SPECTRAL EVIDENCE FOR INTERACTIONS BETWEEN MEMBRANE-BOUND HEMES:

Resonance Raman spectra of mitochondrial cytochrome b–c_1 complex as a function of redox potential

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1. Introduction

Resonance Raman (RR) spectra of many hemeproteins [1–10] and metalloporphyrins [11–16] have been reported since the initial analysis of the scattering enhanced by resonance between the laser frequency and porphyrin π to π^* transitions in cytochrome c by Spiro et al. [17]. Later, more detailed descriptions of the scattering phenomenon in both cytochrome c [18–20] and other metalloporphyrins [15,16,21] were presented and correlations were made between RR band frequencies and oxidation and spin states of the iron in various hemeproteins [6,22,23]. Moreover, the proposal was made that because of its high resolution capabilities, RR spectra could be exploited as a probe of interactions between hemes in functioning biological membranes [24]. A model study on the μ-oxo dimer of tetraphenylporphin indicated that this technique was indeed sensitive to heme aggregation.

Our initial work on the succinate cytochrome c reductase showed it to be a fruitful system for RR study for a variety of reasons: (1) RR spectra of isolated cytochromes exhibit well-defined marker bands [7]; (2) The RR quantum yields of ferrous cytochromes are unusually large (10^{-7}) [25]; (3) The spectroscopic characterization of the RR effect in ferrous cytochromes is highly developed [17–19]. In this mitochondrial fragment which contains two b-type hemes and one c-type heme, we demonstrated [7] that it was possible to identify contributions to the RR spectra of the individual cytochromes by choosing a laser excitation wavelength that was more closely in resonance with one component than the others. In a flowing cell arrangement [26], RR spectra could be recorded while controlling the redox potential in the presence of dye mediators, which showed that RR spectroscopy used in conjunction with potentiometric techniques has the potential to resolve the cytochrome components in a complex mitochondrial preparation.

We report here RR spectra of the cytochrome b–c_1 complex frozen at various redox potentials and excited at 530.9 nm; at lowered temperatures the RR bands are substantially sharper than at room temperature which enables increased resolution of partially overlapping lines. The RR spectra indicate that there are indeed substantial interactions between the hemes in this membranous preparation.

2. Materials and methods

Cytochrome b–c_1 complex was purified from frozen pigeon breast mitochondria by the technique described previously [26]. The final ammonium sulfate precipitate (33–40% saturation) of the complex was suspended in 50 mM phosphate buffer pH 7.2 containing 0.05% lysolecithin (Sigma Chemical

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Co., St. Louis, MO) and dialyzed for 18 h at 4°C against 50 mM phosphate buffer pH 7.2.

Anaerobic potentiometric titrations were carried out as described by Dutton [28] and Wilson and Dutton [29]. At the desired oxidation-reduction potential values, the aliquots were transferred anaerobically to 1 mm i.d. glass, melting point capillaries and frozen by immersion in liquid nitrogen. The following redox mediators were used in the titration: 20 μM diaminodurene, 50 μM of each phenazine methosulfate, phenazine ethosulfate, and ferricyanide. Freshly prepared solution of sodium dithionite was used as the reductant.

Concentration of cytochrome c_1 was determined spectrophotometrically at 553 nm–540 nm using an extinction coefficient (reduced-oxidized) of 17.9 cm^{-1}.

PR spectra in this laboratory are excited by the lines of a Spectra Physics 171-01 high power krypton laser and analyzed by a Jobin Yvon double monochromator based on concave holographic gratings. The optics which couples the sample to the laser and monochromator are provided by a combination of commercial and homebuilt components designed for convenience of sample handling and wavelength interchange. The photomultiplier, an ITT FW 130 (S-20 photocathode), is housed in the standard thermoelectric cooler (Products for Research). Signals are in the form of counts per second. Photoelectric events are amplified in the photomultiplier's dynode chain, and processed by Princeton Applied Research's photon counting system (Models #1105 and 1120). At the present time the signals are integrated and displayed on a strip chart recorder. All components of the system are based on digital electronics which enable easy interfacing with a signal averager or computer control system.

In these experiments samples that had been trapped and frozen in 1 mm glass melting point capillaries were positioned in a quartz dewar and then bathed in cryogenic gas. Temperatures close to the temperature of liquid N_2 were maintained by a rapid flow of cold nitrogen gas. Because the samples are highly scattering in the frozen phase, they are illuminated off axes towards the direction of the entrance slit of the monochromator. The high quality of the monochromator's gratings and reflectors enable measurement of the RR spectra in the presence of large amounts of unshifted laser light.

