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Resonance Raman Spectra of Cytochromes *c* and *b* in *Paracoccus denitrificans* Membranes: Evidence for Heme–Heme Interactions†

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ABSTRACT: Resonance Raman (RR) scattering from cytochromes *b* and *c* in the bacterium *Paracoccus denitrificans* was recorded by exciting with the 568.2-, 530.9-, and 520.8-nm lines of a Kr^+ laser. The main features of the spectra were similar to those of the analogous cytochrome b–c_1 complex derived from pigeon breast mitochondria. Differences in the 1300-cm^{-1} region were interpreted in terms of marker bands for heme type and spectral coupling of the hemes on the membranes. It is difficult to explain the results without invoking sharing of electronic and vibrational wave functions among the hemes. This conclusion documents the potential to study the physics of electron transport in functioning membrane by monitoring the RR spectra.

Since the first reports of the resonance-enhanced Raman spectra of porphyrin in hemoglobin and cytochrome *c* (Brunner et al., 1972; Strekas & Spiro, 1972a,b), resonance Raman (RR) spectroscopy has been developed as a structural and dynamic probe of heme proteins. Rigorous descriptions of the scattering phenomenon in cytochrome *c* and other metalloporphyrins illustrated the elegant spectroscopic effects that this class of materials exhibits (Brunner et al., 1972; Collins et al., 1973; Friedman & Hochstrasser, 1973, 1976; Nafie et al., 1973; Verma et al., 1974; Woodruff et al., 1975; Asher & Sauer, 1976; Shelnutt et al., 1976, 1977; Kitagawa et al., 1976; Spiro & Loehr, 1975). Kinetic experiments on the nanosecond and picosecond time scale have documented differences in the dynamics of rebinding oxygen to hemoglobin and myoglobin following photolysis (Friedman & Lyons, 1980; Terner et al., 1980). More recently, a RR study of flavo-

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cytochrome *c*-552 has documented intramolecular electron transfer from flavin to heme induced by photoreduction (Kitagawa et al., 1980).

In this laboratory we have developed the use of the RR signals from mitochondrial membranes as a probe of interactions between hemes in a functioning system. In an earlier report (Adar & Erecińska, 1974), we have proven the ability of RR spectroscopy to distinguish contributions to the spectra from the individual heme proteins in succinate-cytochrome *c* reductase, using marker bands identified in the spectra of isolated cytochromes. RR spectra recorded from the cytochrome b–c_1 complex as a function of redox potential (Adar & Erecinska, 1977) showed that marker bands from *c*- and *b*-type hemes did not uniformly follow the redox titrations of the optical spectra, indicating that there is substantial coupling between cytochrome c_1 and cytochromes *b*. A subsequent report (Adar & Erecińska, 1978) compared the spectra of the cytochrome b–c_1 complex in whole mitochondria to those from a purified preparation; differences were noted, which were attributed to the increasing organization of the membrane system.

In this paper, we report for the first time the RR spectra of the membrane fragments from the bacterium *Paracoccus denitrificans*, excited in the α- and β-band regions. This microorganism has an electron transport chain similar to that of vertebrate mitochondria; it contains cytochromes $a + a_3$, two *c*-type cytochromes, and at least one *b*-type cytochrome, which are membrane bound (Scholes & Smith, 1968). The RR spectra generated from this system are to be interpreted with the perspectives gained from our previous studies of pigeon breast mitochondria (Adar & Erecińska, 1974, 1977, 1978) but will focus on those details of the spectra that reflect subtle differences between the organization of the two membranes. Such studies can aid in characterizing structural differences of the various membrane systems.

Materials and Methods

Cell Cultivation and Isolation. P. denitrificans was grown aerobically in a New Brunswick psycrotherm rotatory shaker at 30 °C essentially as described previously (Erecińska et al., 1978), except that the manganese salts were omitted from the growth medium. Cultures were harvested in the logarithmic phase of growth, and the sedimented cells were washed twice in 10 mM phosphate buffer, pH 7.4.

