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Resonance Raman Spectra of Whole Mitochondria†

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ABSTRACT: The resonance Raman spectra of reduced cytochromes *b* and *c* and cytochrome oxidase in whole mitochondria have been recorded without any instrument modifications. The contributions of the individual cytochromes have been identified by comparison with the characteristic features observed in partially purified preparations including: (i) the strong dependence of the intensity patterns on excitation wavelength relative to the peak positions of the α, β, and γ absorption bands of the cytochromes; and (ii) the presence of marker bands for heme type. Since the Raman spectra can be used as an intrinsic indicator of interaction between hemes, the ability to record spectra in intact mitochondria opens the possibility to study heme-heme interactions in the functioning membrane in situ.

Reports of the resonance Raman (RR) spectra of hemeproteins have proliferated since the initial investigation of Strekas and Spiro on cytochrome *c* (Strekas & Spiro, 1972a) and hemoglobin (Strekas & Spiro, 1972b) and their elegant analysis of the vibronic origin of the scattering mechanism (Spiro & Strekas, 1972) which is enhanced by resonance between the laser frequency and the porphyrin $\pi \rightarrow \pi^*$ transitions. More detailed descriptions of the scattering phenomenon in both cytochrome *c* (Friedman & Hochstrasser 1973, 1976; Collins et al., 1973; Nafie et al., 1973) and other metalloporphyrins (Verma et al., 1974; Mendelsohn et al., 1975; Woodruff et al., 1975; Asher & Sauer, 1976; Shelnutt et al., 1976, 1977; Kitawagawa et al., 1976; Mayer et al., 1973) have since appeared. Correlations have been made between the RR band frequencies of various heme proteins and (i) their oxidation and spin states (Loehr & Loehr, 1973; Yamamoto et al., 1973; Spiro & Strekas, 1974), (ii) the porphyrins' geometry (Spaulding et al., 1975), and (iii) axial ligands (Kitagawa et al., 1975, 1976). RR spectra of cytochrome oxidase have also been reported (Salmeen et al., 1973; Kitagawa et al., 1977; Salmeen et al., 1978). The observation that the overall RR intensities of heme proteins are inversely correlated with the line widths of the absorption bands was explained by recognizing the role of the iron electronic state in providing paths for radiationless decay out of the porphyrin $\pi \rightarrow \pi^*$ excited state which has the effect of decreasing the probability for the resonance Raman process to occur (Adar et al., 1976). Helium temperature excitation profiles of the intensities of the Raman bands of ferrous cytochromes *c* and b_5 as a function of excitation wavelength demonstrated the usefulness of the technique in making inferences about optically inactive d-d transitions of the iron atom which have direct bearing on the redox properties of the hemes (Friedman et al., 1977). Moreover, in a study of the RR line widths of paramagnetic hemes it has been shown that there is nonradiative coupling between the porphyrin vibrational levels and low lying electronic levels of the iron (Adar, 1978). This latter suggestion may prove important in the study of heme proteins where subtle changes in bonding of axial ligands is thought to have an important role in biological function.

We have proposed that the relatively high resolution capabilities of the Raman technique could be exploited in studying interactions between hemes in biological membranes. A model study of the μ-oxo dimer indicated that this proposal was feasible (Adar & Srivastava, 1975). A spectroscopic analysis of RR data in a series of samples of the purified cytochrome b-c_1 complex from pigeon breast mitochondria, which had been trapped in well-defined redox states, indicated that the effects of membrane organization on some of the Raman bands can be substantial (Adar & Erecińska, 1977). We have since been able to excite RR spectra in whole mitochondria and identify them with the various cytochrome components by exploiting the known wavelength dependence of the intensities and by following marker bands for heme type. These data are reported here.

Materials and Methods

Pigeon breast mitochondria were prepared as described previously (Erecińska et al., 1973) and kept frozen at −30 °C. The frozen mitochondria were rapidly thawed at 30 °C and washed once in 10 mM phosphate buffer, pH 7.4, and twice in 100 mM phosphate buffer, pH 7.4, and finally suspended in the same medium. Final concentration as measured by cytochrome *a* content [$\Delta\epsilon_{mM}$ at 605-630 nm (red-oxid) = 26.4 cm^{-1}] was of the order of 20-30 μM.

