Biochimica et Biophysica Acta, 502 (1978) 80—86
© Elsevier/North-Holland Biomedical Press

BBA 47471

RESONANCE RAMAN SPECTRA OF CYTOCHROME OXIDASE EVIDENCE FOR PHOTOREDUCTION BY LASER PHOTONS IN RESONANCE WITH THE SORET BAND

FRAN ADAR and T. YONETANI

Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania Philadelphia, Pa. 19104 (U.S.A.)

(Received August 15th, 1977)

Summary

Resonance Raman spectra of cytochrome oxidase solubilized in Tween 20 and sodium cholate, and excited at 413.1 nm have been recorded. Differences in the resonance Raman spectra of the two preparations are minimal indicating that the local environment of the hemes is similar in the two preparations. As in the work of Salmeen, et al. (1973) (Biochem. Biophys. Res. Commun. 52, 1100) the strongest band appears at 1358 cm^{-1}. Some of the other bands differ slightly in their band shapes and frequencies when compared to their spectra; these differences can be accounted for by differences in resonance enhancement of the various bands when exciting at 441.6 and 413.1 nm. A study of the region from 1350 to 1380 cm^{-1} as a function of laser intensity (10—130 mW on sample) indicate that the doublet reported by Salmeen, et al. at 1358 and 1372 cm^{-1} is a result of photoreduction of the preparations. In samples to which potassium ferricyanide had been added, broad luminescence bands appear at 476 and 641 nm from which it is inferred that catalytic amounts of flavin in the preparations are photoreduced providing reducing equivalents to cytochrome oxidase.

Introduction

Resonance Raman spectra of many hemeproteins [1—10] and metalloporphyrins [11—16] have been reported since the initial analysis of the enhancement of the Raman scattering by resonance between the laser frequency and the porphyrin π to $\pi\star$ transitions in cytochrome *c* by Spiro, et al. [17], Nafie, et al. [5] discuss the origin of the resonance Raman spectra of cytochrome oxidase, reduced with dithionite and excited at 457.9 nm in terms of resonance with the strong Soret electronic state that results in enhancement of principally

polarized bands, an observation not inconsistent with other theoretical analyses of the resonance Raman effect in porphyrins [18—20]. Salmeen, et al. [4] report resonance Raman spectra of oxidized and reduced cytochrome oxidase (solubilized and in electron transport particles) excited at 441.6 nm. These workers note, in particular, a doublet in the spectrum of the oxidized solubilized enzyme which they argue may indicate spectroscopically-inequivalent hemes.

We report here resonance Raman spectra of cytochrome oxidase solubilized in Tween 20 and sodium cholate. Our data show some differences when compared to those of Salmeen, et al. [4] which, to some extent, are due to the differences in excitation wavelength. We propose, however, that the doublet that occurs at 1358—1372 cm^{-1} can be interpreted in terms of photoreduction of the enzyme. This is based on the empirical correlation of the resonance Raman band in this region with oxidation state of the heme [6,7,21].

It has been proposed that because of its high resolution capabilities, the technique could be exploited as a probe interactions between hemes on functioning biological membranes [22]. A model study of the μ-oxo dimer of tetraphenylporphin indicated that the technique is indeed sensitive to heme aggregation. In this initial report from our laboratory of the spectra of cytochrome oxidase, we summarize differences in resonance Raman bands that can be attributed to exciton splittings of heme dimers. Since the two exciton bands in general will be enhanced differently, their relative intensities may vary as a function of excitation wavelength; this effect can account for the differences in resonance Raman band frequencies observed in the two laboratories.

Methods

Cytochrome oxidase was prepared by the method of Yonetani [23] but modified by replacing the final solubilization in Emasol 4130 with 1% Tween 20 or 1% sodium cholate.

The Raman facility is as described previously [24]. Samples were cooled by contact with cold nitrogen gas. Excitation of the resonance Raman spectra was with the 413.1 Å emission of a large Kr laser. Power levels were typically 300 mW at the laser, 100 mW at the sample. In order to determine whether the laser intensity perturbs the state of the enzyme, Raman spectra were recorded down to 10-fold less laser intensity. The luminescent emission from a sample to which potassium ferricyanide had been added was recorded by scanning the monochromotor rapidly and converting the peak positions from wavenumbers ($1/\lambda$) to wavelengths (λ); the emission is uncorrected for monochromotor response which is rapidly decreasing in the red due to the loss in quantum efficiency of the S-20 photocathode.