Preparation of Membrane Fragments. The washed cells were suspended in 0.5 M sucrose–0.01 M phosphate buffer, pH 7.4, and spheroplasts were prepared following lysis of the cells with lysozyme as described by Scholes & Smith (1968). Membrane vesicles were then purified according to the procedure given by the same authors (Scholes & Smith, 1968).

Samples reduced with sodium dithionite were contained in 1 mm diameter melting point glass capillary tubes. At these high protein concentrations oxygen cannot diffuse into the sample volume at the focused laser beam. The samples were protected from laser heating with a flow of cool nitrogen gas.

Raman spectra of the samples were recorded on a Jobin Yvon Ramanor HG2S monochromator equipped with a cooled GaAs photomultiplier (RCA C31034) and a PAR 1105/1120 counter. The optics coupling laser to sample and then to monochromator were home built. The excitation wavelengths used were 520.8, 530.9, and 568.2 nm, which are available in the Spectra Physics Kr^+ laser (171-01). Peak positions are accurate to ± 2 cm^{-1} and reproducible to better than 0.5 cm^{-1}. Monochromator slit widths were 600 μm, which gives an instrumental line width of ~ 5 cm^{-1} in the yellow and 6 cm^{-1} in the green regions. Spectra were recorded with a polarization analyzer set to measure light with its polarization either parallel or perpendicular to the laser polarization. The instrument calibration was checked daily for each excitation wavelength.

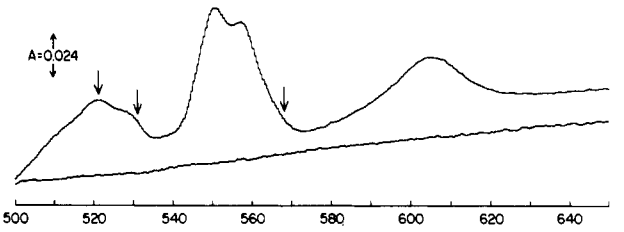

FIGURE 1: Absorption difference spectra of membrane fragments from *P. denitrificans*. *P. denitrificans* membrane fragments were suspended in 50 mM phosphate buffer, pH 7.0, and supplemented with 0.05 mM rotenone and 1 mM ferricyanide. The absorption of the ferricyanide-oxidized sample was recorded and stored in the computer memory. The oxidized–oxidized difference spectrum was then displayed as a straight base line. The reduced–oxidized spectrum was measured after the reduction of the sample with solid dithionite. Cytochrome *a* concentration was about 0.9 μM. The reference wavelength was 575 nm. Arrows indicate positions of the laser excitation relative to the absorption bands.

Results

Optical absorption difference between the reduced and oxidized preparation of the membrane vesicles from *P. denitrificans* in the visible region of the spectrum is shown in Figure 1. The spectrum exhibits features characteristic of three types of cytochromes: cytochromes *c* (absorption peak around 552 nm), cytochromes *b* (absorption around 560 nm), and cytochromes *a* (absorption around 605 nm). If one compares the region between 500 and 580 nm in this spectrum with that of the cytochrome b–c_1 complex isolated from pigeon breast mitochondria (Adar & Erecińska, 1974), one notices the same overall features except that in the *Paracoccus* preparation the absorption characteristic of cytochromes *c* is higher than that of cytochromes *b* whereas the reverse is true for pigeon breast enzyme. This arises from the fact that in the *P. denitrificans* respiratory chain cytochromes *c* appear to be more tightly bound to the membrane than are their vertebrate counterparts (Erecińska et al., 1979) and thus contribute more to the absorption band.

RR spectra excited at 568.2, 530.9, and 520.8 nm of membrane fragments from *P. denitrificans* are shown in Figure 2. The general intensity pattern of the bands in this preparation is strikingly similar to that of the pigeon breast cytochrome b–c_1 complex (Adar & Erecińska, 1974, 1977, 1978); however, closer inspection shows some differences. Because the region between 1290 and 1320 cm^{-1} has been found to be sensitive to heme type and interactions, we will concentrate on the spectral details in this region.

