Resonance Raman Spectroscopy of the Cytochrome *c* Oxidase from *Paracoccus denitrificans*†

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ABSTRACT: Resonance Raman spectra are reported for the fully reduced unliganded and cyanide-bound mixed-valence forms of the cytochrome *c* oxidases from bovine heart and *Paracoccus denitrificans* in both detergent-solubilized forms and within their natural membrane environments. Comparison of the vibrational patterns observed for these enzymes indicates that overall the heme environments are similar for both. The only major differences seen between the spectra of these two enzymes are for vibrations associated with the low-spin bis(histidine)-coordinated heme cytochrome *a*. The data reported here serve to further illustrate the close structural and functional relationship between these evolutionarily distant enzymes. However, the data also demonstrate specific differences between the nature of the heme–protein interactions in the cytochrome *a* binding pocket which may be of mechanistic importance with regard to intramolecular electron transfer in these enzymes.

Oxidative phosphorylation is fueled by a cascade of respiratory electron-transfer events that terminate in the enzyme cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1). The mammalian form of this enzyme has been well studied. Cytochrome *c* oxidase is a multisubunit, metalloenzyme that utilizes two heme A cofactors (cytochrome *a* and cytochrome a_3) and two copper ions (Cu_A and Cu_B) to facilitate electron transfer from ferrocytochrome *c* to molecular oxygen (Sarraste, 1990). In the process of intramolecular electron transfer among these metal cofactors, the enzyme also functions as a transmembrane proton pump. It is now known that the mammalian enzyme is a member of a superfamily of terminal oxidases that all function as electron-transfer-driven proton pumps. What the common structural determinants of these catalytic activities are within this superfamily remains a question of current research interest.

To help identify common features of these enzymes that are required for catalysis, one would wish to compare the proteins from evolutionarily distant species in terms of their amino acid sequences, metal ion content, catalytic activities, and other physicochemical properties. The most well-studied enzyme in this superfamily is that isolated from bovine cardiac tissue; this enzyme thus serves as a useful benchmark for comparative studies with enzymes from different sources. Another well-studied enzyme of this superfamily is the cytochrome *c* oxidase isolated from the bacterium *Paracoccus denitrificans*. Despite the great evolutionary distance between the bovine and *Paracoccus denitrificans* sources, these two enzymes share many common structural and mechanistic features [see Ludwig (1987) for a review]. These similarities notwithstanding, some differences in the physicochemical properties of these two enzymes have been reported. For example, the absorption spectra of the oxidized and reduced forms of the two enzymes are qualitatively similar, but differ in quantitative detail. Comparing the spectra of the resting states of these enzymes, one finds that the Soret band maximum is red-shifted by as much as 4 nm for the *Paracoccus denitrificans* enzyme relative to the bovine enzyme. For the reduced states, one observes red-shifts of both the Soret and the α band when comparing the spectra of the *Paracoccus denitrificans* enzyme to that of the bovine enzyme (Ludwig & Schatz, 1980). The cause of these spectral differences has not been fully elucidated.

In this paper, we compare the resonance Raman spectra of reduced and partially reduced forms of the enzymes from *Paracoccus denitrificans* and bovine sources, in an attempt to further define structural differences between the heme group environments of the two enzymes. We also compare the spectra for the enzymes isolated in detergent solutions and within their natural membranes (i.e., submitochondrial particles for the mammalian enzyme and spheroplasts for the bacterial enzyme) to evaluate any structural changes that might attend release of the enzymes from their natural venues.

MATERIALS AND METHODS

Bovine cardiac mitochondria were isolated, and the cytochrome *c* oxidase from them was purified by the method of Hartzell and Beinert (1974). Submitochondrial particles were prepared as previously described (Ragan et al., 1987). Spheroplasts were prepared from whole cells of *Paracoccus denitrificans* by lysozyme/DNase treatment, as described by Ludwig and Schatz (1980). The enzyme from *Paracoccus denitrificans* was purified by a combination of ion-exchange chromatography on Q Sepharose FF and lysine affinity chromatography (Felsh et al., 1992). A detailed description of the purification method for this and other prokaryotic oxidases will be presented separately (Horvath et al., unpublished results). This purification method yields a highly active enzyme that retains all three of its subunits, in contrast to the earlier purification methods that yielded a two-subunit enzyme (Ludwig & Schatz, 1980).

