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Resonance Raman Spectroscopy of Cytochrome Oxidase Using Soret Excitation: Selective Enhancement, Indicator Bands, and Structural Significance for Cytochromes *a* and a_3[†]

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ABSTRACT: Resonance Raman studies of oxidized and reduced cytochrome oxidase and liganded derivatives of the oxidized enzyme have been performed by using direct-Soret excitation at 413.1 and 406.7 nm, as well as near-Soret excitation (457.9 nm) and α-band excitation (604.6 nm). The Soret results clearly show selective enhancement of Raman modes of the hemes of cytochromes *a* and a_3, depending upon the excitation wavelength chosen. For the preparations employed in this study, photoreduction of cytochrome oxidase in the laser beam was not a significant problem. Resonance Raman frequencies sensitive to oxidation state and spin state or core expansion of the *a* and a_3 hemes are identified and correlated with those previously identified for other heme proteins. An unusual low-frequency (<500 cm^{-1}) spectrum is observed for oxidized high-spin cytochrome a_3, which may be due to axial nonheme structures in this cytochrome.

Resonance Raman spectroscopy (RRS)[1] has proven to be an extremely informative probe for the structures and electronic states of hemes, both in their heme protein environments (Spiro, 1975) and as protein-free metalloporphyrins (Spiro & Burke, 1976; Spaulding et al., 1975; Kitagawa et al., 1975). Information available from heme protein RRS includes oxidation state (Spiro & Strekas, 1974), ligation state (Spiro et al., 1979), and spin state (Spiro & Strekas, 1974) of the iron atom, macrocyclic "hole size" associated with the equatorial nitrogen donors of the heme (Spaulding et al., 1975; Spiro et al., 1979), and perhaps effects of protein tertiary and quaternary structure (Shellnut et al., 1979). Clearly, such information on cytochrome *c*/dioxygen oxidoreductase (cytochrome oxidase) would be invaluable in elucidating the heme structures involved in the stable forms of the enzyme and in identifying the heme species associated with the various transients in the electron-transfer sequence of reacting cytochrome oxidase.

Cytochrome oxidase has, however, proved more resistant to analysis by RRS than other heme proteins. Although cytochrome oxidase was one of the first proteins to have been examined by RRS (Nafie et al., 1973) and has been the subject of several RRS studies since (Salmeen et al., 1973, 1978; Adar & Yonetani, 1978; Babcock & Salmeen, 1979; Bocian et al., 1979; Ondrias & Babcock, 1980), the general phenomenology of cytochrome oxidase RRS is far from established, the catalogue of RR spectra of its various oxidation states and derivatives is quite incomplete, and entirely satisfactory interpretations of even the data which exist are lacking. There are several reasons for this situation. Primarily, preparations of cytochrome oxidase which are suitable for RRS study are even

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[1] Abbreviations used: RRS, resonance Raman spectroscopy; RR, resonance Raman; HRP, horseradish peroxidase; Hepes, *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

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more difficult to obtain than preparations suitable for characterization by most other techniques. For example, RRS samples must have good optical properties, be resistant to photodegradation, and be minimally fluorescent in addition to the commonly applied purity criteria. Another difficulty is that reports from different laboratories of RRS of the same cytochrome oxidase species have sometimes been directly contradictory, even with regard to the frequencies of the major Raman peaks. Finally, facile photoreduction of some preparations of oxidized cytochrome oxidase (Adar & Yonetani, 1978; Bocian et al., 1979) has prevented observation of RRS of the oxidized enzyme and its derivatives when excitation directly within the Soret absorption is employed.

The RRS of oxidized cytochrome oxidase is particularly intriguing in view of the evidence (Vanneste, 1966; Babcock et al., 1976) that the broad Soret electronic absorption of the oxidized enzyme contains contributions from cytochroms *a* and a_3 at different (although unresolved) wavelengths. Thus, one may hope, by using laser excitation at different wavelengths within the Soret envelope of oxidized oxidase, to observe selectively the RR spectra of cytochromes *a* and a_3 and thus to monitor the structure of the heme of one cytochrome independently of the other.