Results

Resonance Raman spectra between 600 and 1700 cm^{-1} of cytochrome oxidase excited at 413.1 nm and solubilized in Tween 20 and sodium cholate are shown in Fig. 1. The spectra exhibit only very small differences. Between 200 and 650 cm^{-1}, our spectra are featureless in contrast to those of Salmeen,

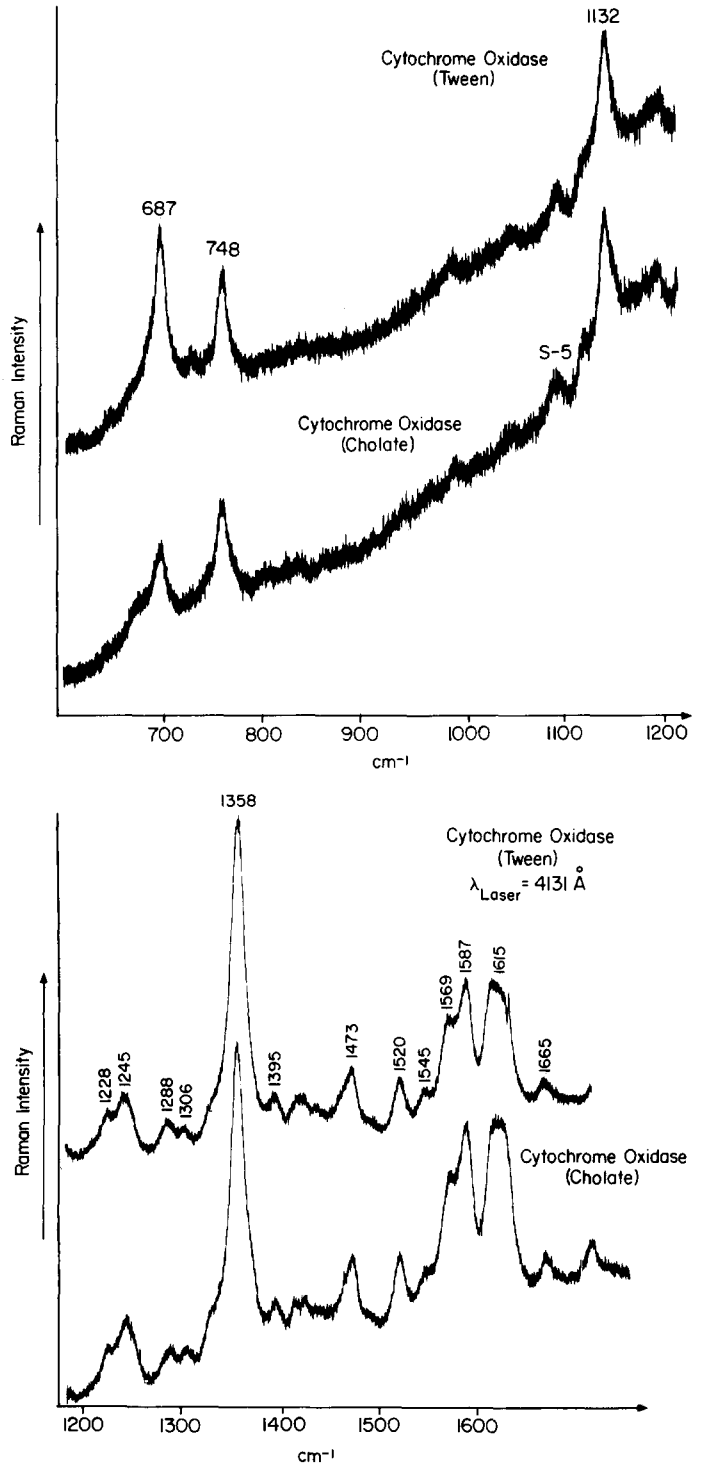

Fig. 1. Resonance Raman spectra of cytochrome oxidase (a) between 600 and 1200 cm^{-1}, and (b) between 1200 and 1700 cm^{-1}, excited at 413.1 nm. Sample concentration was 200 μM. Laser power was 280 mW. Spectrometer resolution was 5 cm^{-1}; scan rate was 10 cm^{-1}/min; time constant, 1".