Samples reduced with dithionite were contained in 1-mm melting point glass capillaries. At these high protein concentrations, oxygen cannot diffuse into samples contained in the sealed capillaries. To prevent denaturation by the focused laser beam, the samples were cooled with a flow of cool nitrogen gas. (Previous experience with particulate preparations has indicated that samples will not denature if adequately cooled.) It was assumed that RR spectra can be attributed to the reduced

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TABLE I: Resonance Raman Marker Bands for c and b Type Hemes.

Cyt c (cm^{-1})	Cyt b_5 (cm^{-1})	polarization properties	symmetry type
1315	1306 1342	anomalously polarized	A_{2g}
1548	1538 1564	depolarized	B_{1g} or B_{2g}

species because the quantum yields have been documented to be 10 times higher in the ferrous rather than the ferric forms (Adar et al., 1976).

The Raman system consists of a Jobin Yvon Ramanor HG2S double monochromator, a Spectra Physics 171-01 krypton laser, a Liconix HeCd laser, a cooled RCA GaAs photomultiplier, and a PAR photon counter. The optics coupling laser to sample to monochromator were home built.

Rationale

In order to exploit the RR spectra of cytochromes in the mitochondrial matrix as an intrinsic probe of interactions between hemes, we have constructed a hierarchy of effects with which to interpret the porphyrin's vibrational spectra.

(i) Early studies of purified cytochromes c and b_5 identified clear marker bands that enable one to distinguish the two types of hemes (Adar & Erecińska, 1974; Adar, 1975). These bands are summarized in Table I.

(ii) Profiles of the enhancement of the RR spectra as excitation wavelength is varied show that the normalized intensities of the bands are proportional to the absorption bands (Friedman et al., 1977). However, the characteristic enhancement patterns are different for resonance with the α and β bands. The RR processes for laser in resonance with these two visible bands are depicted in Figure 1. The arrows pointing up indicate the electromagnetic coupling between the laser photon and the visible (α and β) transitions of the heme. Excitation profiles (Friedman et al., 1977) recorded with a liquid dye laser indicate that the intensities of all the vibrational bands enhanced by photons in resonance with the α band follow the α band envelope uniformly; this is indicated on the left side of the figure by uniformly darkened arrows pointing down. When the exciting wavelength is in the vibronic envelope (the β band), however, all bands are not uniformly enhanced. For the sake of explanation we will assume that there is a unique one-to-one correspondence between vibrations in the excited (β) state and ground state. (Vibronic coupling and orthonormalization of the vibrations in the excited state will relax this condition somewhat; these effects have not been observed to be very large in hemes, although the data is yet sparse.) A particular RR vibration will exhibit a peak in its excitation profile when the laser is in resonance with the corresponding vibrational component of the β band (i.e., the corresponding vibronic level). As shown below points (i) and (ii) can be used to assign bands to the RR spectra of the cytochrome b-c_1 complex to c or b type hemes (Adar & Erecińska, 1974).

An understanding of the relationship between the laser excitation wavelengths which are available for recording heme spectra in this work and the absorption bands of cytochromes aids in the interpretation of the RR spectra of mitochondria and their subfractions. In mitochondria the peak positions of the visible bands of the various b and c cytochromes vary by 10–15 nm (see Figure 2 in Adar &Erecińska, 1974). Therefore one would expect that RR spectra excited at 568.2 nm will arise

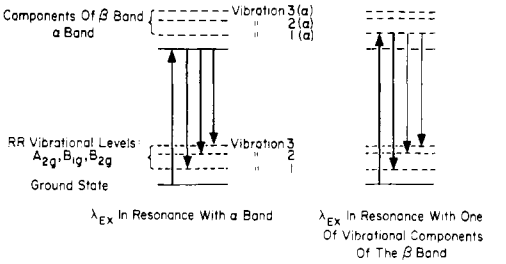

FIGURE 1: Resonance Raman process in hemes excited in their visible bands.