3. Results

3.1. *Characteristics of the cytochrome* b–c_1 *complex*

Cytochrome b–c_1 complex used in this work [30] contains four one-electron redox components in equimolar concentrations: cytochrome c_1 ($E_{m_{7.0}}$ = 275 ± 10 mV) Rieske iron-sulfur protein ($E_{m_{7.2}}$ = 280 ± 10 mV) and two b cytochromes: b_{561} ($E_{m_{7.2}}$ = 95 ± 10 mV) and b_{566} ($E_{m_{7.2}}$ = −5 ± 10 mV) [27,31]. It has been shown previously [31] that these half-reduction potential values, which were determined using the anaerobic potentiometric titrations technique, combined with optical spectroscopy at room temperature are, within experimental error, equal to those obtained by the EPR studies at the temperatures of liquid helium. Therefore, the same half-reduction potential values were used for discussion presented in this work.

3.2. *Resonance Raman spectra of cytochrome* b–c_1 *complex at various oxidation-reduction potentials*

A RR spectrum of cytochrome b–c_1 complex at an E_h of −105 mV (a potential at which all cytochromes are reduced) and excited at 530.9 nm is shown in fig.1. (A spectrum recorded on a sample reduced with dithionite but without dye mediators is indistinguishable from this spectrum [result not shown].) This excitation wavelength overlaps with the β bands of all three cytochromes. The differences between the laser frequency (in units of cm^{-1}, $\tilde{\nu}(cm^{-1}) = 1/\lambda(cm)$) and the frequency at which the individual α-bands peak, determine the relative intensities of the various Raman bands [18,19,32].

The trace of the RR spectrum between 600 cm^{-1} and 1650 cm^{-1} from the excitation in fig.1 shows many bands. By comparison with band frequencies that occur in the RR spectra of purified cytochromes c and b_5 [7], some of these bands can be tentatively assigned to the b-type or c-type components of the preparation as labelled in the figure. (It should be remembered that RR spectra generated by a different laser line will enhance differently the relative contributions of the various cytochromes. For example, in this spectrum excited by laser light at 530.9 nm, the 750 cm^{-1} band can be attributed to cytochrome c_1 whereas the spectrum excited at 568.2 nm [unpublished data, manuscript in preparation] exhibits a

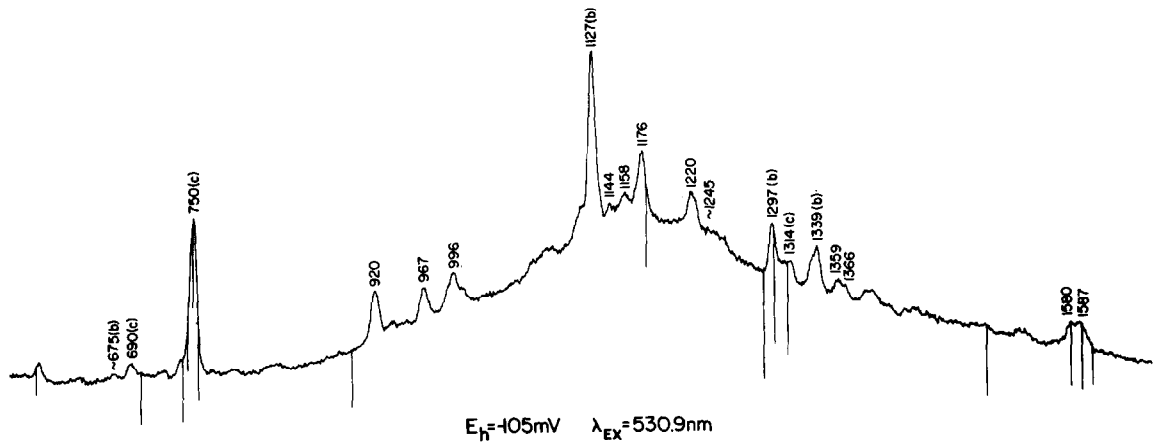

Fig.1. Resonance Raman spectra of succinate cytochrome *c* reductase trapped at $E_h = -105$ mV. Concentration of cytochrome c_1 was 66 μM. Excitation was 300 mW at 530.9 nm.

comparable line at 746 cm^{-1} which we attribute to cytochromes *b*.)

In the present communication we choose to focus on the RR bands between 1270 and 1350 cm^{-1}; an earlier RR study of ferrous cytochromes [7] as well as a model study of the RR spectra of pure hemes incorporated into cytochrome b_5 [33] showed that it was possible to distinguish the Raman bands of hemes *c* and *b* in this region. The RR band of heme *c* appears at 1315 cm^{-1}. In the RR spectra of heme *b*, there are two bands at 1306 and 1342 cm^{-1}; these numbers are invariant (to 1 or 2 cm^{-1}) for other hemeproteins containing heme *b* [2,23].

