RESONANCE RAMAN AND SURFACE-ENHANCED RESONANCE RAMAN STUDIES OF CYTOCHROME cd_1

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1. Introduction

Cytochrome cd_1 is the dissimilatory nitrite reductase found in many facultative, anaerobic denitrifying bacteria. It is an oxidation–reduction enzyme that accepts reducing equivalents from donor *c*-type cytochromes and transfers these to nitrite, reducing it to nitric oxide predominantly [1]. It reduces oxygen to water [2]. The native enzyme is a dimer composed of two identical subunits each of which is ~60 000 M_r and contains one *c*-type and one d_1-type heme [3]. Because of the spectral properties of the prosthetic groups, visible [4–6], electron paramagnetic resonance [7,8] magnetic circular dichroism [9] and emission [10] spectroscopy have been used to provide structural and physical information about the enzyme. In addition, resonance Raman (RR) spectroscopy is an invaluable technique for providing detailed insights into the structure and environment of heme in hemoproteins. Here we present resonance Raman data for cytochrome cd_1 and illustrate the scope of this approach.

Surface-enhanced resonance Raman spectroscopy (SERRS) was first applied to biomolecules in [11]. This technique utilizes the combined enhancement resulting from the resonance Raman effect (10^3–10^6) together with that observed for molecules adsorbed to Ag electrodes (an additional 10^3–10^6) to determine spectra on very dilute solutions (~10^{-6} M). In [11] horse cytochrome *c* and whale myoglobin were studied. Here, we also show that SERRS is a highly appropriate technique for enzymes of 120 000 M_r.

Cytochromes cd_1 have been purified from *Pseudomonas aeruginosa* [7,12–14], *Paracoccus denitrificans* [15,16], *Alcaligenes faecalis* [17] and *Thiobacillus denitrificans* [18]. Although all homologous, differences have been reported in physicochemical properties among these proteins. SERRS has been used here to obtain preliminary heme structure/environment comparisons between *Pseudomonas* and *Paracoccus* cytochromes cd_1. Because of the following points of similarity, cytochrome cd_1 can be viewed as an analogue for mitochondrial cytochrome aa_3: cd_1 is a cytochrome *c* oxidase; it can reduce O_2 in vitro; heme d_1 is spectroscopically related to heme *a*. Spectra reported here for cd_1 will be compared to data available for *c* and aa_3 to test the extent of analogy.

2. Experimental

Pseudomonas aeruginosa (ATCC 19429) was cultured and its cytochrome cd_1 was isolated as in [14]. *Paracoccus denitrificans* (ATCC 13543) was cultured in a standard nitrate medium [19]. The isolation of its cytochrome cd_1 will be described elsewhere; the protein used in this study was identical in properties to that described as component I in [16]. Samples for solution resonance Raman were 0.55 mM in protein in 0.1 M phosphate buffer (pH 7.5). Reduced protein was prepared by adding a 10-fold excess of sodium ascorbate as a neutralized, concentrated solution and sealing in 5 mm glass sample tubes under N_2. Concentrated stock protein solutions were diluted with the electrolyte just before adsorption.

Resonance Raman and surface-enhanced resonance Raman spectra were obtained using the 457.9 and 514.5 nm lines of an Ar^+ laser (Coherent Radiation, model CR-3). Raman-scattered light was collected in the 180° back-scattering geometry and focussed with an *f* 1.6 camera lens onto the slits of the double monochromator (Spex 1400-II). The band pass was 5 cm^{-1} for solution spectra and 2 cm^{-1} for the SERR spectra. An RCA C31034A cooled photomultiplier and standard low level threshold photon-counting

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methods were used to measure the Raman signals. Data were collected and processed on-line with a Raytheon 500 minicomputer interfaced to the Raman spectrometer. Other details of the instrumentation appear in [20].