Frequencies observed in the two membranous preparations and in the purified cytochromes *c* and b_5 are presented in Table I. In the *P. denitrificans* membranes the principle band frequencies observed with all three excitations appear at about 1306 and 1314 cm^{-1}, i.e., at values very close to those in purified cytochromes b_5 and *c*. When the preparations are excited at 568.2 nm, the most intense band is that of a *b*-type heme at 1307 cm^{-1}. In the spectrum excited at 530.9 nm bands at 1305 and 1314 cm^{-1} have comparable intensity, whereas with 520.8-nm excitation the *c*-type band at 1314 cm^{-1} has more intensity than the *b*-type shoulder at 1306 cm^{-1}. These results are consistent with the predictions of intensities from the excitation-wavelength dependence of the RR spectra developed in the mitochondrial preparations (Friedman et al., 1977). In our earlier work on pigeon breast mitochondria

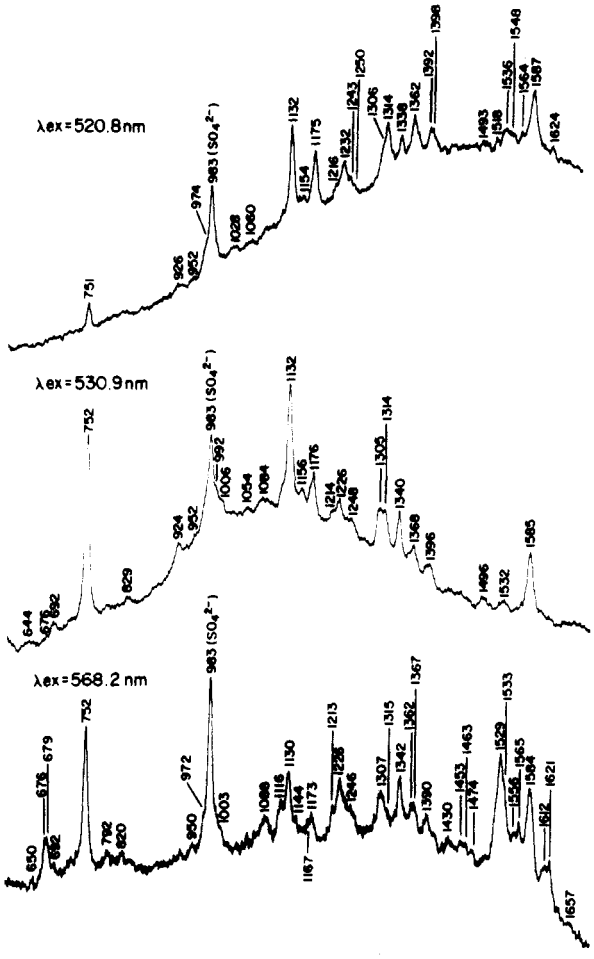

FIGURE 2: Resonance Raman spectra of membrane fragments from *P. denitrificans* excited at 568.2, 530.9, and 520.8 nm. Samples were reduced with minimal amounts of dithionite and cooled with nitrogen gas. Slits were 600 μm. Spectra were scanned at 50 cm^{-1} min with a 1-s time constant. Heme *a* concentration was about 20 μM.

Table I: Frequencies of Heme Marker Bands in Membrane Vesicles from *P. denitrificans* and Pigeon Breast Mitochondrial Cytochrome b–c_1 Complex

excitation wavelength (nm)	*P. denitrificans* (cm^{-1})	pigeon breast cytochrome b–c_1 complex[a] (cm^{-1})	cytochrome c (cm^{-1})	cytochrome b_5 (cm^{-1})
568.2	(1298 sh)[b]/ 1307/ (1315 sh)	1299 (s)/ (1315 br, sh)	–[c]	1306/1340
530.9	1305/1314	1298 (s)/1314	1315	1306/1340
520.8	(1306 sh)/ 1314	1298 (s)/ 1316 (s, br)	1315	1306/1340

[a] Taken from Adar & Erecińska (1974, 1978). [b] sh = shoulder; s = strong; br = broad. [c] Not observable with these concentrations.

