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RESONANCE RAMAN RESOLUTION OF *a*-, *b*- AND *c*-TYPE CYTOCHROMES IN MEMBRANE VESICLES OF ALKALOPHILIC BATERIA

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Resonance Raman spectroscopy has been used to obtain complete spectra of each individual cytochrome type – *a*, *b* and *c* – in the reduced state within membrane vesicle preparations from two species of obligately alkalophilic bacteria: *Bacillus alcalophilus* and *Bacillus firmus* RAB. The vibrational spectra, in the range 250–1700 cm^{-1}, were obtained with tunable dye laser excitation in the wavelength range 550–600 nm tuned to resonance with the appropriate reduced alpha band maximum for the cytochrome type of interest. The spectra reveal details which serve to characterize the specific type of cytochrome as well as to confirm the similarity of the heme prosthetic group to previously well-characterized cytochromes of the the *a*- *b*- or *c*-type. Preliminary evidence in support of heterogeneity of *b*-type, and possibly *a*-type cytochromes, or of heme-heme interaction within the membrane is presented.

Introduction

The cytochrome content of membranes of two obligately alkalophilic bacteria *Bacillus alcalophilus* and *Bacillus firmus* RAB has been shown [1] to be unusually high (5.5 nM/mg membrane protein). Spectrophotometric studies [2,3] of the midpoint redox potentials of the cytochromes present in membrane vesicles of these bacteria have revealed a high degree of heterogeneity with respect to the potentials of the *b*-type cytochrome population and pH dependent midpoint potentials for both *b*- and *a*-type cytochromes. Membrane vesicles of the non-alkalophilic strains contain significantly less cytochrome showing little heterogeneity. It has been conjectured that the unusual quantity and redox potential range of the membrane cytochromes of these alkalophiles may be an adaptation of the respiratory chain, which facilitates these organisms in meeting the stringent bioenergetic requirements of life at high pH.

Resonance Raman spectroscopy of heme containing proteins is well established [4–6] as a method which yields specific structural information about the heme prosthetic group and its interaction with the polypeptide chain. Because of the availability of tunable dye lasers, multicomponent systems can be studied via this technique with the resonance Raman spectrum of each component dominating the spectrum as the excitation wavelength matches the absorption maximum of that component. Previous studies of mitochondria [7,8] and bacterial membranes [9] have demonstrated the feasibility of such studies especially the ability to resolve the spectra of cytochromes by type. These studies relied on the use of fixed frequency krypton ion lasers, however. Maximum separation should be attainable by using a tunable dye laser to match exactly the sharp reduced alpha band maximum of each cytochrome type.

The membrane vesicles of *B. alcalophilus* and *B. firmus* RAB with their high cytochrome content appeared to be an ideal case for such a study. In the reduced state, the sharp alpha band (or Q

(0–0) band) of the different cytochrome types *a* (600 nm), *b* (560 nm) and *c* (550 nm) are separated enough to make selective dye laser excitation of each feasable. The resulting spectra for each type of cytochrome contain features which allow identification of the heme prosthetic group via comparison to resonance Raman spectra of previously well-characterized [3–5,7] cytochromes such as eucaryotic cytochrome *c* and b_5. Spectral detail is present which may provide evidence of heterogeneity among specific types or of heme-heme interaction within the membrane vesicles.

Materials and Methods

B. alcalophilus and *B. firmus* RAB were grown, and membrane vesicles prepared as previously described [1–3]. For *B. alcalophilus* vesicles, cytochrome *c* content was approx. 2.5 nM per mg of membrane protein, with heme ratioes *c/b/a* of 4.9 : 4.3 : 1. *B. firmus* RAB vesicles contained approx 3.8 nm of cytochrome *c* per mg of membrane protein, with heme ratioes *c/b/a* of 6.7 : 4.7 : 1.

Raman spectra were recorded using an Argon ion laser pumped tunable dye laser (Coherent 590) with sodium fluorescein (550–560 nm) or Rhodamine 6G (600 nm). The spectrometer has been described previously [10].

Samples of membrane vesicles which had been stored at −20°C. and thawed were run at room temperature in 1.0 mm i.d. glass capillary tubes. The vesicles were suspended in 100 mM potassium carbonate buffer (pH 9) containing 10 mM magnesium carbonate. Crystals of sodium dithionite (Sigma) were added prior to sealing the samples in the capillary tube. The Raman spectra themselves are diagnostic of the state of reduction, and sample degradation was monitored by repeating scans of the spectra. Reported spectra showed no changes on the time scale of hours of continuous laser exposure with incident powers of 10–25 mW.