In this membranous preparation, however, the lower frequency band is significantly and reproducibly shifted to 1297 cm^{-1}. (At one point, we did observe the band close to 1305 cm^{-1} in a sample that had been reduced with dithionite one day before the spectrum was recorded.)

The RR spectra generated in this region as a function of redox potential between −105 mV and +276 mV are shown in fig.2. It is clear that the intensities of the RR bands decrease as the samples become more oxidized which is a consequence of the difference in RR quantum yields for oxidized and reduced cytochromes. (In an earlier work that documented the relationship between the linewidths of the visible absorption bands and the RR intensities in hemeproteins, we showed that the quantum yield for RR

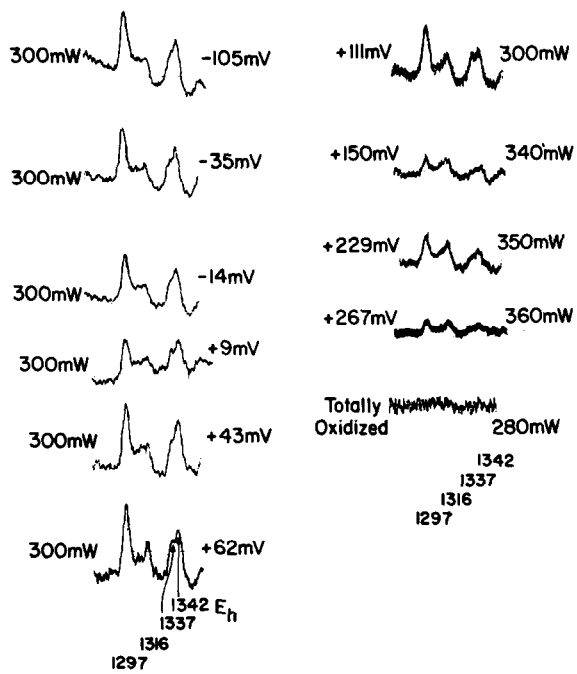

Fig.2. Resonance Raman spectra of the bands between 1290 and 1350 cm^{-1} as a function of redox potential. Anaerobic potentiometric titration and trapping of the samples is described in the Methods. Concentration of cytochrome c_1 was 66 μM.

in ferrous cytochrome b_5 is about an order of magnitude larger than in the ferric cytochrome [25]. Therefore the spectra presented here are attributed entirely to reduced cytochromes.)

The redox state as presented in the optical data [27,30,31] can be used to predict the appearance of the various RR marker bands. However, because of self-absorption of the scattered light which changes with redox state along with the intrinsic enhancement of the bands, the intensities of the RR bands will not be expected to yield entirely smooth Nernst curves.

All three cytochromes are expected to contribute to the RR spectra of samples trapped at −105 and −35 mV. Contributions from cytochrome b_{566} should be minimal in RR spectra of samples trapped at 43 mV and higher. At 150 mV and higher potentials, no *b*-type cytochrome is reduced so its contributions are expected to be negligible.

Inspection of the data in fig.2 indicates that the contribution from cytochrome c_1 at 1316 cm^{-1} is relatively constant over all spectra where it is fully reduced. However, in contrast to predictions based on the titration of the optical bands, at potentials where the optical spectra indicate that only cytochrome c_1 is reduced (267 mV, 229 mV, 150 mV), marker bands for *b*-type cytochromes (1297 cm^{-1} and ∿1340 cm^{-1}) are clearly observed. In all spectra where signal to noise is adequate, the 1340 cm^{-1} band exhibits structure. While it can be argued that at potentials below +30 mV such structure is due to the presence of two inequivalent ferrous *b*-type hemes (cytochromes b_{561} and b_{566}), this cannot be the case in samples trapped at 43 mV and higher. Yet, the structure at 1340 cm^{-1} persists, and becomes even more resolved as the potential is increased.

The contribution of the *b*-type band at 1297 cm^{-1} is plotted as a function of redox potential in fig.3 (corrected for constant incident intensity at 300 mW). If the two *b* cytochromes were contributing to this RR band, one would expect a sigmoid titration curve characteristic of two components with half-reduction potentials of −5 mV and 95 mV [27,31]. It can be seen from fig.3 that although initially the intensity of this band is lost as cytochrome b_{566} becomes oxidized, at an E_h of between 0 mV and 50 mV, the intensity abruptly increases in spite of the fact that cytochrome b_{566} is continuing to be oxidized. Then, at about 100 mV and above, the intensity