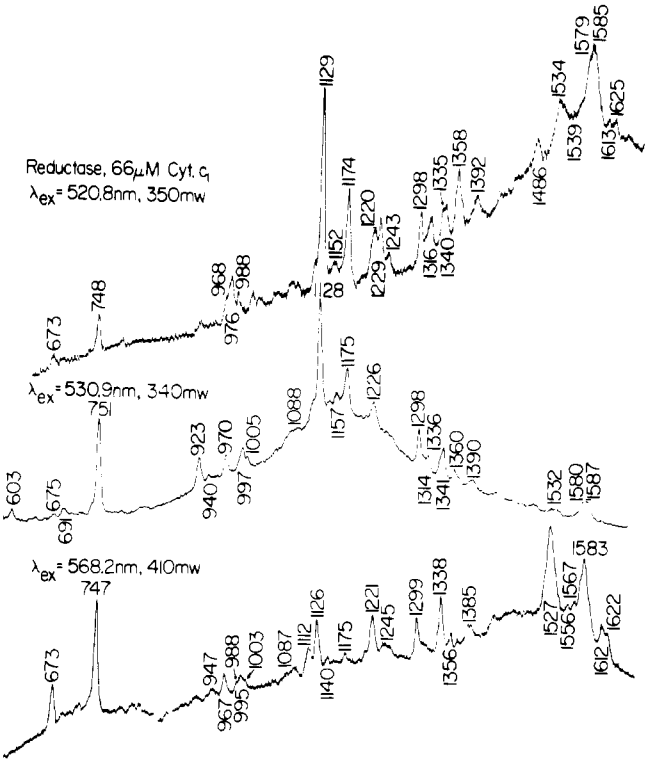

FIGURE 2: Resonance Raman of cytochrome b-c_1 complex excited at 568.2, 530.9, and 520.8 nm. Samples were reduced with minimal amounts of dithionite and cooled with nitrogen gas. Slits were 600 μm giving instrumental resolution of 4–6 cm^{-1}. Spectra were scanned at 50 cm^{-1}/min with a 1-s time constant.

almost completely from cytochrome b_{566} (Adar & Erecińska, 1977). On the other hand when exciting at 530.9 nm, both the b and c type hemes are enhanced. The data obtained at this wavelength shown in Figure 2 are explained by reference to the resonance enhancement patterns cited above (Friedman et al., 1977). The RR bands of cytochromes b in the region around 1100 cm^{-1} (±200 cm^{-1}) are preferentially enhanced over their lower frequency bands, while the RR bands of cytochrome c_1 around 800 cm^{-1} are enhanced over its higher frequency bands. The following examples illustrate the assignments that can be made. The band at 751 cm^{-1} is attributed to cytochrome c_1 (Adar & Erecińska, 1974, 1977); scattering from b type hemes at 748 cm^{-1} would broaden this band if it were appreciable. In the region around 1300 cm^{-1} where clear marker bands for heme type have been identified (Adar, 1975), the contributions from b type hemes at 1298 and 1340 cm^{-1} are much stronger than those of heme c at 1314 cm^{-1}. In the high 600 cm^{-1} region, the band at 675 cm^{-1} is assigned to b type hemes because of its appearance in the spectrum generated at 568.2 nm and because of the studies of purified cytochromes (Adar & Erecińska, 1974).

TABLE II: Resonance Raman Band Frequencies (cm^{-1}) of Cytochrome Oxidase.

λ_{ex} = 441.6 nm (purified enzyme)[a]	λ_{ex} = 441.6 nm (whole mitochondria)	λ_{ex} = 413.1 nm (purified enzyme)[b]
215	214	
268	265–275	
343	340	
364	365	
398	394	
441	435	
585	564, 583	
634	632	
661	658	
682	680	687
714	713	
750	747	748
	788	
	850	
	935, 960, 977	
	1038	
	1088	
	1116	
1134	1132	1132
	1183	
1230 (sh)	1227	1228
1250	1248	1245
	1288	1288
	1305	1306
	1331	
1360	1357	1358
	1393	1393
	1492	1473
	1519	1520
1576	1568	1545, 1569
1590	1585	1587
1616, 1628	1610, 1623	1615 (broad)
1670	1665	1665

[a] From Salmeen et al. (1978). [b] From Adar & Yonetani (1978).

When exciting at 520.8 nm the bands of the *b* type hemes around 1500 cm^{-1} and those at 1200 cm^{-1} of cytochrome *c* are most effectively enhanced. The spectrum at this excitation wavelength (Figure 2) shows comparable intensities in the marker band region at 1300 cm^{-1} for bands of hemes *c* and *b*. In the mid 1500 cm^{-1} region a pronounced band appears at 1534 cm^{-1}, whereas the comparable band excited at 568.2 nm is at 1527 cm^{-1} (the intensity in this region excited at 530.9 nm is fairly weak). These frequencies are low compared with both purified cytochrome *c* and b_5 (1548, 1538 cm^{-1} respectively) and the bands may not be easily identifiable with individual components.