Solution resonance Raman spectra were recorded at room temperature using 5 mm pyrex tubes as sample containers. The tubes were rotated in a spinner to minimize thermal heating of the sample during data acquisition. The SERR spectra were recorded using the cell in [21]. The electrolyte solution consisted of 0.1 M Na_2SO_4 in distilled and deionized (Milli-Q, Millipore Corp.) water. Degassing of the electrolyte and cell was accomplished by sparging with high purity nitrogen gas for at least 30 min. Following the degassing procedure, aliquots of the protein solution were added from a Hamilton microliter syringe. The aliquot size was adjusted so that the final bulk concentration of the protein was $1-3 \times 10^{-6}$ M in the electrolyte solution. The working electrode consisted of a Ag wire epoxied in glass. It was mechanically polished and anodized in the protein solution. The anodization procedure consisted of an oxidation at +0.45 V (*vs* SCE) followed by a reduction at −0.6 V. The total charge passed in the oxidation step was equivalent to 25 mC/cm^2. Following the anodization procedure, the electrode potential was held constant at various values during the Raman experiments. A description of the electrochemical instrumentation may be found in [21].

3. Results and discussion

Cytochrome cd_1 is ideal for resonance Raman investigations since it contains two heme components which have distinctive electronic absorption properties. The spectral properties of the protein, as well as the isolated chromophores have been well documented [4,22,23] and from these studies it appears that it should be possible to excite selectively RR scattering from either heme *c* or heme d_1 in the intact complex. For example, the 514.5 nm line of an Ar^+ laser is in a region of the absorption spectrum where reduced heme *c* is the principal absorber; hence, RR scattering from this chromophore should predominate when this line is used as an excitation source. On the other hand, reduced heme *d* has a broad absorption band near 460 nm and, therefore, excitation with the 457.9 nm Ar^+ line would be expected to produce enhancement of this chromophore in the protein complex. The results to be discussed below support the selective enhancement of heme *c* or heme d_1.

Due to the difficulties encountered in preparing sufficient quantities of cytochrome cd_1 from some organisms, we have explored the possibility of using the surface-enhanced effect observed for molecules adsorbed on Ag electrodes to study the RR spectra of these complexes in dilute solutions. In [11], the combined enhancement resulting from the surface and resonance effects permitted the observation of RR spectra of cytochrome *c* and myoglobin adsorbed on Ag from bulk solution levels of $\leqslant 1$ μM. Both of these proteins are much smaller than the cytochrome cd_1 complex, however, and the success of this approach could not be predicted a priori. Therefore, the initial set of experiments was designed to test the feasibility of using this method for the study of cytochrome cd_1