Results and Discussion

Spectra

The spectral region which is most informative for heme proteins (1100–1700 cm^{-1}) is displayed in Figs. 1–3. This region contains the most strongly enhanced vibrations of the porphyrin macrocycle,

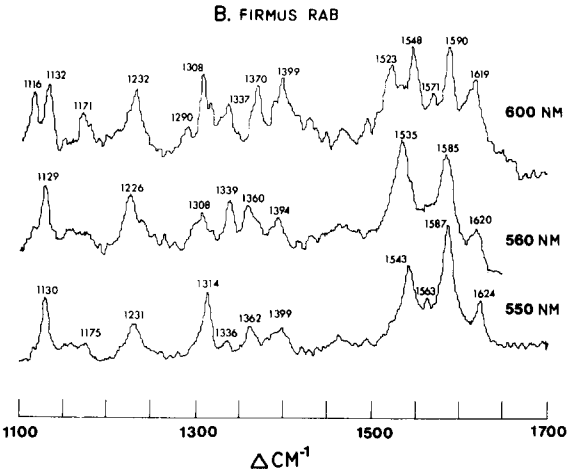

Fig. 1. Resonance Raman spectra (1100–1700 cm^{-1}) of *B. firmus* RAB membrane vesicles at pH 9 with added sodium dithionite. Spectral conditions: (top) 600 nm excitation, 40 mW laser power, 4 cm^{-1} spectral slit width; (middle) 560 nm excitation, 30 mW, 3.5 cm^{-1}; (bottom) 550 nm excitation, 30 mW, 3.5 cm^{-1}.

which have been demonstrated [6,7] to be diagnostic of the peripheral substitution pattern of *a*-, *b*- and *c*-type hemes.

Fig. 1 shows the resonance Raman spectra for *B. firmus* RAB vesicles with each of three excitation wavelengths used: 600 nm for heme *a* (top); 560 for heme *b* (middle) and 550 for heme *c*

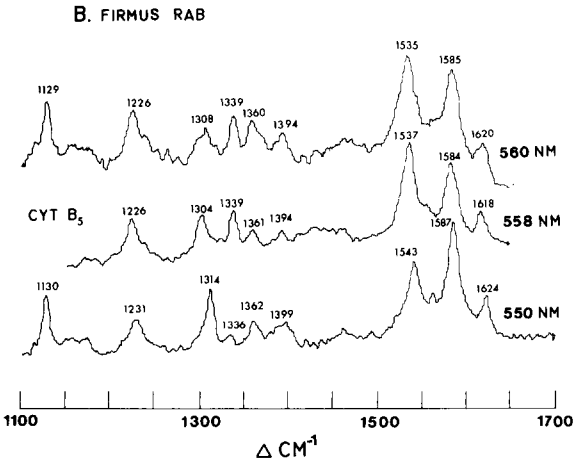

Fig. 2. Resonance Raman Spectra of *B. firmus* RAB membrane vesicles (top and bottom) as in Fig. 1, compared to 558 nm excitation spectrum (middle) of dithionite-reduced cytochrome b_5 from rabbit liver. Middle spectrum: 40 mW, 3.5 cm^{-1} slit width.

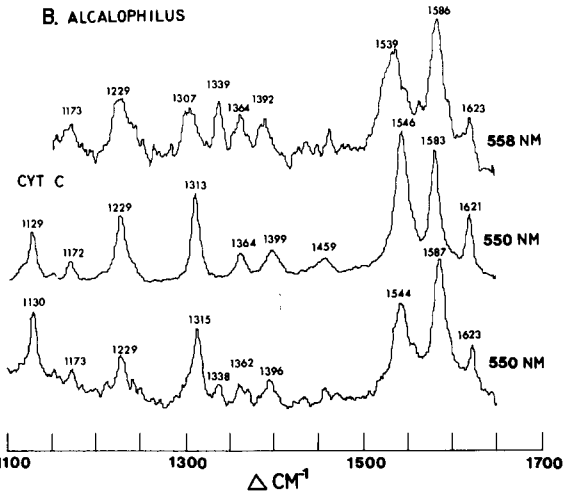

Fig. 3. Resonance Raman spectra (1100–1700 cm^{-1}) of *B. alcalophilus* membrane vesicles at pH 9 with added sodium dithionite, compared to spectrum of dithionite reduced horse cytochrome *c*. Spectral conditions: (top) 558 nm excitation, 20 mW, 4 cm^{-1} slits: (middle) 550 nm excitation, 30 mW, 3.5 cm^{-1}; (bottom) 550 nm excitation, 25 mW, 4 cm^{-1}.

(bottom). These wavelengths correspond as well to wavelengths used in analysis of the redox titrations which have been reported [2,3]. The spectra are of high quality despite a large amount of scattering from these vesicle preparations, and spectral detail is comparable to that obtained from solutions of purified cytochromes (see Figs. 2 and 3).