(iii) Following identification of RR spectral features in the cytochrome b–c_1 complex, based on marker bands and RR excitation profiles, we examined the RR spectra of this complex in order to identify details that could be attributed to interaction between hemes in the lipoprotein matrix. In earlier reports it was noted that the marker bands for heme *b* in the low 1300 and mid-1500-cm^{-1} range are low and the frequency of the latter varies as the excitation wavelength is changed. One is at 1527 cm^{-1} when excited at 568.2 nm, at 1532 cm^{-1} when excited at 530.9 nm, and at 1534–1539 cm^{-1} when excited at 520.8 nm (Table II). The comparable bands of cytochromes *c* and b_5 appear at 1548 and 1538 cm^{-1}. The other marker band occurs at 1315 in cytochrome *c* and 1307 cm^{-1} in cytochrome b_5. In addition to the marker for heme *b* occurring almost 10 cm^{-1} lower in the cytochrome b–c_1 complex

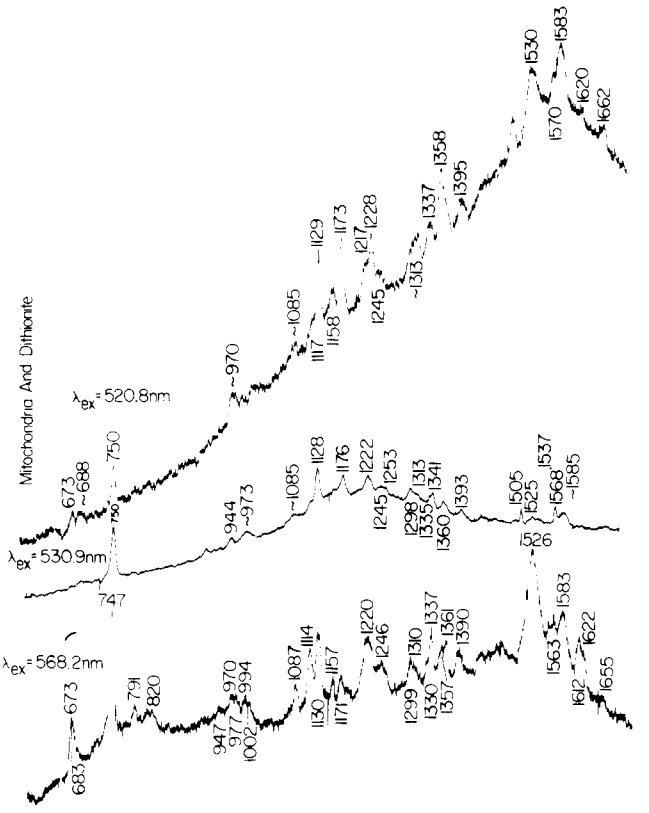

FIGURE 3: Resonance Raman spectra of whole mitochondria (20–30 μM cytochrome *a*) recorded as in Figure 2.

(1298 cm^{-1}) than in purified cytochrome b_5, a second band that occurs simultaneously in *b* type hemes at 1340 cm^{-1} is consistently split in mitochondrial complexes. The observation of band frequencies that are split, that vary with excitation wavelength, and that are consistently lower than those of purified cytochromes are reconcilable with resonant Raman scattering onto exciton components of a multiheme complex.

(iv) In this report we show RR spectra of the same cytochromes but here they are located in intact mitochondria. The highest level of interpretation of these RR data involves identification of differences between the spectra of the isolated b–c_1 complex, and the same cytochromes in whole mitochondria. Because of the enormous enhancement of the Raman scattering of hemes by the available laser wavelengths, we assume that no other mitochondrial components contribute to the spectra. The similarity of the spectra of the purified preparations and whole mitochondria supports this assumption. The slight differences indicate the potential use of the RR effect as an intrinsic monitor of the biophysical condition of the mitochondrial matrix. In particular, it is known that midpoint reduction potentials of the cytochromes are affected by preparative procedures (Leigh & Erecińska, 1975), and the ability to phosphorylate adenosine diphosphate is found only in membranous preparations. With the development of the RR technique as a monitor of interactions between hemes, it will be possible to define the role of the geometrical arrangement of hemes in their biological function.