In this paper, we report the RR spectra of oxidized cytochrome oxidase and its cyanide and formate derivatives, using laser excitation directly within the Soret absorption at 413.1 and 406.7 nm. Our observations indicate that extensive photoreduction of oxidized oxidase with Soret excitation is not an intrinsic property of the enzyme and is a minor effect for the preparations that we employ. In addition, we demonstrate that selective enhancement of the RR spectra of cytochromes *a* and a_3 does indeed occur as a function of excitation wavelength within the Soret envelope. A similar finding has recently been reported by other workers (Ondrias & Babcock, 1980). Finally, we present a preliminary catalogue of RR indicator bands which are sensitive to oxidation and/or spin state of the iron atom in cytochrome oxidase and relate these heme a–a_3 modes to those previously determined for other heme proteins and the heme structures involved.

Experimental Procedures

Samples of beef heart cytochrome oxidase were prepared by the methods described elsewhere (Babcock et al., 1976) and examined in aqueous buffer/detergent solution (0.05 M Hepes, pH 7.4, containing 0.5% Brij 35). The preparations were frozen and stored in liquid nitrogen temperature until immediately prior to dilution for RRS study. Total heme concentrations in RR samples were 125 μM for oxidized and reduced cytochrome oxidase, 160 μM for the cyano derivative, and 108 μM for the formato derivative. Reduced cytochrome oxidase was prepared from the oxidized preparation by the addition of a slight excess of solid sodium dithionite under a N_2 atmosphere. The oxidation state of the hemes in the RR samples was confirmed by periodically scanning the RR oxidation state marker peak in the 1350–1380-cm^{-1} region. Cyanide and formate derivatives were prepared by previously reported methods (Babcock et al., 1976).

Resonance Raman spectra were obtained by using a SPEX Ramlog EU spectrometer with a cooled RCA C31034A photomultiplier and an ORTEC 9300 series photon counting system. Laser excitation at 413.1 and 406.7 nm was provided by a Spectra-Physics 171-01 krypton laser, at 457.9 nm by a S-P 164 argon laser, and at 604.6 nm by a S-P 375 dye laser (Rhodamine 6G). Samples for RR study were contained in a 1-mm path-length spectrophotometer cuvette thermostated at 14 °C. The identical sample and cuvette were used to monitor the UV–visible absorption spectra before and after RR spectra were obtained. Provisions were available for stirring the samples during laser irradiation, but RR results were identical whether or not the samples were stirred. Raman scattering was observed in 135° backscattering geometry in a plane perpendicular to the polarization of the laser beam. The following spectral acquisition conditions were the same for all samples (406.7 or 413.1-nm excitation): scan speed, 0.2 cm^{-1}/s; spectral slit width, 5 cm^{-1}; laser power at sample, 75 mW (except for reduced cytochrome oxidase, 50 mW); photon count intervals, 2 s between 900 and 1700 cm^{-1}, 3 s between 100 and 900 cm^{-1}.

Results and Discussion

Figure 1 shows the spectra of oxidized and reduced cytochrome oxidase obtained with laser excitation at 413.1 nm. This wavelength is virtually the same as that assigned as the cytochrome a_3 Soret maximun of oxidized oxidase (414 nm) and near the Soret maximum of oxidized cytochrome *a* (427 nm) (Vanneste, 1966; Babcock et al., 1976). For reduced oxidase, the position of the Soret maximum is more remote (443 nm), although a weak magnetic circular dichroism (MCD) feature associated with reduced cytochrome *a* appears at 413.5 nm (Babcock et al., 1976). Despite the positions of the oxidized and reduced Soret transitions relative to the 413.1-nm laser line, the experimentally observed RR intensities of correlatable *high-frequency* (>1000 cm^{-1}) heme modes are essentially the same in the oxidized and reduced enzyme (see Figure 1). This outcome is surprising at first glance, and indeed cannot be accounted for by the simple single-state resonance enhancement expression as commonly used:

$$\text{intensity} \propto \frac{\nu_0^4 \epsilon^2}{[(\nu_e - \nu_0)^2 + \Gamma^2]^2} \quad (1)$$

