ~4 cm^{-1}.

result from a keto group, its large shift upon the addition of cyanide could result from either a significant change in heme electron density or a change in heme/protein interaction. Formyl or keto substituents were not detected in a chemical analysis of heme *d* extracted from *Aerobacter aerogenes* [24]. However, in [25] spectral differences between the heme *d* isolated from *A. aerogenes* [24] and that from *P. aeruginosa* [25] were indicated.

The spectrum of the high frequency region of the oxidized enzyme obtained with 457.9 nm excitation shown in fig.4 is interesting for the following reasons:

(i) While the number of Raman active modes is comparable to that of the reduced protein there are numerous changes in both position and intensity of the modes;

(ii) The mode at ~1715 cm^{-1} in the reduced spectra is also present in the oxidized spectrum at 1726 cm^{-1} with increased intensity, indicating that the group giving rise to this mode still interacts strongly with the chlorin electronic system in the oxidized protein.

These data illustrate that the good separation obtainable in the resonance Raman spectrum by selective excitation frequency allows independent studies of the heme *c* and heme d_1 in *Pseudomonas* cytochrome oxidase. Extension of these investigations to other forms of the enzyme and to other regions of the spectrum is underway and should provide insight into the mechanism of oxidase function.

Acknowledgements

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