TABLE I

RESONANCE RAMAN BAND FREQUENCIES EXCITED AT 413.1 AND 441.6 nm

413.1 nm (cm^{-1})	441.6 nm (cm^{-1})
687	683
748	750
1132	1134
1228	
1245	1253
1288	
1306	
1358	1358
1395	
1473	
1520	
1545	1530
1569	
1587	1580
1615 *	1622
1665	1660

* Very broad.

et al., who excited at 441.6 nm. Table I summarizes the resonance Raman bands observed in this laboratory when exciting at 413.1 nm and those observed by Salmeen, et al. when exciting at 441.6 nm. Any differences greater than 2 cm^{-1} are considered significant. Inspection of the table shows that there are bands with positive and negative frequency differences eliminating the possibility of a systematic difference between the monochromotors' calibrations.

Fig. 2 shows the band(s) between 1350 and 1380 cm^{-1} as a function of laser intensity. Just before each scan the sample was removed and stirred. At 400,

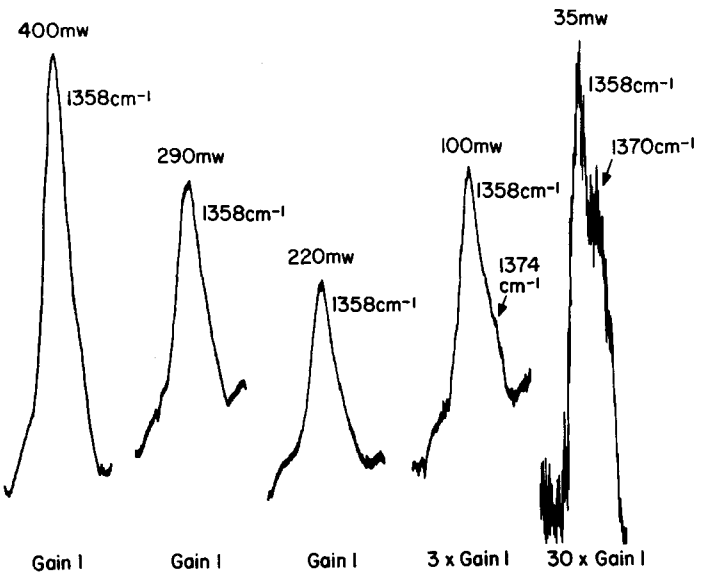

Fig. 2. Resonance Raman Band(s) between 1350 and 1380 cm^{-1} for laser intensities 400, 290, 220, 100 and 35 mW (the intensities at the sample were about 30% of these values).

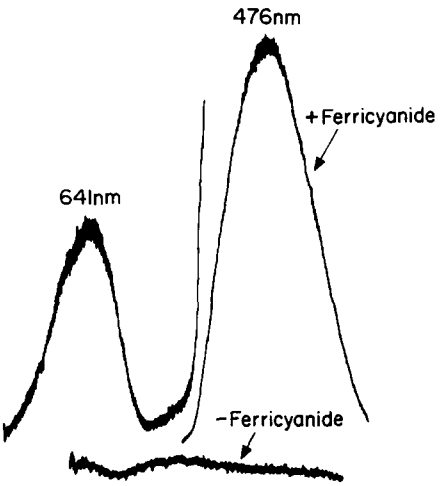

Fig. 3. Emission from sample of cytochrome oxidase to which ferricyanide had been added.

290 and 220 mW there is a single peak at 1358 cm^{-1}. At 100 mW, there is a shoulder at approx. 1374 cm^{-1}. At 35 mW there is a definite, reproducible doublet at 1358 and 1370 cm^{-1}, similar to that reported by Salmeen, et al.