This equation in various forms has often been used to predict RR intensities in resonance with a single, strong, and isolated electronic transition such as the Soret band (Babcock & Salmeen, 1979; Albrecht & Hutley, 1971; Nafie et al., 1976; Strekas et al., 1973). In this equation, ν_0 is the laser frequency, ν_e is the frequency of the resonant electronic transition, Γ is the damping factor associated with the electronic transition (commonly taken to be the half-width at half-maximum for a structureless absorption band), and ϵ is the extinction coefficient. Equation 1 assumes that the absolute frequency of the Raman scattering, ν_r, is insignificantly different from ν_0, and, therefore, $\nu_e - \nu_0$ is approximately equal to $\nu_e - \nu_r$. This approximation fails when ν_0 is within a few Raman shifts ($\Delta\nu = \nu_0 - \nu_r$) of ν_e and is particularly poor in the present case wherein ν_0 is greater than, but close to, ν_e. A more general single-state resonanc expression can be derived from eq 5 of Nafie et al. (1976), viz.:

$$\text{intensity} \propto \frac{\nu_r^4 \epsilon^2}{[(\nu_e - \nu_0)(\nu_e - \nu_r) + \Gamma^2]^2 + (\Gamma\Delta\nu)^2} \quad (2)$$

which correctly predicts the present observation that the Raman scattering intensities of oxidized and reduced cytochrome oxidase are nearly equal when 413.1-nm excitation is employed.[2] An amusing consequence of eq 2 arises when the laser frequency, ν_0, and the Raman frequency, ν_r, are equally spaced on either side of the electronic transition frequency by an amount equal to Γ; then the damped resonant

[2] Resonance Raman intensity calculations (eq 2) for oxidized and reduced derivative of cytochromes *a* and a_3 employ the electronic transition frequencies (ν_e) and damping factors (Γ) determined by Vanneste (1966).