The purified enzymes were flash-frozen in a dry ice/ethanol bath and stored at −80 °C until use. The fully reduced and cyanide-inhibited mixed-valence forms of these enzymes were prepared in low ionic strength buffer [50 mM 4-(2-hydrox-

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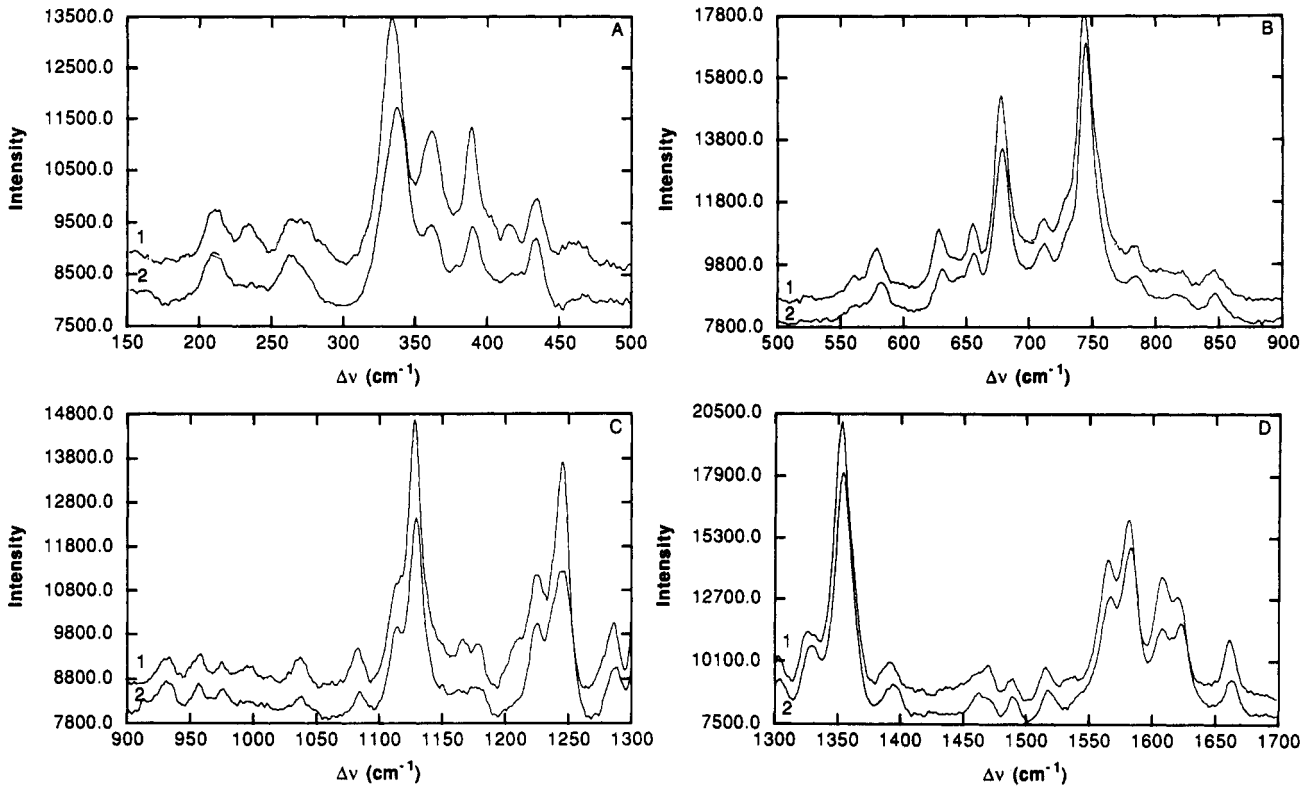

FIGURE 1: Resonance Raman spectra of detergent-solubilized cytochrome *c* oxidase from *Paracoccus denitrificans* (top spectrum 1) and bovine heart (bottom spectrum 2) in their fully reduced unliganded forms.

yethyl)-1-piperazineethanesulfonic acid (HEPES)[1]/0.5% dodecyl β-maltoside, pH 7.4] using either sodium dithionite or sodium ascorbate and TMPD as reductants (Ishibe et al., 1991). All other reagents were the highest quality commercially available.