Results

The Raman spectra of mitochondria reduced with minimal amounts of dithionite and excited at 568.2, 530.9, 520.8, and 441.6 are presented in Figures 3–4. In order to allow us a precise correlation of the results obtained on whole mito-

chondria with those on purified preparations, we display RR spectra of the purified cytochrome b–c_1 complex also excited at 568.2, 530.9, and 520.8 nm (Figure 2). Clear marker bands for hemes c and b have been identified and are summarized in Table I. The other bands are similar from heme to heme so clear assignments are not being made at the present time.

The RR band frequencies of purified cytochrome oxidase excited at 441.6 and 413.1 nm have been derived from Salmeen et al. (1978) and Adar & Yonetani (1978). The spectrum of mitochondria excited at 441.6 nm is esentially identical with that of the purified preparation.

Comparison of RR of the purified cytochrome b–c_1 complex and of whole mitochondria is presented.

568.2 nm: The clearest differences lie in the 1300-cm^{-1} region. The intensity of the shoulder at 1310 cm^{-1} relative to the peak at 1299 cm^{-1} is higher in the whole mitochondria, possibly because of the presence of residual cytochrome c as well as the tightly membrane-bound cytochrome c_1. Whereas the peak at 1338 cm^{-1} in the b–c_1 complex is relatively symmetric, that at 1337 cm^{-1} in mitochondria has a distinct shoulder at 1330 cm^{-1}. Mitochondria exhibit bands at 1357 and 1361 cm^{-1}, whereas the b–c_1 complex exhibits only one band at 1356 cm^{-1}. One would not expect contributions from cytochrome oxidase at this excitation wavelength.

530.9 nm: The mitochondria show unusually high intensity at 1568 and 1505 cm^{-1} as well as possible structure around 1530 cm^{-1}. The first and last of those have been identified as regions for markers of heme b; however, 1530 cm^{-1} is significantly lower than 1538 cm^{-1}, the analogous band in cytochrome b_5.

520.8 nm: The largest differences occur around 1230 and 1300 cm^{-1}. Because the 1230-cm^{-1} region is complex even in a purified cytochrome, this structure is difficult to interpret. However, the loss of a well-defined b-type band at 1299 cm^{-1} in whole mitochondria and a shift of the c-type band (1316 cm^{-1}) could be significant. In addition, there is appreciable intensity at 1662 cm^{-1} in whole mitochondria. Previously, a band in this region was only reported in cytochrome oxidase (Salmeen et al., 1973, 1978).

441.6 nm: At this excitation wavelength only cytochrome oxidase is expected to contribute appreciably to the Raman spectra. This is based on the effective overlap between the exciting wavelength and its Soret band. Salmeen et al. (1973) documented that this is indeed the case.

The scattering mechanism that accounts for the RR effect in hemes when excited in the Soret region differs from that presented earlier and has been discussed elsewhere (Friedman et al., 1973; Nafie et al., 1973; Salmeen et al., 1973). Under these conditions a different set of vibrations (A_{1g} symmetry type) will be active and has been identified by the polarization properties.

Data collected from cytochrome oxidase and whole mitochondria are presented in Table II. Some band frequencies measured in purified oxidase in the Ford Motor laboratory (Salmeen et al., 1973, 1978) differ from the values that we measure in whole mitochondria at the same excitation and those that we reported earlier for purified cytochrome c oxidase preparation excited at 413.1 nm (Adar & Yonetani, 1978). Before analyzing the differences in detail it should probably be established that the same preparation yields the same frequencies, when the samples are excited by HeCd laser line at 441.6 nm in the two laboratories. After the limits of calibration accuracy have been established, recorded differences can be attributed to differences in preparations and biophysical state. At the present time we are presenting these data to demonstrate the ability to acquire signals as good as those in isolated preparations. Detailed analysis will be presented elsewhere (manuscript in preparation).

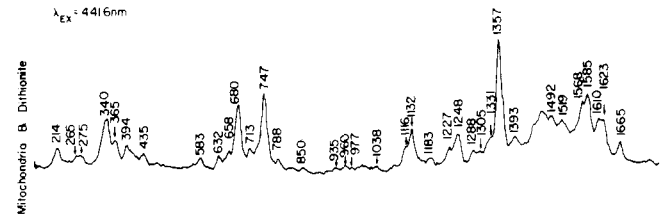

FIGURE 4: Resonance Raman spectrum of whole mitochondria excited at 441.6 nm. Samples were reduced with minimal amounts of dithionite and cooled with nitrogen gas. Slits were 500 μm (6-cm^{-1} band width). Scan speed 100 cm^{-1}/min; 1-s time constant.