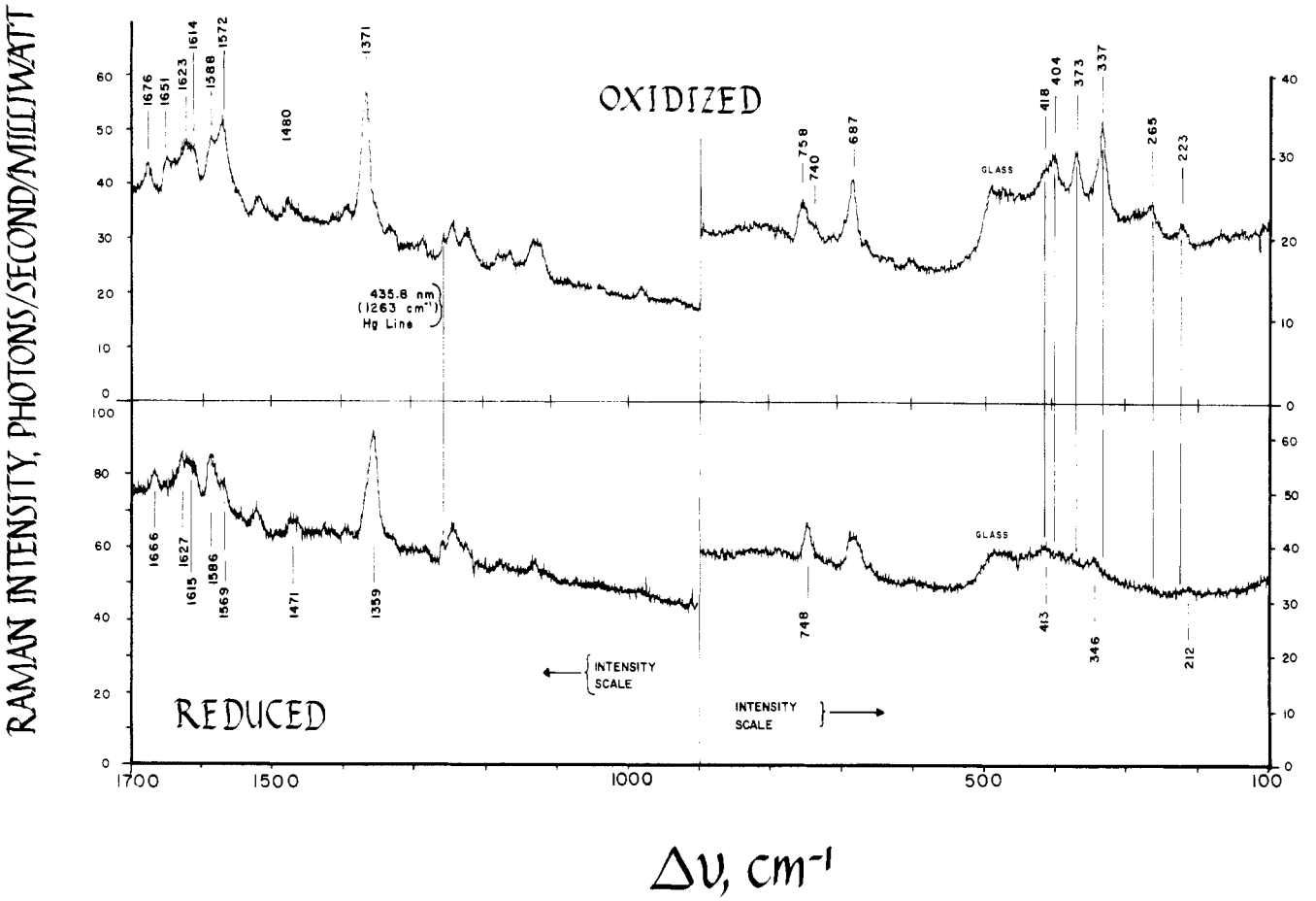

FIGURE 1: Resonance Raman spectra of oxidized (unliganded) and reduced cytochrome oxidase obtained by using 413.1-nm excitation. The intensity scales are correctly normalized for laser power and photon-count interval, and the zero of each intensity scale is the true zero of the signal. The spectral region below 900 cm^{-1} was recorded by using a 3-s photon-count interval as opposed to 2 s for the region above 900 cm^{-1}. The adventitious mercury emission line at 1263 cm^{-1} is the 435.8-nm line of the fluorescent room lighting, providing a positive frequency reference.

term in the denominator vanishes, and the quotient is saved from becoming infinite only by the "fraternity term" $\Gamma\Delta\nu$.

The low-frequency region of the oxidized enzyme shows several prominent peaks below 500 cm^{-1} while this region in the reduced protein shows only weak features. Equation 2, which correctly predicts the relative intensities of high-frequency heme modes in oxidized and reduced cytochrome oxidase, fails to account for the virtual disappearance of low-frequency peaks in the (413.1 nm excited) reduced spectrum. Thus, for example, eq 2 predicts that a 300-cm^{-1} mode of heme *a* or a_3 should be approximately one-third as intense in reduced oxidase as *the analogous mode* in oxidized enzyme. The observed intensity difference is at least a factor of ten, comparing the most intense low-frequency modes in the oxidized and reduced spectra. This apparent inconsistency may be explained if the low-frequency modes of the oxidized enzyme are presumed to be either absent, Raman inactive, or decoupled from the resonant chromophore(s) in the reduced form.

Figure 2 shows the spectra of oxidized (unliganded) cytochrome oxidase excited by 406.7- and 413.1-nm laser lines, and the spectrum of the oxidized cyano derivative excited at 413.1 nm. The spectrum of the oxidized formate derivative (inset, Figure 2) differs from the unliganded spectra only in that the modes which we ascribe to the a_3 heme (vide infra) are fractionally more intense.