Resonance Raman spectra were obtained using 441.6-nm excitation from a helium/cadmium laser (Liconix) as previously described (Ishibe et al., 1991). The relative intensities of vibrational bands in the spectra were referenced to the ν_4 heme vibration at ca. 1355 cm^{-1}; in separate experiments, the relative intensity of this band was in turn referenced to a nearby laser spike at ca. 1420 cm^{-1}. The monochromator was calibrated daily for frequency accuracy using the 459-cm^{-1} Raman band of carbon tetrachloride.

RESULTS

Resonance Raman spectra of the enzymes from both species were obtained with laser excitation at 441.6 nm, in direct resonance with the Soret band of the ferrous heme A cofactors of these proteins. Figure 1 compares the spectra of the fully reduced, unliganded forms of the enzymes from the two organisms. For clarity, we present these spectra in four panels representing four distinct frequency ranges of the Raman spectra. The *y* axes for these four panels are arbitrarily scaled to best illustrate the vibration bands in that frequency region. Qualitatively, the spectra are quite similar; however, some clear differences can be discerned.

In the low-frequency region (150–500 cm^{-1}), the bacterial enzyme displays a moderately intense band at 234 cm^{-1} that is absent in the spectrum of the mammalian enzyme. The strong band at 338 cm^{-1} in the spectrum of the bovine enzyme is shifted down by 4 cm^{-1} in the bacterial enzyme, and the weak shoulder at 417 cm^{-1} in the spectrum of the bovine enzyme shows augmented intensity in the spectrum of the *Paracoccus denitrificans* enzyme. There is also a noticeable broadening on the high-frequency side of the ca. 260-cm^{-1} band in the case of the bacterial enzyme.

The mid-frequency regions (500–900 and 900–1300 cm^{-1}) show only minor differences between the spectra from the two enzymes. In the high-frequency region (1300–1700 cm^{-1}), the most dramatic change observed between the spectra is a large augmentation of the intensity of the 1608-cm^{-1} band for the bacterial enzyme relative to the mammalian enzyme.

Figure 2 compares the spectra of the fully reduced enzymes within their natural biological membranes. The mammalian enzyme spectra were obtained using submitochondrial particles, while those of the bacterial enzyme were for samples of spheroplasts. The spectra of the enzymes within membranes display more noise than the corresponding spectra of the detergent-solubilized enzymes. Otherwise, however, the spectra are very similar, and show many of the same species differences as do the spectra of the detergent-solubilized proteins. The only notable difference between the spectra in Figures 1 and 2 is a diminution of intensity for the cytochrome a_3 formyl band (ca. 1663 cm^{-1}) of the bovine enzyme within the membrane. Other than this, the data indicate litte disruptions of the heme environment upon detergent extraction of either enzyme from their biological membranes.

Figure 3 compares the resonance Raman spectra of the cyanide-inhibited mixed-valence forms of the enzymes from bovine and *Paracoccus denitrificans*. In this form of the enzyme, cytochrome a_3 remains ferric and bound by cyanide while cytochrome *a* is reduced to its ferrous form. Because of the excitation wavelength used in this study, only vibrational modes associated with ferrous cytochrome *a* are strongly enhanced in these spectra. The differences seen between the spectra of the two enzymes here are complementary to those seen for the fully reduced enzymes in Figure 1. One sees, for

[1] Abbreviations: EPR, electron paramagnetic resonance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