(Adar & Erecińska, 1974), it was shown that the 568.2-nm line excites the long-wavelength cytochrome component(s), i.e., cytochromes *b*. The shorter wavelength lines, 530.9 and 520.8 nm, excite all hemes by coupling to their respective β bands, but the relative intensities of the bands are determined by the energy of the laser photon relative to the respective vibrations of the hemes' excited states. Thus, cytochrome *c* is most effectively enhanced in the 520.8-nm spectrum (Adar & Erecińska, 1978).

Comparison of the results in Table I also shows that the positions of the RR marker bands obtained at pH 7 in the *P. denitrificans* preparation differ from those in the mitochondrial

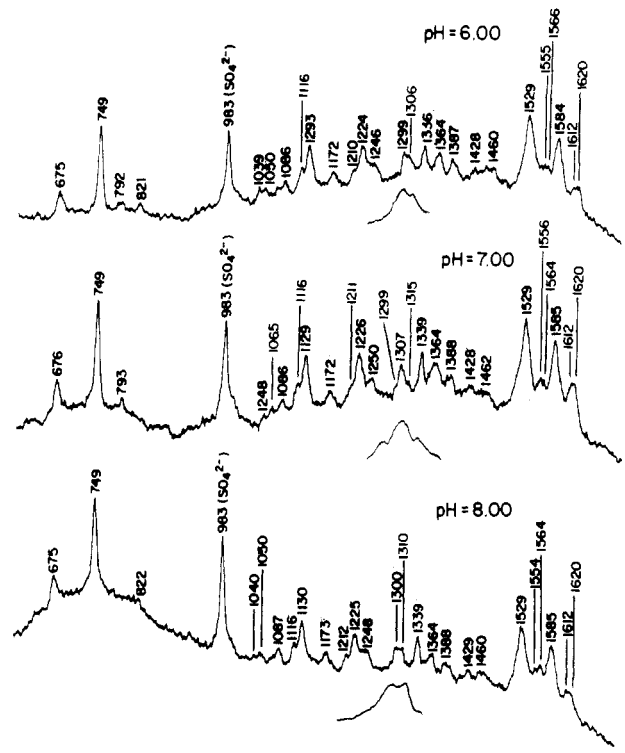

FIGURE 3: Resonance Raman spectra of membrane fragments from *P. denitrificans* at pH 6.0, 7.0, and 8.0 excited at 568.2 nm. Membrane fragments were suspended in 50 mM phosphate buffer of the appropriate pH value and recorded as described in the legend to Figure 2.

Table II: pH Dependence of Heme Marker Bands Excited at 568.2 nm

pH	cm^{-1}
6.0	1299/1306
7.0	(1299 sh)/1307/(1315 sh)
8.0	1300/1310

cytochrome b–c_1 complex. In the latter, the *b*-type heme band at 1298 cm^{-1} is significantly shifted from the position observed in the purified heme where it appears at 1305–1306 cm^{-1}.

Figure 3 shows the pH dependence of the RR spectra of *P. denitrificans* excited at 568.2 nm. Comparison of the positions of the heme marker bands seen at pH 6 and 8 with those observed at pH 7 (Table II) suggests significant reorganization of the hemes on the membrane. At pH 6, there are two *b*-type heme marker bands with almost equal intensities, at 1299 and 1306 cm^{-1}. The former of those is assignable to a membranous *b*-type cytochrome (Adar & Erecińska, 1974, 1977, 1978). At pH 8, there are also two bands, at 1300 and 1310 cm^{-1}. The second band at 1310 cm^{-1} appears at a frequency that is usually not observed in cytochromes *c* or *b*. The only other case where a feature was observed at about 1310 cm^{-1} was in a spectrum of whole mitochondria also excited at 568.2 nm, where a shoulder appeared at 1310 cm^{-1}. It should be noted, however, that in the spectrum of *P. denitrificans* at pH 8 this feature at 1310 cm^{-1} has intensity comparable to that of the 1300-cm^{-1} band.