A comparison of the unliganded oxidase spectra recorded at 406.7 and 413.1 nm reveals that the intensities of some modes are nearly independent of laser wavelength whereas others are considerably weaker at 406.7 nm than at 413.1 nm. Thus, selective Raman intensity enhancement clearly occurs between these two excitation wavelengths. For example, the shoulder at 1588 cm^{-1} shows approximately one-half intensity at 406.7 nm compared to 413.1 nm, and the peaks below 500 cm^{-1} are approximately one-third less intense at 406.7 nm than at 413.1 nm. The doublet at 1612–1624 cm^{-1} also shows different intensities between 406.7 and 413.1 nm, but this feature is not observed in the oxidized preparations of Ondrias & Babcock (1980) (vide infra). Since no identifiable vibronic sideband or other transition of comparable intensity appears in conjunction with or near the Soret transition in the electronic spectra of cytochrome oxidase or *a*-type hemes, vibronic coupling (Spiro & Strekas, 1972) and interference effects (Nafie et al., 1976) are ruled out as probable sources of selective resonance Raman enhancement. It appears clear that the selective enhancement is due to different resonance conditions for cytochromes *a* and a_3 caused by the different Soret peak positions and extinction coefficients of the two hemes in oxidized oxidase. Equation 2 should adequately describe this selective enhancement mechanism because, in the absence of heme–heme electronic interactions, cytochrome *a* vibrations will be enhanced only by the cytochrome *a* Soret and a_3 vibrations only by the a_3 Soret transitions. Therefore, the Raman intensity patterns at 406.7 and 413.1 nm, in addition to previously published 441.6-nm data (Babcock & Salmeen, 1979), can serve to identify Raman modes that are uniquely associated with cytochrome *a* or a_3, or are common to both.