Discussion

The original proposal for use of the RR spectra of cytochromes in functioning biological preparations was based on the μ-oxo dimer of iron tetraphenylporphin (Adar & Srivastava, 1975). Results obtained on the b–c_1 complex were presented as more convincing evidence of the potency of this proposal (Adar & Erecińska, 1977). In this paper we show the following. (1) It is possible to obtain high quality RR data of cytochromes in mitochondrial preparations of poor optical quality without any instrumental modification. (2) Differences can be seen between the RR spectra of purified preparations and the same components in whole mitochondria which can be used to monitor physical and biochemical state of the hemes and their environments.

As we discussed earlier the precise mechanism(s) that accounts for the splittings and mixing of vibrations is not well-defined so we are as yet unable to calculate distances between hemes and their relative orientations from these data. There is precedent for these effects, however. In a review of Raman work in biological materials (Thomas & Kyogoku, 1977) it has been observed that there is substantial interchain splitting of the amide I band in crystalline polyglycine I which arises from two peptides groups from each of the two chains in the unit cell.

In our model describing the interactions between hemes in the mitochondrial membrane, we visualize units of lipids and proteins in which the electron transport chain and enzymes necessary for phosphorylation are embedded in a geometrical array appropriate for biochemical function. These units are repeated in the "two-dimensional" space defined by the membrane. In analogy to molecular crystals, the units of protein and lipids form the unit cell, which when repeated over the membrane, form a "two-dimensional" crystal. Since, even in perfect crystals, the Raman effect is usually insensitive to the complete space group lattice (i.e., wave vector conservation fixes the scattering to Brillouin center vibrations) analysis of selection rules is confined to the unit cell. Thus in the case of the cytochromes in mitochondria we need only be concerned with the biological functioning unit and not the entire membrane surface with its variations in curvature.

Analysis of molecular aggregation appropriate to our situation treats first the effects of the crystal field on molecular vibrations, and then the effects of the correlation field between similiar vibrations on different molecular sites (White, 1975; Carter, 1976). The crystal field has a symmetric contribution which can shift the vibrational levels, and a distortional part which can split levels that are degenerate in the case of the isolated molecule. Because the RR vibrations in hemes are all nondegenerate, the second effect is not relevant. The correlation field which gives rise to the Davydov (1962) or factor

group splitting is the source of the splittings that will be useful in monitoring biological function of these membranes.

The anomalies in RR bands that we have noted (Adar & Erecińska, 1977; Adar & Yonetani, 1978) are consistent with these effects of molecular aggregation. Both RR bands that have been identified as markers for *b* type heme (low 1300 and mid-1500 cm^{-1}) are shifted to lower frequency in the cytochrome b–c_1 complex; this can be explained by the symmetric part of the crystal field. In addition, the frequency of the band in the mid-1500-cm^{-1} range is strongly dependent on the excitation wavelength and the 1340-cm^{-1} marker band for heme *b* is split. This suggests that the correlation field has split these bands and the various components experience different enhancement properties (excitation wavelength dependence).

In the cytochrome b–c_1 complex there is another region experiencing both a shift in center of gravity and splitting. Whereas the RR spectra of purified cytochrome *c* and b_5 show the highest frequency band at 1622 and 1618 cm^{-1}, respectively, in the cytochrome b–c_1 complex a doublet appears at 1612 and 1622 cm^{-1}.

In cytochrome oxidase the data are more sparse but do support the proposition that the hemes interact spectrally. The presence of multiple peaks with low centers of gravity in several regions (high 600, low to mid 1500, low 1600 cm^{-1}) is consistent with the model. What is lacking are data (including polarization behavior) at several excitation wavelengths of the same preparation.

On the other hand, the possibility still exists that these spectral details reflect effects of the protein matrix rather than interactions between hemes (exciton coupling) in the membrane. In fact, Burke et al. (1978) present data on the μ-oxo dimer that bring the exciton proposal into question in this system. In order to determine whether the observed spectral effects can be attributed to heme–heme interactions or to differences in the hemes' environments in the mitochondrial preparations, Raman data of mitochondria need to be taken under conditions where there are well-characterized changes in function that are related to the lipoprotein matrix. We believe that exciton interactions are possible because it is difficult to explain the shifts in centers of gravity of the bands by differences in the lipoprotein matrix.

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