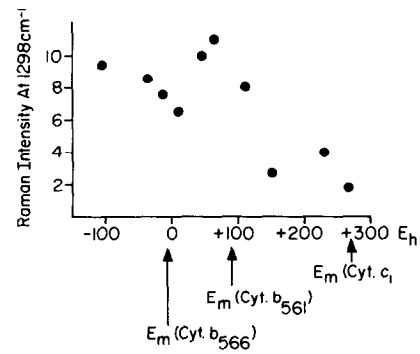

Fig.3. Intensity of the 1297 cm^{-1} band as a function of redox potential. Experimental results are those of fig.2.

decreases, concomitantly with oxidation of cytochrome b_{561}; we emphasize that the intensity of this band does not drop to zero. The increase in intensity observed around 0 mV can be accounted for by the absorption changes which accompany the oxidation of cytochrome b_{566}. With the disappearance of the α band of ferrous cytochrome b_{566}, the absorption losses at the wavelength of the scattered light are substantially decreased. (The excitation at 530.9 nm corresponds to 18 837 cm^{-1}. The Raman band is 1297 cm^{-1} lower at 17 540 cm^{-1} which is equivalent to 560 nm). Thus, although the absolute intensity of scattered light of the marker band for heme *b* may decrease as cytochrome b_{566} is oxidized, the apparent intensity may increase quantitatively (this will depend on the sample concentration and the optical configuration).

We have considered the possibility that the laser excitation is itself interacting with the samples to produce reducing equivalents that could be transferred to the cytochromes. A plot of the ratio of the RR intensities at 1297 and 1316 cm^{-1} showed that between 100 and 400 mW of radiation, no dependence on the input power was observed, which argues against photoreduction of the cytochromes under the experimental conditions used.

4. Discussion and conclusion

The RR spectra of succinate cytochrome *c* reductase exhibit three anomalous features. (1) Relative to

the RR spectra of other hemeproteins containing heme *b* (including hemoglobin, myoglobin, and cytochrome b_5), the peak position of one of the marker bands for heme type is low (1297 cm^{-1} vs. 1305–1308 cm^{-1}). (2) The other marker band for heme *b* at 1340 cm^{-1} shows persistent reproducible structure that is present even when one of the cytochrome *b* is oxidized (and therefore not scattering with appreciable quantum yields). (3) Intensity for heme *b* marker bands persist even when only cytochrome c_1 is reduced.

Since coupling between hemes would be expected to shift the centers of gravity of the RR bands, and to mix the bands and split them into as many components as there are hemes [34], we are interpreting these three observations as evidence for interactions between the *b* and *c* type hemes in the membranous preparation of the cytochromes b–c_1 complex. Each of the features mentioned above is consistent with this description. The band at 1297 cm^{-1} that is tentatively assigned to heme *b* is substantially shifted from the frequency observed in other preparations. The second and third observations are to be understood by assuming that because of the heme–heme interactions, these RR bands no longer reflect pure vibrations of the individual hemes. For instance, the doublet at 1340 cm^{-1} can possibly be attributed to mixing of cytochromes c_1 and b_{561}. The persistence of scattering by 'heme b' at 1297 and 1340 cm^{-1} even when only cytochrome c_1 is reduced can also be explained by quantum mechanical mixing of the vibrational levels.

Similar interactions between cytochromes *b* and *c*, have been suggested on the basis of circular dichroism studies (B. Hess, personal communication).

The physical mechanism that promotes the interaction between hemes is not at present clear. According to Davydov's multipole expansion in lowest approximation, the energy levels of excitons (interacting molecular aggregates) are mixed and split by the dipole interaction [34]. But the resonance enhanced Raman active vibrations in heme are of symmetry A_{1g}, A_{2g}, B_{1g}, B_{2g}; they are, therefore, infrared inactive and have no associated dipole moment. In the absence of the dipole coupling term, the exciton states would then be split by quadrapole interactions, but the quadrapole term is expected to be small. Davydov argues [34], however, that in large polyatomic molecules there may be infrared active modes that are accidentally degenerate with Raman modes; the splittings of the infrared active excitons can then induce splittings of the Raman active excitons via an indirect dipole coupling term.

On the other hand Davydov's multipole expansion of the exciton interaction would be expected to be invalid when the distances between hemes are comparable to the hemes themselves, a condition which may occur often in biological preparations. For the present, it is clear that firm assignments of geometry between hemes cannot be made until theoretical analysis can formulate predictions. From the experimentalist's point of view, it is our task to collect data at other excitation wavelengths, and in preparations in which the hemes are modified by well-known procedures. It is evident from the present information that enough data collected from well-designed experiments in conjunction with rigorous theoretical analyses, may yield information relating the functioning of hemes in the electron transport chain to their geometry in the mitochondrial membrane.

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