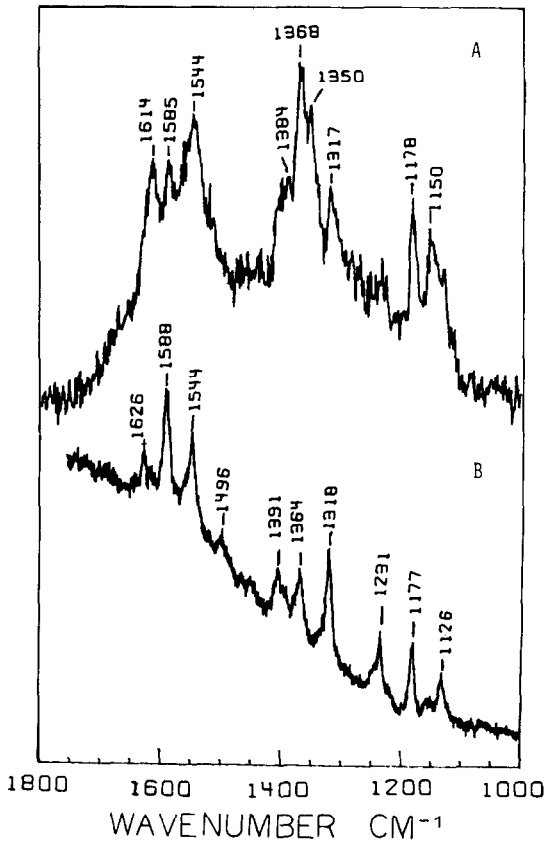

Fig.1. RR spectra of *Pseudomonas aeruginosa* cytochrome cd_1 complex, 514.5 nm excitation. (A) SERR spectrum, 3×10^{-6} M in 0.1 M Na_2SO_4, adsorbed on Ag at −0.6 V *vs* SCE. Laser power was 20 mW; monochromator slit width was 2 cm^{-1}. (B) Solution RR spectrum of reduced complex; 5.5×10^{-4} M; laser 20 mW; monochromator slit width = 2 cm^{-1}.

from two sources. This communication details the results of the initial study and shows that it is indeed possible to observe strong SERR signals from the proteins adsorbed on Ag. Due to the better quality spectra which result for the reduced protein, both in solution and adsorbed on the electrode, spectra will be presented for this oxidation state only at this time.

Fig.1A depicts the RR spectrum of spontaneously adsorbed cytochrome cd_1 at a Ag electrode, which was maintained at a potential of −0.5 V following the anodization procedure. The complex was isolated from *Pseudomonas* and the bulk solution concentration was 3×10^{-6} M. This spectrum can be compared with that in fig.1B, the RR spectrum of a 5.5×10^{-4} M solution of the reduced protein. In both cases 20 mW at 514.5 nm was used to excite RR scattering. The enhancement which results at the electrode is appreciable considering that the bulk solution concentration was ~200-fold less than that used in the solution spectrum. An accurate determination of the surface enhancement would require knowledge of the surface coverage. In the case of cytochrome *c*, assuming monolayer coverage, an increase in signal intensity of $\sim 10^5$ was estimated for the protein adsorbed on a Ag electrode. With cytochrome cd_1, the absolute signal intensity appears to be somewhat less than that observed for cytochrome *c* or myoglobin. However, if the much larger size of this protein is taken into account, the relative enhancement factors are probably quite similar. Other variables which may affect the

Table 1
Comparison of RR vibrational frequencies of cytochrome cd_1 adsorbed on a Ag electrode with those in solution[a]

514.5 nm Excitation		Cyt. *c*[b] solution	457.9 nm Excitation		Cyt. oxidase[c] solution
−0.6 V	Solution		−0.6 V	Solution	
				1719	
			1674	1686	1664
	1640				
	1626	1622	1622, sh	1625	1622
1614	1615, sh		1611	1613, sh	1610
1585	1588	1583	1578	1579	1584
1554, sh	1554		1557	1559	1569
1544	1544	1547	1544	1547, sh	
			1512, sh	1511	1520
	1496	1492			
				1472	
	1462	1457			
	1446			1455	
1397, sh	1400	1401	1394, sh	1397	
1384	1391			1389	
	1373			1376	
1368	1369			1367	
	1364	1364	1364		1358
1350			1342	1343, sh	
				1336	
1317	1318	1314			1322
			1300	1299	
				1280	
	1245, sh	1242		1247	1248
1231, vw	1231	1230	1229	1229	1226
1178	1177	1176	1172	1177	
1150	1154				
			1140	1146	
1125, sh	1126	1130	1130, sh	1129, sh	1130
			1039	1046	

[a] Peak positions are in cm^{-1}; sh = shoulder; vw = very weak; [b] from [24]; [c] from [25]

enhancement ratio include the orientation and distance of the chromophore from the electrode surface. The effects of these parameters are not well understood even in the case of small molecules at Ag electrodes and, therefore, their role with respect to cytochrome cd_1 cannot be determined at this time. Nonetheless, the important point to be noted here is that the surface enhancement effect is significant even in the case of a large protein such as cytochrome cd_1.