In Fig. 2, the spectrum of purified reduced rabbit liver cytochrome b_5 (558 nm excitation) is compared to spectra of *B. firmus* RAB vesicles obtained with 560 nm (top) and 550 (bottom) excitation. The close similarity of the 560 nm vesicle spectrum and that of the cytochrome b_5 is most evident if one compares the three spectra in the 1300–1350 cm^{-1} and 1520–1560 cm^{-1} spectral regions. Other regions are similar, as anticipated, since the majority of the resonance Raman bands are not sensitive to peripheral substitution patterns (e.g., protoheme vs. mesoheme). Other features of the spectra which are of interest are the broadened 1308 and 1620 cm^{-1} bands in the 560 nm membrane spectrum as compared to the purified cytochrome b_5 spectrum.

Fig. 3 compares spectra of *B. alcalophilus* membrane vesicles excited at 558 (top) and 550 nm (bottom) with purified horse cytochrome *c* (reduced) excited at 550 nm. In this case, the 550 nm membrane spectrum is in close correspondence to the cytochrome *c* spectrum. Note the single strong band at 1315 cm^{-1} (1313 for cytochrome *c*) and the band at 1544 cm^{-1} (1546 for cytochrome *c*). Again note the broadened 1307 cm^{-1} band for 558 nm excitation in the top spectrum.

Fig. 4 shows spectra in the low frequency region (250–450 cm^{-1}) for *B. firmus* RAB membrane vesicles with three excitation wavelengths. The differences among the three spectra are more striking in this region, which is especially sensitive to the peripheral substitution pattern of the porphyrin. Note the greatest amount of detail which is evident in the 550 nm excitation spectrum emphasizing the cytochrome *c* low frequency vibrations. This is consistent with previous findings [10] for the cytochrome *c* low frequency resonance Raman spectrum.

Reference to Fig. 1 and the preceding discussion of Fig. 2 and 3 indicate that the bands in the 600 nm excitation spectrum (top) which are most characteristic of heme *a* are at 1116, 1290, 1370

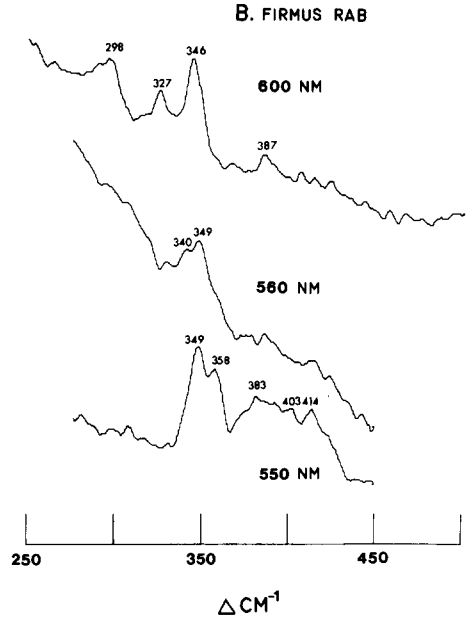

Fig. 4. Resonance Raman spectra (250–450 cm^{-1}) of *B. firmus* RAB membrane vesicles as in Fig. 1. Spectral conditions: (top) 600 nm excitation, 40 mW, 4 cm^{-1} slit width; (middle) 560 nm excitation, 30 mW, 3.5 cm^{-1}; (bottom) 550 nm excitation, 30 mW, 3.5 cm^{-1}.

and 1523 cm^{-1}. In addition, since contribution from no other heme is possible using 600 nm excitation, the 1548 and 1571 cm^{-1} peaks as well as the doublet near 1308 cm^{-1}, with another band at 1337 cm^{-1} are also characteristic of heme *a* as found in the *B. firmus* RAB membrane vesicles.

Analysis

Resonance excitation at the heme alpha, or Q (0–0) band maximum is expected [3–6] to result in greatest enhancement for non-totally symmetric heme vibrations (B_{1g}, B_{2g} and A_{2g}), especially above 600 cm^{-1}. In the low-frequency region, the totally symmetric vibrations (A_{1g}) are dominant, especially below about 500 cm^{-1}. These are nevertheless weaker, due to a lower degree of enhancement for alpha resonance. In addition, the alpha resonant scattering for the reduced hemes will fall off sharply as the excitation wavelength varies from exact coincidence with the characteristically sharp reduced alpha band maximum. Interference from oxidized cytochrome is negligible, since the quantum efficiency of resonant scattering [7] is one-tenth or less that of the reduced form in the visible region. A second maximum in resonant scattering will be observed for a vibration of, e.g., 1000 cm^{-1} using an excitation wavelength 1000 cm^{-1} to the high frequency side of the alpha band. This is the so-called beta band, or vibronic resonance.