The only previous report of the RRS of fully oxidized cytochrome oxidase obtained by using near-Soret excitation (Babcock & Salmeen, 1979) employed the 441.6-nm line of the helium–cadmium laser. This line is near the maximum

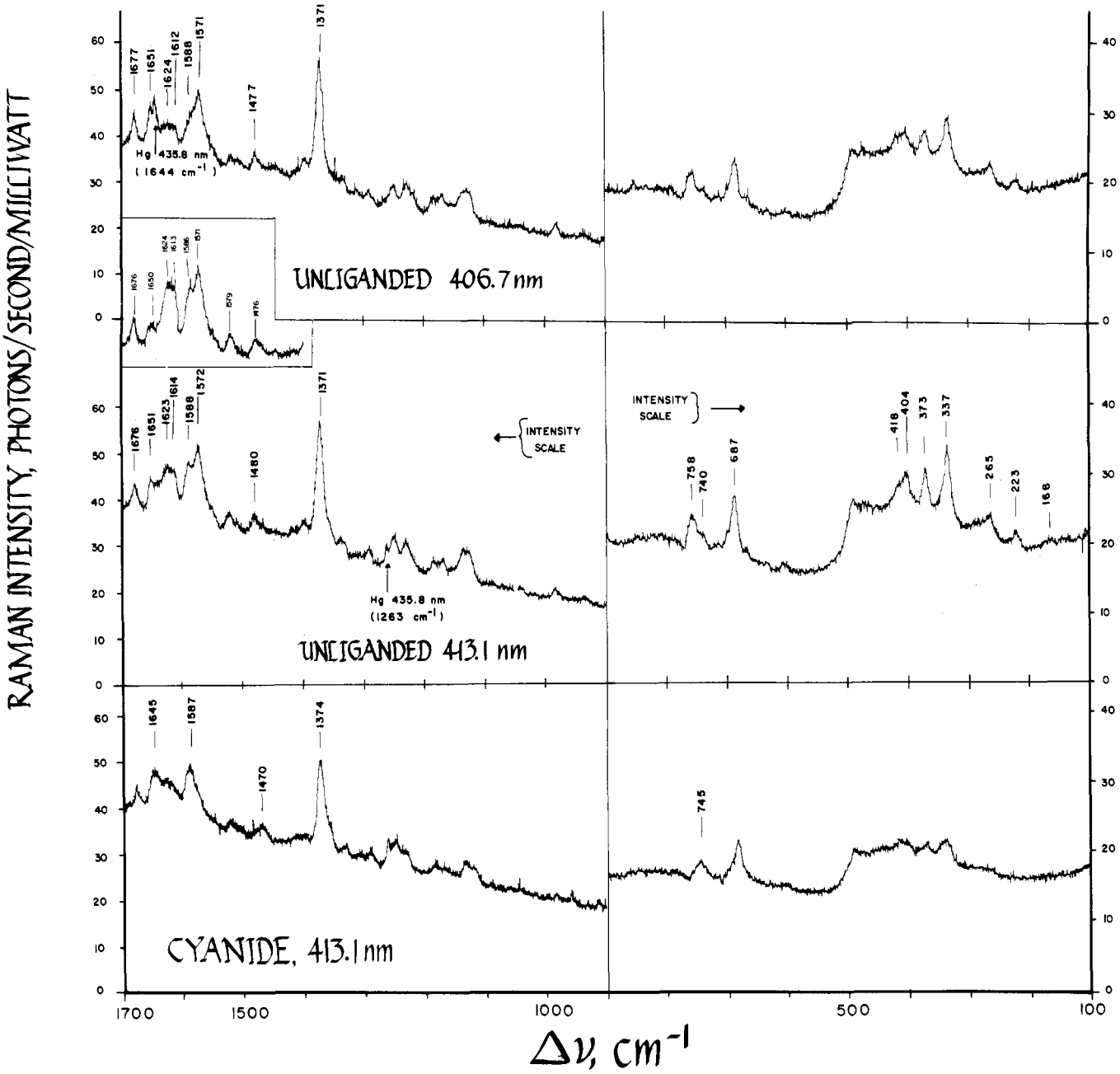

FIGURE 2: Resonance Raman spectra of oxidized, unliganded cytochrome oxidase obtained by using 413.1- and 406.7-nm excitation (middle and top full spectra, respectively). The spectra of the cyanide (bottom spectrum) and formate (inset) inhibitor complexes of the oxidized enzyme using 413.1-nm excitation are also shown. General statements regarding intensity scales and acquisition conditions are the same as for Figure 1. In the 406.7-nm-excited spectrum, the 435.8-nm mercury emission line appears at 1644 cm^{-1}.

of the *reduced* oxidase Soret (443 nm), but somewhat remote from resonance with the *a* component of the oxidized Soret (427 nm) and even farther removed from the a_3 Soret maximum (414 nm). Equation 2 clearly predicts that cytochrome *a* peaks of oxidized oxidase will dominate the RR spectrum under the 441.6-nm excitation condition, retaining one-third of the intensity that they exhibit when 413.1-nm excitation is employed, while the a_3 modes will be a factor of thirty less intense than with 413.1-nm excitation. We may thus safely ascribe any heme modes in the RR spectrum of oxidized cytochrome oxidase to cytochrome *a*, under the 441.6-nm excitation condition, in agreement with Babcock & Salmeen (1979).

In the previous report, three major peaks were observed in the high-frequency region at 1374, 1597, and 1651 cm^{-1}. Two or three weaker features with poorly reproducible peak positions were also reported. Our results with 413.1-nm excitation agree precisely as to the position of the oxidation state marker band at 1374 cm^{-1}, making the correct comparison between (low-spin) cytochrome *a* resonance observed with 441.6-nm excitation and the low-spin cyanide derivative of oxidized oxidase in our present experiments. The two higher frequencies reported previously, however, appear at first glance to have no counterparts in our spectra. Instead, we observe our strongest high-frequency peaks at 1587 and 1645 cm^{-1}, lower than the previous values by 10 and 6 cm^{-1}, respectively. In an earlier version of this paper, arguments based upon results from several laboratories led us to suggest that this discrepancy was due to a systematic error of +7 (±1) cm^{-1} (for frequencies above 1400 cm^{-1}) in the spectrometer used in the previous study (Babcock & Salmeen, 1979). This conclusion has recently been confirmed (G. T. Babcock, unpublished experiments). Applying this correction of 7 cm^{-1} to the reported frequencies for oxidized oxidase, one obtains frequencies of

Table I: Indicator Band Frequencies (cm^{-1}) of Oxidized Cytochrome Oxidase Compared to Protoheme-Containing Proteins

band designation (see text)	resting cyt a_3 (high spin)[a]	aquomet-Hb (high spin, 6-coordinate)[b]	resting HRP (high spin, 5-coordinate)[c]	cyt a and cyt a_3·CN- (low spin)[a]	met-Hb cyanide (low spin)[c]
I	1371	1373	1375	1374	1374
II	1480	1481	1495	1480	1508
III	*d*	1565	1565	*d*	1564
IV	1572	1561	1572	1588	1588
V	1651	1610	1632	1645	1642
VI	1676	*e*	*e*	1676[f]	*e*

[a] Present work. [b] Spiro et al. (1979). [c] Spiro & Burke (1976). [d] Not observed in cytochrome oxidase. [e] Not observed in protoheme proteins. [f] Cytochrome a_3 only (Salmeen et al., 1978).