A comparison of the cytochrome cd_1 spectrum on the electrode surface with that in solution shows a close correspondence in band positions in most cases. The peak values are listed in table 1. Small shifts (3–4 cm^{-1}) are observed for some bands at the electrode surface, as was also found in the cytochrome *c* and myoglobin study. However, there are marked differences in the band intensities, with the most notable being the increase in bands at 1614, 1544, 1368, 1350 and 1150 cm^{-1} in the −0.6 V spectrum. The band at 1231 cm^{-1}, however, is much weaker on the surface. Small band shifts and marked intensity variations are characteristic phenomena observed for both small molecules as well as the heme proteins examined thus far (see [11] and literature cited therein).

The vibrational frequencies for the solution and surface spectra of cytochrome cd_1 shown in fig.1 are quite similar to those in [24] for cytochrome *c* using 514.5 nm excitation. These latter values are also listed in table 1. Thus, the oxidation and spin state markers indicate the heme *c* component of this complex is in its low spin, ferrous state. However, there are numerous vibrations, some of which are relatively weak, which are not observed in cytochrome *c*. These include bands at 1615, 1554, 1446, 1391, 1373, 1350 and 1154 cm^{-1}, and are tentatively assigned to heme d_1. Moreover, the 1614 and 1350 cm^{-1} bands are especially strong in the surface spectrum which could imply that heme d_1 may be closer to the electrode. The 1614 cm^{-1} band is only weakly enhanced in the solution spectrum and that at 1350 cm^{-1} is not observed at all.

Spectra recorded for *Pseudomonas* cytochrome cd_1 adsorbed on a Ag electrode at −0.6 V (fig.2A) and in solution (fig.2B) using the 457.9 nm Ar^+ line support the above heme d_1 assignments. A comparison of fig.2 with fig.1 shows there are many more bands in the former. In addition, the overall signal intensity is greater as would be expected on exciting closer in resonance with the strong Soret transitions. Some modes assignable to the *c*-type heme are also

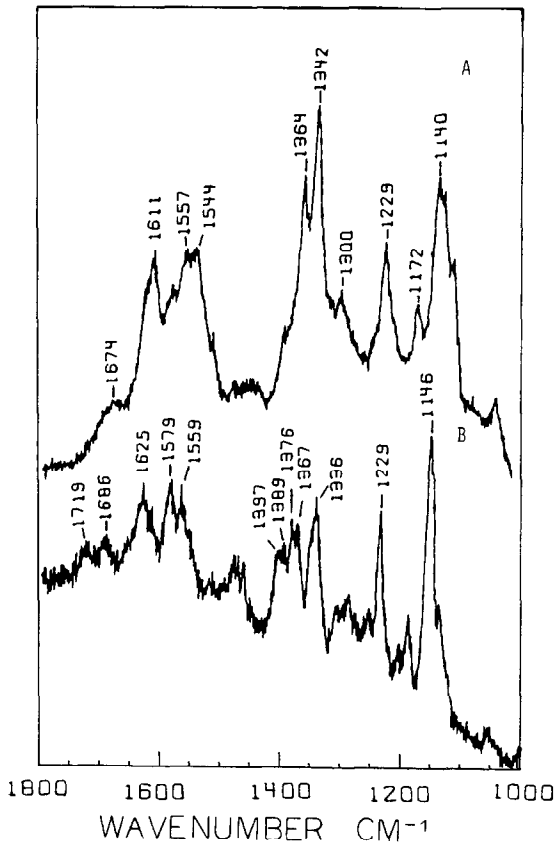

Fig.2. RR spectra of *Pseudomonas aeruginosa* cytochrome cd_1 complex, 457.9 nm excitation. (A) SERR spectrum, 3×10^{-6} M in 0.1 M Na_2SO_4, adsorbed on Ag at −0.6 V *vs* SCE. Instrumental parameters the same as fig.1A. (B) Same as in fig.1B.