For a multicomponent system such as the membrane vesicles studied here, this means that 600 nm excitation will produce resonance Raman spectra for heme *a* only. For 560 nm excitation one might expect heme *b* Raman bands to dominate the spectrum with heme *a* bands neaar 1200 cm^{-1} possibly contributing. For 550 nm excitation, heme *a* bands near 1500 cm^{-1} might appear, in addition to heme *c* bands and low-frequency heme *b* bands. Fig. 1 shows little evidence of heme *a* contribution to the 560 nm excitation spectrum (no 1116 cm^{-1} band) or the 550 nm excitation spectrum (no 1523 cm^{-1} band). The vibronic enhancement must therefore be much less than the heme *b* or *c* alpha enhancement.

For 560 nm excitation then, heme *b* is the major contributor. Note that a prominent heme *c* band near 1315 cm^{-1} (Fig. 1, bottom) is not evident for 560 nm (Fig. 1) or 558 nm (Fig. 3) excitation.

Finally, for 550 nm excitation, heme *c* contribution dominates, but it appears that some heme *b* contribution is evident since both 550 nm excitation spectra (Fig. 1–3) show a heme *b* band near 1336 cm^{-1}, although with only about one-fifth the intensity of the corresponding heme *c* band near 1315 cm^{-1}.

Assignment of observed heme vibrations for hemes *b* and *c* in the membrane vesicles is tabulated in Table I. The labels are from Ref. 6 and indicate the symmetry of the mode in the ideal D_{4h} point group of a 4-fold symmetric metalloporphyrin as well as the molecular coordinates which contribute most to the normal mode. The *B. firmus* RAB modes which correlate with the characteristic modes of well-known cytochromes b_5 and *c* are listed. The corresponding modes for the *B. alcalophilus* membrane vesicles are all within 1 or 2 cm^{-1} (see figures). For heme *a*, more Raman

TABLE I

FREQUENCIES AND ASSIGNMENTS FOR PRINCIPAL RESONANCE RAMAN BANDS OF MEMBRANE CYTOCHROMES *B. FIRMUS* RAB VESICLES

Mode [a]	cyt *c*	cyt b_5	550 nm	560 nm	600 nm
ν_{10} (C_a-C_m), B_{1g}	1621	1618	1624	1620	1619
ν_{19} (C_a-C_m), A_{2g}	1583	1584	1587	1585	1590
ν_{11} (C_b-C_b), B_{1g}	1546	1537	1543	1535	1523
ν_4 (C_a-N), A_{1g}	1364	1361	1362	1360	1370
$\delta = CH_2$ [2], A_{2g}	–	1339	–	1339	1337
ν_{21} (δC_mH), A_{2g}	1313	1304	1314	1308	1308
ν_{13} (δC_mH), B_{1g}	1229	1226	1231	1226	1232
ν_{22} (C_a-N), A_{2g}	1129	1130	1130	1129	1132

[a] From Ref. 6, principle contributor to normal mode designated, and symmetry in D_{4h} point group.

bands are observed (see Fig. 1) as expected due to the lower symmetry of the porphyrin (4-vinyl, 8-formyl) which is induced by strong conjugative effects of the unsaturated substituents.

The redox potential studies of these vesicles have revealed the presence of three to four *b*-type cytochromes and two *a*-type. The *a*-type show in addition pH dependent midpoint redox potentials.

The resonance Raman spectra for both species excited at the reduced *b*-type cytochrome alpha maximum show that the 1308 cm^{-1} band characteristic of heme-*b* is broadened to a frequency that is lower (i.e., not due to *c*-type contribution). Compare in Fig. 2 the spectrum of cytochrome b_5 and the membrane vesicles (top), both with the same resolution. Previous studies of isolated mitochondrial b-c_1 complex [7], whole mitochondria [8] and *Paracoccus denitrificans* membranes [9] have shown that this *b*-type cytochrome band is especially sensitive to the disposition of the cytochrome within the membrane. In particular, an especially lowered frequency (near 1298 cm^{-1}) and/or splitting of this band has been associated with the membrane mediated heme-heme interaction with other *b* or *c* cytochromes. It appears that this is the case for the *b*-type cytochromes in the membrane vesicles of the two species of alkalophilic bacteria studied here. The unresolved low-frequency shoulder observed here may well be indicative of such an interaction which in turn may relate to the observed heterogeneity of *b*-type cytochromes and their redox potentials in the membrane vesicles.

Further studies of this system will look at pH dependence of the resonance Raman spectra, as well as the membranes of the non-alkalophilic strains.

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