In an attempt to maintain the sample chemically oxidized we added potassium ferricyanide to a sample. The signal itself deteriorated because of absorption by the ferricyanide, but the peak was centered at approximately 1365 cm^{-1} (spectrum not shown). Perhaps even more significant was the luminescent emission from the sample containing ferricyanide. The emission is shown on Fig. 3; there are very broad peaks (2500 and 3500 cm^{-1}) centered at 476 nm and 641 nm.

Discussion and Conclusions

The most important conclusion to be drawn from these data is the apparent photoreduction of cytochrome oxidase, possibly via reducing equivalents generated by flavin impurity. It is based on three points of evidence. (i) Previous studies [6,7,21] have shown that the strong polarized band of ferrous heme appears at approx. 1360 cm^{-1} and shifts to approx. 1372 cm^{-1} in the ferric state. (ii) The relative intensities of the bands at 1358 and 1372 cm^{-1} is a function of incident laser intensity; higher incident powers favor the 1358 cm^{-1} band, i,e., the ferrous species. (iii) Addition of potassium ferricyanide was accompanied by the appearance of flavin luminescence bands at 476 and 641 nm.

It has already been reported that NADH and NADPH reductase appear as consistent contaminants of purified beef heart cytochrome oxidase [25] and it is known that these contaminants contain one mole equivalent of flavin (see the review by Hatefi, for example, ref. 26). Since flavin fluoresces only in its oxidized state, there apparently are indogenous reducing equivalents present in

the preparations which prevent laser illumination from building up a significant population of oxidized flavin. However, the addition of excess ferricyanide allows build-up of photo-oxidized flavin which is detectable by its fluorescence bands.

In the sample to which ferricyanide had been added the resonance Raman band appeared at approx. 1365 cm^{-1}. We have observed, however, that even cytochrome *c* in the presence of ferricyanide can be reduced by violet excitation (unpublished results) so we choose at the present time not to characterize the oxidation state of cytochrome oxidase in the presence of ferricyanide by its marker band. In fact, a clear characterization of the oxidation state of cytochrome oxidase by the resonance Raman spectra will require recording spectra under well defined chemical conditions. In order to determine the photochemical reaction we will repeat these experiments on anaerobic samples; this will establish whether oxygen is required to produce the photochemical reactions that we observe.

The second significant finding in this study, is that spectra of the Tween 20 and sodium cholate-solubilized enzymes were nearly identical leading to the conclusion that the heme environments are indistinguishable at least by the resonance Raman technique in these two preparations.

The last issue to be discussed concerns the possibility that the resonance Raman spectra of cytochrome oxidase can aid in characterizing its optical spectrum. Differences between the optical spectrum of cytochrome oxidase and that of isolated heme have not been accounted for [27]. It would be useful to determine whether the hemes are spectrally and chemically distinguishable, and we believe that the resonance Raman technique has the potential to do so.

Inspection of band frequencies in Table I shows significant differences in peak positions recorded in this laboratory and in that of Salmeen, et al. [4] with the major exception of the strong polarized band which appears at 1358 cm^{-1} in both laboratories. These differences in band frequencies can be attributed to non-equivalent, non-interacting hemes, or to interacting hemes (equivalent or non-equivalent).

In the exciton model of interacting hemes, both the electronic and vibrational energy levels interact via a resonance-type coupling [28]. Although in the simplest formulation it is presumed that the two molecular entities are identical, this is not an absolute requirement. Thus, hemes *a* and a_3 could still be inequivalent, but exhibit energy levels close enough to allow a (near) resonance interaction. Under conditions where the hemes are coupled, the resonance Raman bands observed may reflect the coupling. Because the electronic states are also effected by exciton interactions, the enhancement of the Raman vibrations at a particular excitation wavelength will not be equivalent. This effect explains the differences in the bands listed in the table.

At the present it is not completely clear how to relate these data to the relative orientation of the two hemes in cytochrome oxidase. In particular, Davydov's multipole expansion [28] of the exciton interaction would be expected to be inaccurate when the distance between chromophores is comparable to the size of the chromophore itself. This problem has been discussed previously [24]. The data thus far generated, however, are consistent with the excition proposal and we are proceeding to test the model with additional data

gathered at other excitation wavelengths and in samples well-characterized by their biochemistry and electron spin resonance signals.