evident (1544 and 1364 cm^{-1} on the electrode and 1625, 1579, 1547 and 1367 cm^{-1} in solution). The surface spectrum appears less complex overall because, as was noted in the 514.5 nm spectrum, the heme d_1 modes are more strongly enhanced. The most intense of these are at 1611 (1613), 1557 (1559), (1389), (1376), 1342 (1336) and 1140 (1146) cm^{-1}, where the numbers in parentheses are the frequencies observed in the solution spectrum. These modes may be compared with those in cytochrome oxidase (table 1) which contains heme *a* and heme a_3 chromophores [25]. These are reportedly spectroscopically similar in absorption properties to heme d_1 and may have common peripheral substituents. The exact substituents of heme d_1 have not yet been obtained through a chemical structure determination, but on the grounds of visible spectroscopy they are probably

similar to, but not necessarily the same, as for heme *d* [22]. There are two major differences in the structures of hemes *a* and *d*. Heme *d* is reduced in ring IV and, hence, is a chlorin. It is also believed to contain a methyl group on carbon 8, rather than a formyl group as in heme *a* [26]. The reduction of the pyrrole ring appears to shift some of its frequencies to lower values, which are closer to those observed in the chlorophylls [27]. These include the 1613 and 1342 cm^{-1} bands. A series of overlapping bands between 1370 and 1400 cm^{-1} in the solution spectrum are also analogous to those in the spectra of the chlorophylls. The 1342–1336 cm^{-1} band of heme d_1 can be assigned to its oxidation state sensitive band; it is at 1348–1350 cm^{-1} in the oxidized complex.

The 457.9 nm spectra of cytochrome cd_1 also contain bands which are in a region where carbonyl modes are found. These include the 1674 band in the surface spectrum and the 1719 and 1686 cm^{-1} bands in the solution spectrum. That at 1674 (1686) cm^{-1} is close to the formyl mode in cytochrome oxidase [25], and also in common with this protein, it is not observed in spectra produced by excitation within the β-band (514.5 nm). Thus, it is tempting to assign this mode to a similar carbonyl substituent on the periphery of the heme d_1 macrocycle. However, previous attempts to detect C=O substituents in heme *d* by chemical methods have yielded negative results [22]. The presence of a carbonyl may be a major difference between heme d_1 and heme *d* [22]. A definitive assignment of this band will require additional RR studies, chemical analyses, and NMR spectroscopy on the isolated chromophore. The weak band at 1719 cm^{-1} in the solution spectrum is close to the region where ester or carboxylic acid stretching modes are observed. It seems unlikely that the carboxylic acid side chains are resonance-enhanced since they are not conjugated to the macrocylic π-electron system. However, ester modes are observed in chlorophyll *b* aggregates [27]. Thus, this possibility cannot be excluded, and further RR studies on the isolated chromophore are needed.

As noted in section 1, cytochromes cd_1 have been isolated and purified from several different organisms and found to exhibit differences in their physicochemical properties. If these differences result from structural properties of the hemes, RR and SERR spectroscopy may be used to determine their origin. Thus, cytochrome cd_1 from *Paracoccus denitrificans* was examined by SERRS and compared to the results obtained on *Pseudomonas*, as shown in fig.3A and 3B, respectively. Only the SERRS of *Paracoccus* could be recorded at this time, due to the limited quantity of the protein which was available at the time of the experiments. Note that the spectra show many similarities, but the *Paracoccus* protein spectrum has a lower signal-to-noise ratio. The bulk solution concentration was only one-half that used in the *Pseudomonas* case, and, therefore, the surface coverage is probably lower. Important differences between the two spectra include two regions of broad and poorly resolved bands at 1500–1600 cm^{-1} and 1360–1400 cm^{-1}. Because of the poor resolution it is difficult to determine peak positions accurately, but it does