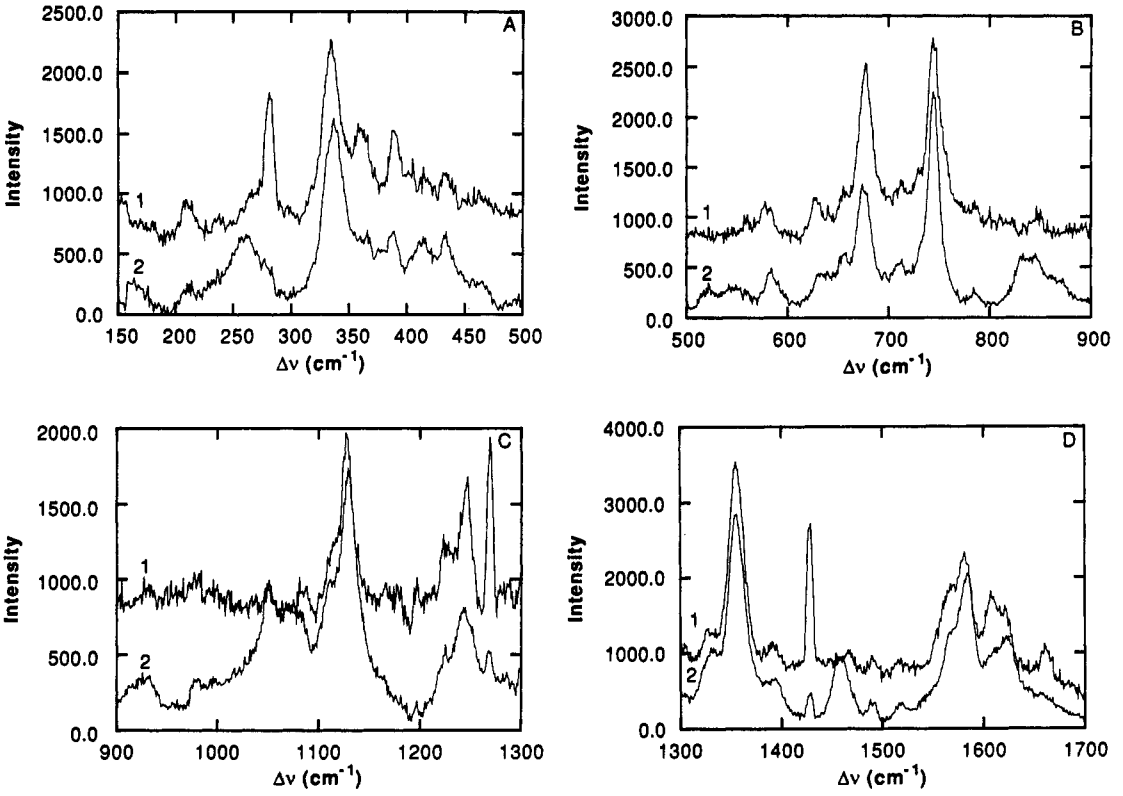

FIGURE 2: Resonance Raman spectra of membrane-bound cytochrome *c* oxidase from *Paracoccus denitrificans* (top spectrum 1) and bovine heart (bottom spectrum 2) in their fully reduced unliganded forms. The spectrum of the bacterial enzyme was obtained from spheroplasts, while that of the mammalian enzyme was obtained from submitochondrial particles.