Acknowledgements

This work has been supported by grants from NSF (BMS 75 07355 and PCM 77 00811) and NIH (HL 14508).

References

1 Strekas, T.C. and Spiro, T.G. (1972) Biochim. Biophys. Acta 278, 188—192
2 Strekas, T.C. and Spiro, T.G. (1972) Biochim. Biophys. Acta 263, 830—833
3 Brunner, H., Mayer, A. and Sussner, H. (1972) J. Mol. Biol. 70, 153—156
4 Salmeen, I., Rimai, L., Gill, D., Yamamoto, T., Palmer, G., Hartzell, C.R. and Beinert, H. (1973) Biochem. Biophys. Res. Commun. 52, 1100—1107
5 Nafie, L.A., Pezolet, M. and Peticolas, W.L. (1973) Chem. Phys. Lett. 20, 563
6 Yamamoto, T., Palmer, G., Gill, D., Salmeen, I.T. and Rimai, L. (1973) J. Biol. Chem. 248, 5211—5214
7 Loehr, T.M. and Loehr, J.S. (1973) Biochem. Biophys. Res. Commun. 55, 218—223
8 Adar, F. and Erecinska, M. (1974) Arch. Biochem. Biophys. 165, 570—580
9 Kitagawa, T., Kyogoku, Y., Iizuka, T. and Saito, M.I. (1976) J. Am. Chem. Soc. 98, 5169—5173
10 Felton, R.H., Romans, A.Y., Yu, N.T. and Schonbaum, G.R. (1976) Biochim. Biophys. Acta 434, 82
11 Woodruff, W.H., Adams. D.H., Spiro, T.G. and Yonetani, T. (1975) J. Am. Chem. Soc. 97, 1695—1698
12 Verma, A.L., Mendelsohn, R. and Bernstein, H.J. (1974) J. Chem. Phys. 61, 383
13 Mendelsohn, R., Sunder, S., Verma, A.L. and Bernstein, H.J. (1975) J. Chem. Phys. 62, 37—44
14 Spaulding, L.D., Chang, C.C., Yu, N.-T. and Felton, R.H. (1975) J. Am; Chem. Soc. 97, 2517—2525
15 Shelnutt, J.A., O'Shea, D.C., Yu., N.-T., Cheung, L.D. and Felton, R.H. (1976) J. Chem. Phys. 64, 1156—1165
16 Asher, S. and Sauer, K. (1976) J. Chem. Phys. 64, 4115—4125
17 Spiro, T.G. and Strekas, T.C. (1972) Proc. Natl. Acad. Sci. U.S. 69, 2622—2626
18 Friedman, J.M. and Hochstrasser, R.M. (1973) Chem. Phys. 1, 457—467
19 Friedman, J.M. and Hochstrasser, R.M. (1976) J. Am. Chem. Soc. 98, 4043
20 Collins, D.W., Fitchen, D.B. and Lewis, A. (1973) J. Chem. Phys. 59, 5714—5719
21 Spiro, T.G. and Strekas, T.C. (1974) J. Am. Chem. Soc. 96, 338—345
22 Adar, F. and Srivastava, T.S. (1975) Proc. Natl. Acad. Sci. U.S. 72, 4419—4424
23 Yonetani, T. (1960) J. Biol. Chem. 235, 845—852
24 Adar, F. and Erecinska, M.E. (1977) FEBS Lett. 80, 195—200
25 Penniall, R., Elliot, W.B., van Buuren, K.J.H. and van Gelder, B.F. (1976) J. Bioenerg. Biomembranes 8, 271—292
26 Hatefi, Y. (1976) in the Enzymes of Biological Membranes, vol. 4, Electron Transport Systems and Receptors (Nartonosi, A., ed.), pp. 3—42, Plenum Press, N.Y.
27 Adar, F. (1977) in The Porphyrins (Dolphin, D., ed.) academic Press, New York
28 Davydov, A.S. (1962) Theory of Molecular Excitions, McGraw Hill, New York