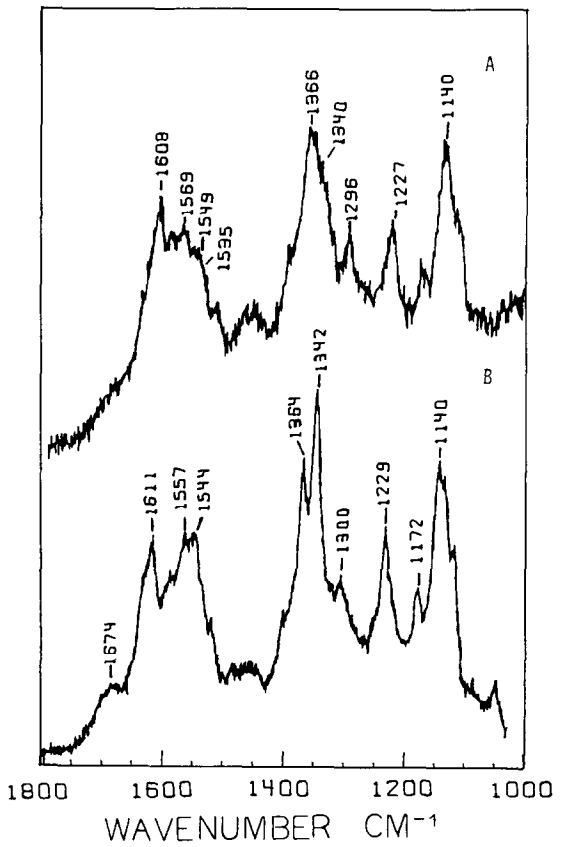

Fig.3. Comparison of the RR spectra of cytochrome cd_1 complex from *Paracoccus denitrificans* with that from *Pseudomonas aeruginosa*, 457.9 nm excitation. (A) SERR spectrum, 1.4×10^{-6} M cytochrome cd_1 from *Paracoccus* in 0.1 M Na_2SO_4, adsorbed on Ag at −0.6 V *vs* SCE. Instrumental parameters the same as in fig.1A. (B) SERR spectrum, 3.0×10^{-6} M cytochrome cd_1 from *Pseudomonas* in 0.1 M Na_2SO_4, adsorbed on Ag at −0.6 V *vs* SCE. Instrumental parameters the same as in fig.1B.

appear that some of the bands are shifted in the 1500–1600 cm^{-1} region with respect to *Pseudomonas:* 1569 (–9), 1549 (–8) and 1535 (–9) cm^{-1}. Also, new bands are present at 1590 and 1354 cm^{-1} in the *Paracoccus* spectrum. Intensity differences may be noted in the oxidation state sensitive bands, with the 1366 cm^{-1} band exhibiting more intensity than the 1340 cm^{-1} band in the *Paracoccus* spectrum which is the reverse of the situation found with *Pseudomonas.* Finally, the broad 1674 cm^{-1} band, which may represent a formyl mode, is noticeably absent in the *Paracoccus* spectrum. Hence, these results suggest there are indeed differences in the heme chromophores in these proteins, and these differences may even include peripheral substituents.

The results show how SERRS and RRS can be used to study heme *c* and heme d_1 in the cytochrome cd_1 complex. Tentative assignments have been made, based upon the differences observed for β and Soret band excitation. A comparison between the SERR spectra of the cd_1 protein from two different sources showed substantial differences, and also illustrated the utility of the SERR method for studying samples which are extremely scarce. The success of these results now affords opportunities for further structural studies. Our long range goals include Raman spectroscopy on different oxidation states, including partially reduced states, in which the electrode potential will be used to control and adjust heme states. The optical resolution of heme *c* and heme d_1 absorption bands will be exploited by using selective excitation in other regions of the spectrum to emphasize heme d_1 or *c* vibrations. Also, SERRS will be used together with RRS to study proteins reconstituted with porphyrins other than heme d_1 [28]. Resonance Raman spectroscopy will be used to examine the isolated heme d_1 chromophores from different organisms.

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