Discussion

At first inspection, the RR spectra of *P. denitrificans* membranes excited in the bands of cytochromes *b* and *c* are very similar to the spectra of the mitochondrial cytochrome b–c_1 complex derived from pigeon breast mitochondria. However, there are significant differences in the positions and intensities of the marker bands in the vicinity of 1300 cm^{-1}.

We shall attempt to interpret these results on the basis of the following observations, which were documented in detail in previous publications (Adar & Erecińska, 1974, 1977, 1978): (1) There are marker bands for *c*- and *b*-type cytochromes (at 1315 and 1307/1340 cm^{-1}, respectively) that were established from examination of purified cytochromes *c* and b_5. (2) Changes in the position of the marker bands in membranous preparations may be indicative of interactions between the hemes. This suggestion was based on the appearance of the band of cytochrome *b* at 1299 cm^{-1} in the cytochrome b–c_1 complex, i.e., at a frequency that was much lower than that observed in any purified heme protein containing a *b*-type heme where the analogous band always appeared at 1307 cm^{-1}. (3) Intensity patterns of samples at a fixed reduction potential differ from predictions based on amounts of particular hemes present in the reduced state [which has a RR quantum yield ~10 times higher than that of the oxidized state (Adar et al., 1976)]. These anomalies have been explained by invoking electronic coupling between hemes on the membrane. Specifically, at a potential where only cytochrome c_1 was reduced, marker bands for cytochrome *b* appeared in the spectra (Adar & Erecińska, 1977). It was inferred that vibrational bands of reduced cytochromes *b* were enhanced in these spectra via resonance enhancement with excited states composed of excitons of cytochromes *c* and *b*.

Aided by these observations and their interpretations, we suggest that the characteristic features of RR spectra of *P. denitrificans* described in detail above show evidence for coupling between the hemes. Comparison of the RR spectra of *P. denitrificans* to those of pigeon breast mitochondria suggests that the details of the coupling in the bacterial membrane differ from those of the vertebrate mitochondria. As described above, the frequency of cytochrome *b* at pH 7 and the relative intensity patterns at the three excitation wavelengths are different in the two systems. While coupling between the optical bands explains the RR intensity patterns, coupling of the vibrations accounts for the shifts of the RR bands that have been noted. The dependence of frequencies on pH implies a reorganization of the membrane or hemes within the membrane at different pH values. At pH 6 and 8 the band at 1300 cm^{-1} that has been a marker for membranous cytochrome *b* appears quite clearly, even though it is only a shoulder at pH 7. At pH 8 a band appears at 1310 cm^{-1}, which is too high for cytochrome *b* and too low for cytochrome *c*. Its intensity is comparable to that of cytochrome *b* at 1300 cm^{-1}. It is difficult not to invoke substantial spectral coupling between *c*- and *b*-type hemes to account for this band. Not only will such coupling account for its frequency but also for its intensity, which cannot be derived from direct excitation of cytochrome *c* with 568.2-nm excitation. It is known that the midpoint potentials of both cytochromes *b* shift with pH (Wilson et al., 1972). The conclusion that can be drawn from the data is that the shifts in the midpoint potentials are a result of changes in the geometry of the hemes on the membrane matrix.

How does one interpret this coupling microscopically? If the hemes are close enough together, electronic transitions of one heme can be delocalized onto the other hemes. Vibrational excitations will also experience delocalization due to resonance interactions. Not only will Raman spectra be affected by heme interactions but optical transitions will also be perturbed. However, the technique used to separate the optical spectra of the various individual hemes will probably suppress details arising from heme interactions. Thus the ability of the RR effect to monitor spectral interactions between hemes on the membrane provides a unique direct means of probing variations in the membrane geometry that must determine the biochemical interactions between the components.

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