Table II: Indicator Band Frequencies (cm^{-1}) of Reduced Cytochrome Oxidase Compared to Protoheme-Containing Proteins

band designation (see text)	cyt a_3 (high spin)[a]	deoxy-Hb (high spin)[b]	cyt a (low spin)[a]	ferro-cyt c (low spin)[b]
I	1359	1358	1359	1362
II	1471	1473	1471	1493
III	*c*	*d*	*c*	1548
IV	1569	1552	1586	1584
V	(~1620)[e]	1607	(~1620)[e]	1620
VI	1666	*f*	*f*	*f*

[a] Present work. [b] Spiro et al. (1979). [c] Not observed in cytochrome oxidase. [d] Observed by band IV. [e] Estimated position. Peak obscured in cytochrome oxidase. [f] Cytochrome a_3 peak only (Salmeen et al., 1978).

1590 and 1644 cm^{-1} for the nonconforming values. These are in satisfactory agreement with the frequencies that we observe in the oxidized cyanide derivative, viz., 1587 and 1645 cm^{-1}.

All of the RR peaks observed in the cytochrome oxidase spectra excited at 413.1 nm are polarized ($\rho \approx 0.3$), as is the case with most reported heme a modes regardless of excitation wavelength (Kitagawa et al., 1975). Thus, the polarization information which is so useful in correlating heme vibrations among various derivatives in other heme proteins is absent in cytochrome oxidase. This may be because heme a lacks the strict 2-fold equivalency of opposite pairs of pyrroles, necessary for electronic degeneracy of the π systems along the x and y in-plane axes containing the nitrogen atoms. Under this conditions, the α and Soret bands of heme a have nondegenerate transition symmetry rather than E_u symmetry as is the case with protoheme and mesoheme. In this nondegenerate situation, only symmetric vibrations are resonance enhanced. Thus, the correspondence between the structure-sensitive bands identified in this paper and the previously identified protoheme and mesoheme "indicator bands" (Spiro et al., 1979) is not necessarily rigorous, except for the modes that are symmetric in all of the heme systems (viz., the 13XX-cm^{-1} oxidation-state marker). Regardless of this caveat, however, there are several self-consistent indicator bands that can be identified for cytochrome oxidase, and some useful structural information can be extracted even at this preliminary stage.

Two oxidation-state indicators can readily be identified. One is the "traditional" heme protein oxidation-state marker which was previously identified for cytochrome oxidase (Adar & Yonetani, 1978; Babcock & Salmeen, 1979), occurring at 1359 cm^{-1} in the fully reduced enzyme and at 1371 cm^{-1} in the unliganded oxidized preparation. We note here that, in contrast to previous reports (Babcock & Salmeen, 1979; Adar & Erecinska, 1979), we only encountered minimal difficulty with our preparations undergoing photoreduction in the laser beam. With the position and line shape of the 1371-cm^{-1} peak as the criterion for oxidation state, photoreduction was less than 10% in all cases.[3] Indeed, we encountered some difficulty in maintaining the dithionite-reduced preparation in its fully reduced state, as evidenced by the slight broadening on the high-frequency side of the 1359-cm^{-1} peak in Figure 1, lower trace. The other peak whose frequency responds to oxidation state only is the highest frequency feature, which appears at 1676 cm^{-1} in the oxidized enzyme and at 1666 cm^{-1} in the fully reduced protein. This feature has been assigned as the C=O stretch of the formyl group of cytochrome a_3 (Salmeen et al., 1978).

One RR feature in the high-frequency region exhibits frequency shifts which are primarily sensitive to spin state. This is the doublet that appears at 1588–1572 cm^{-1} in the unliganded oxidized enzyme spectrum. Upon reduction, the doublet remains although the relative intensities change and both peaks shift to slightly lower frequency (1586–1569 cm^{-1}). The 1572-cm^{-1} peak disappears, however, when the low-spin oxidized cytochrome a_3 cyanide adduct is formed. In addition, the previously reported oxidized enzyme spectra (which show only low-spin cytochrome a peaks due to 441.6-nm excitation) exhibit only one discernible peak in this frequency region at 1590 cm^{-1} (corrected frequency, vide supra). We assign the 1588–1572-cm^{-1} pair as the heme a analogues of "band IV" (Spiro et al., 1979), the spin state or (more correctly) core expansion marker peak (Spaulding et al., 1975; Spiro et al., 1979) of cytochromes a and a_3, respectively.