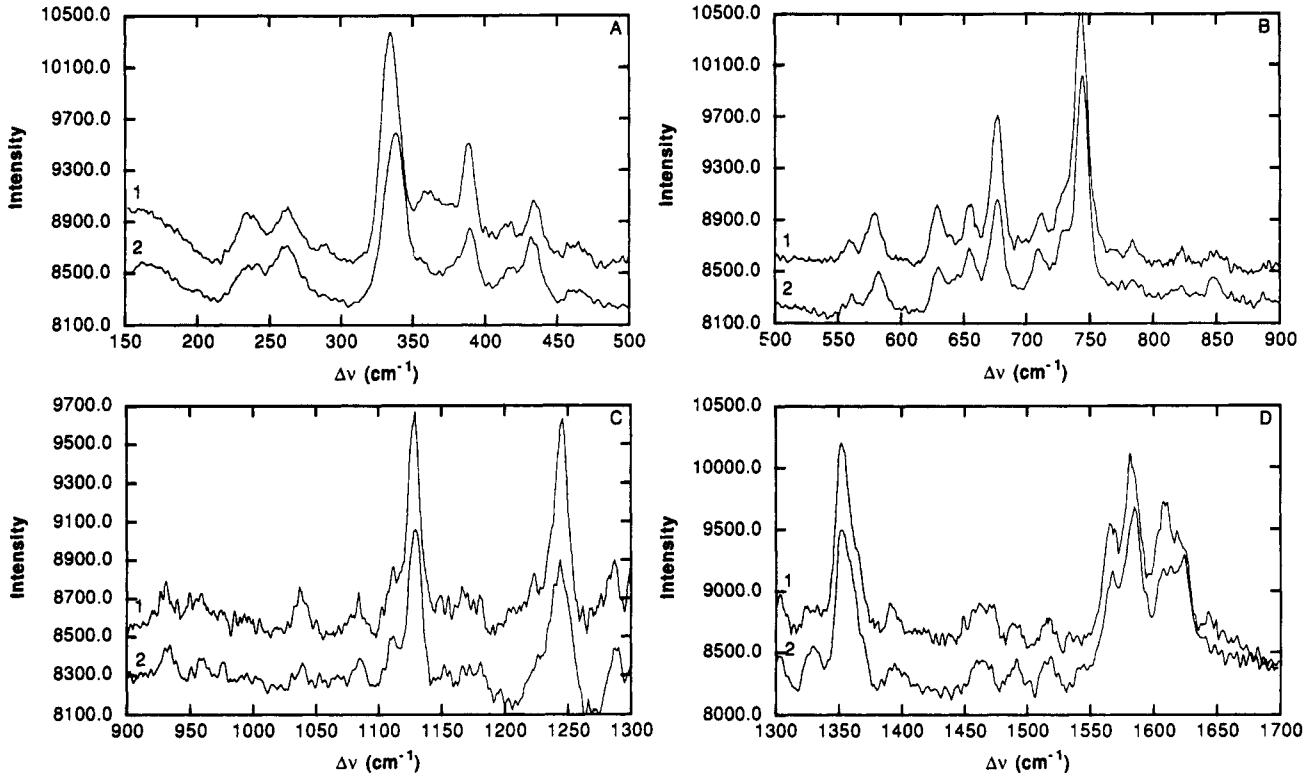

FIGURE 3: Resonance Raman spectra of detergent-solubilized cytochrome *c* oxidase from *Paracoccus denitrificans* (top spectrum 1) and bovine heart (bottom spectrum 2) in their cyanide-bound, mixed-valence forms.

example, the same frequency shifting of the ca. 336-cm^{-1} band and the augmented intensity of the 1606-cm^{-1} bands as seen in Figure 1. The spectra of both enzymes show a band at about 233 cm^{-1} in their cyanide-inhibited mixed-valence forms. The intensity of this band, relative to the nearby 260-cm^{-1} band, is slightly greater for the bacterial enzyme. It is not clear, however, from these spectra whether or not the additional band at 234 cm^{-1}, seen in the spectrum of the fully reduced bacterial enzyme, is responsible for this augmented intensity. As with the fully reduced enzymes, the same set of species

Table I: Assignments for the Vibrational Bands Observed in the Resonance Raman Spectra of the Cytochrome *c* Oxidase from Bovine Heart and *Paracoccus denitrificans*

bovine reduced	*Paracoccus* reduced	bovine CN mixed valence	*Paracoccus* CN mixed valence	assignment[a]
210	211			ν_{Fe-N}
	234	233	234	?
262	263	260	263	pyr tilt
		285	289	γ_{CmCa}
338	334	336	335	ν_8
361	361		362	$2\nu_{35}$
390	389	388	389	γ_{CbS}
417	415	418	418	$\delta_{CbCaC(1)}$
434	435	432	434	pyr fold
466	463	464	465	(470-pyr fold)
501	502	500	502	(506-pyr fold)
519	521	514	515	(524-pyr fold)
563	561	559	559	ν_{49}
582	579	580	579	ν_{48}
631	628	627	629	$\delta_{C=O}$
656	655	652	656	?
679	678	675	677	ν_7
712	712	708	713	?
745	744	742	743	ν_{16}
785	782	782	784	ν_{32}
814	821	821	823	?
847	847	845	846	γ_{CmH}
931	933	928	926	ν_{46}
957	958	959	959	?
976	975	974	974	$\nu_{20} + \nu_{35}$
1038	1037	1037	1037	?
1084	1084	1083	1084	?
1114	1113	1110	1112	?
1129	1128	1127	1129	$\nu_6 + \nu_8$
1163	1167	1165	1166	ν_{30}
1177	1177	1179	1180	?
1226	1224	1224	1223	ν_{CbCHO}
1247	1246	1242	1246	$\nu_5 + \nu_9$
1287	1286	1288	1287	ν_{42}
1304	1303	1302	1303	ν_{21}
1330	1326	1327	1325	$\delta_{S=CH2(2)}$
1354	1353	1350	1352	ν_4
1395	1391	1392	1391	$\nu_{29/20}$
1462	1463	1457	1461	ν_{28}
1469	1469			?
1484	1490	1489	1488	ν_3
1518	1515	1516	1517	ν_{11}
	1539	1543	1545	ν_{38Y}
1567	1565	1566	1569	ν_{38X}
1583	1581	1583	1581	ν_2
1608	1607	1606	1608	$\nu_{C=O(a)}$
1623	1620	1622	1618	$\nu_{C=C}$
1663	1661			$\nu_{C=O(a_3)}$

[a] Assignments taken from Choi et al. (1983).

differences were observed for the enzymes imbedded within their natural biological membranes (data not shown).

The frequencies of the Raman bands observed in these spectra and their likely assignments are summarized in Table I. The assignments listed here are based on previous studies of the mammalian enzyme (Choi et al., 1983).

DISCUSSION

Low-Frequency Vibrations. In the low-frequency region, one observes several differences between the spectra of the prokaryotic and mammalian enzyme. The most striking difference one observes is the presence of a band at ca. 234 cm^{-1} for the bacterial enzyme that is absent, or very weak, in the spectrum of the bovine protein. A weak band at ca. 240 cm^{-1} has been previously noted in the spectrum of the reduced bovine enzyme (Choi et al., 1983; Ching et al., 1985), but its intensity is minimal compared to that of the bacterial enzyme (Figure 1). A band at similar frequency is observed for both enzymes in their CN-inhibited mixed-valence forms, but it is not clear that this band has the same molecular origin as that seen in the spectrum of the prokaryotic reduced unliganded enzyme. This new band has been observed in the spectra of other prokaryotic cytochrome *c* oxidases as well. It is observed in the resonance Raman spectrum of the aa_3–ubiquinol oxidase from *Bacillus subtilis* (Lauraeus et al, 1992; Copeland et al., unpublished data), and Hosler et al. (1992) have observed a vibrational band of similar frequency in the resonance Raman spectrum of the reduced enzyme from *Rhodobacter sphaeroides*. The origin of this band is unclear at present. Its frequency suggests that this vibration could be associated with one of the axially coordinated histidine groups of the heme cofactors. However, Ogura et al. (1983) have studied the RR spectra of the cytochrome *c* oxidase from *Thermus thermophilus HB8* grown on ^{56}Fe and ^{54}Fe. The isotopic substitution at the iron should result in a frequency lowering for the iron–nitrogen stretch associated with the axial histidine ligand. Indeed, the ca. 212-cm^{-1} band assigned to this stretch experiences a 1.8-cm^{-1} upshift for the lighter isotope, in excellent agreement with the theoretically expected isotopic shift of 2.1 cm^{-1}. In contrast, however, the ca. 238-cm^{-1} band does not show an iron isotope sensitivity. These data do not preclude assignment of this band to an axial histidine vibration, but do exclude the possibility that this band represents the iron–histidine stretch of a conformational variant of cytochrome a_3. Recently Einarsdottir et al. (1989) reported the Raman spectra of oxidized and reduced forms of cytochrome ba_3 from *Thermus thermophilus*. With excitation at 442.5 nm, these workers obtained a Raman spectrum which was mainly composed of vibrational bands from the reduced cytochrome a_3 center. No vibration at ca. 234 cm^{-1} was observed in this spectrum. These data suggest either that the cytochrome a_3 center of this enzyme is very different from that of other prokaryotes or that the 238-cm^{-1} band observed here and elsewhere for other prokaryotic oxidases is associated with the reduced cytochrome *a* cofactor.

Other differences between the low-frequency spectra of the *Paracoccus denitrificans* and bovine enzymes include a ca. 4-cm^{-1} downshift of the 338-cm^{-1} band, and a significant augmentation of intensity for the 361-cm^{-1} band for the prokaryotic enzyme. These bands are assigned respectively to the the porphyrin deformation mode ν_8 and an overtone of ν_{35} (Choi et al., 1983). Both of these vibrations have been previously shown to be sensitive to changes in the vicinity of the cytochrome *a* formyl substituent (Copeland & Spiro, 1986). These data suggest that, with respect to the heme cofactors, the enzymes from both species are quite similar, the only differences being associated with the peripheral groups on the cytochrome *a* heme. This conclusion is consistent with the optical spectral differences between the two enzymes. Thus, Ludwig and Schatz (1980) have reported that while the optical spectra of the two enzymes are qualitatively similar, the *Paracoccus denitrificans* enzyme displays slightly red-shifted Soret and α-band maxima relative to the bovine enzyme. Comparison of the second-derivative absorption spectra from the two species suggests that these differences can be associated with cytochrome *a* exclusively (Horvath et al., unpublished data). As will be discussed below, the high-frequency region of the RR spectra also suggest minimal differences between the two enzymes, except for differences associated with the cytochrome *a* formyl group.

Middle- and High-Frequency Regions. The spectral region between 500 and 1300 cm^{-1} is rich in vibrational modes of the

porphyrin macrocycle, and assignments for most of these vibrations have been made for heme A model compounds and cytochrome *c* oxidase. The spectra of the two enzymes in this frequency region are remarkably similar with little, if any, significant differences observed here. In the high-frequency region, between 1300 and 1700 cm^{-1}, one observes a number of vibrational modes that are diagnostic of the valence and spin state of the central heme iron atom, and that provide information on the environment of the heme peripheral groups. The majority of spectral features in this frequency region are similar for the two enzymes studied here, suggesting an overall similarity between the heme cofactors in both proteins. The only notable exception to this is the intensity difference observed for the cytochrome *a* formyl stretch at ca. 1608 cm^{-1}. The intensity of this band is significantly (~32%) greater in the spectrum of *Paracoccus denitrificans* cytochrome *c* oxidase as compared to that for the bovine enzyme. An even greater intensification of this band is seen in the RR spectrum of the reduced enzyme from *Rhodobacter sphaeroides* (Hosler et al., 1992). In all of these enzymes, the frequency of this mode is significantly lower than that for the corresponding vibration of cytochrome a_3 or for heme A model compounds in aprotic solvents. The low frequency observed for this mode has been attributed to strong hydrogen bonding between the formyl oxygen and a hydrogen donor from the surrounding polypeptide (Babcock & Callahan, 1983) or interactions with a immobilized water molecule (Sassaroli et al., 1989). The frequency of this mode varies little from species to species, but its intensity does vary considerably. These intensity changes most likely reflect differences in the angle between the carbonyl group of the formyl and the plane defined by the porphyrin macrocycle. The closer to coplanar these two π systems become, the greater the degree of π cloud delocalization that can occur between them. As the porphyrin π system becomes more mixed with that of the formyl carbonyl, one would expect greater resonance enhancement of the carbonyl vibration with excitation under a π–π^* electronic transition of the heme. A similar argument has been put forth to explain the anomalously weak intensity of the 1608-cm^{-1} band in the Raman spectra of certain plant cytochrome *c* oxidases and the accompanying blue-shifts of the heme absorption bands for these enzymes, relative to the bovine enzyme (dePaula et al., 1990). If this explanation for the increased relative intensity of the cytochrome *a* formyl band is correct, it implies that the carbonyl group attains greater planarity with the porphyrin macrocycle in the *Paracoccus denitrificans* and *Rhodobacter sphaeroides* enzyme relative to the bovine protein.

An alternative explanation for the apparent increase in intensity for this cytochrome *a*-specific vibration is that the extinction coefficient for cytochrome *a* relative to cytochrome a_3 is greater at the excitation wavelength used here (441.6 nm) for the bacterial enzyme compared to that for the bovine enzyme. If this were the case, one would expect greater resonance enhancement for those vibrations associated with cytochrome *a* relative to those associated with cytochrome a_3. As discussed earlier, there is good reason to expect relative extinction coefficient differences for the heme groups of the enzymes from these two species. This explanation seems less likely to be correct, however, in view of the fact that one does not observe a systematic increase in relative intensity for all cytochrome *a*-associated vibrational modes; rather, the effect seems to be limited to the formyl band exclusively.

SUMMARY

The Raman spectra of the reduced and partially reduced cytochrome *c* oxidases from bovine heart and *Paracoccus denitrificans* are very similar to one another when excited in resonance with the reduced heme Soret transition. The only major differences between the spectra of the enzymes are all associated with vibrations originating from the cytochrome *a* cofactor. These data further exemplify the similarities between these two enzymes, but also point to specific differences between the protein–cofactor interactions at the cytochrome *a* site for the two species. We note that previous optical (Ludwig & Schatz, 1980) and EPR (Erecinska et al., 1979) spectroscopic comparisons between these two enzymes have also suggested differences in the vicinity of cytochrome *a*.

The low-spin heme cytochrome *a* is the obligatory electron donor to the oxygen binding site of cytochrome *c* oxidase and other terminal oxidases. As such, it plays a crucial role in the electron-transfer activity of these enzymes, and may play a role in controlling the rate of electron-transfer-driven proton translocation. The differences observed between the cytochrome *a* environments of the two enzymes studied here, while minor, may reflect subtle differences in the interactions of this cofactor with the surrounding protein that may be of mechanistic importance.

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