In addition to the indicator bands noted above, which respond only to oxidation state or to spin state, several additional peaks are sensitive to both oxidation and spin state (as in the case with other heme proteins). Thus, the 1651-cm^{-1} peak of the unliganded enzyme not only shifts to 1645 cm^{-1} in the cyano adduct, proving it to be a cytochrome a_3 spin-state marker, but also apparently disappears upon dithionite reduction. We assign this peak as the heme a equivalent of "band V" (Spiro et al., 1979), the highest frequency depolarized feature in most heme protein spectra. We suggest that

[3] Other workers (Ondrias & Babcock, 1980; G. T. Babcock, unpublished experiments) take the ratio of the intensities of the 1651-cm^{-1} peak to that of the 1620-cm^{-1} doublet as an indicator of the extent of photoreduction. This criterion yields significantly different conclusions regarding the fraction of enzyme reduced; i.e., the intensity of the "reduced" 1620-cm^{-1} peak does not track with that of the 1359-cm^{-1} oxidation-state marker. Spectra obtained in our laboratories with a vidicon Raman spectrograph show the intensity of the 1620-cm^{-1} doublet in our oxidized preparations essentially unchanged when laser exposure times for spectrum acquisition are reduced to 1.64 s. We find this fact, combined with the positions of the 1371- and 1676-cm^{-1} peaks, difficult to rationalize with the assignment of the 1620-cm^{-1} doublet as a photoreduction artifact. Moreover, recent results from our laboratories demonstrate that the 1620-cm^{-1} intensity is sensitive to the identity and concentration of the ionic strength medium (or internal Raman intensity reference) employed; i.e., we observe results essentially identical with those of Ondrias & Babcock (1980) when 0.1 M $NaClO_4$ is employed as a reference for Raman intensities. We also interpret this result as inconsistent with the assignment of the 1620-cm^{-1} doublet as a photoreduction artifact. The reasons for the variations in the 1620-cm^{-1} intensities are being investigated.

this peak apparently disappears in reduced oxidase because it shifts to a position under the borad feature centered at 1620 cm^{-1}.

The RR peak at 1480 cm^{-1} in resting oxidase is also sensitive to both spin state and oxidation state, shifting to 1471 cm^{-1} in reduced oxidase and to 1470 cm^{-1} in the cyanide adduct. The oxidation-state shift is analogous to that of "band II" in other heme proteins (Spiro et al., 1979), but the spin-state shift (as with the 1651-cm^{-1} peak) is opposite in direction and smaller in magnitude than the usual observation for heme proteins.

Our interpretations of the high-frequency RR indicator bands in cytochrome oxidase are summarized in Tables I and II. In general, the indicator bands may be correlated with those previously determined (Spiro & Burke, 1976; Spaulding et al., 1975; Spiro et al., 1979) for other heme proteins. Exceptions are "band III", the depolarized oxidation-state marker near 1570 cm^{-1} for which we find no analogue in cytochrome oxidase, and the peak which we designate "band VI", the oxidation-state marker near 1670 cm^{-1} which may be unique to *a*-type hemes. The general phenomenology of the cytochrome a_3 RR frequencies in *resting* oxidase is similar to the "anomalous" 5-coordinate iron(III) heme proteins, resting horseradish peroxidase and cytochrome *c'* (although the cytochrome a_3 band V is higher in frequency and band II lower by 15–20 cm^{-1}). The oxidation-state shifts of all bands (except band IV of cytochrome a_3, vide supra) and the spin-state shifts of band II are similar to the previously observed behavior of heme proteins. The spin-state shifts of bands II and V, however, are opposite to the usual direction. Thus, cytochrome a_3 represents a third class of heme protein with regard to position and shifts of high-frequency indicator bands, in addition to the two previously identified classes, hemoglobin/myoglobin and HRP/cytochrome *c'*.

We may now identify reasonable alternatives as to the heme structures which are associated with the spin states of oxidized cytochrome oxidase. The direct, linear relationship between the frequency of band IV and the metalloporphyrin center-to-nitrogen distance, Ct–N, the averave distance between the center of the plane bounded by the four pyrrole nitrogen atoms and the nitrogen atoms themselves, is now established beyond question for all metalloporphyrins (having methine proteins) that have been examined so far (Spaulding et al., 1975; Spiro et al., 1979; Huong & Pommier, 1977). It is reasonable to suppose that a similar, self-consistent relationship exists between and "band IV" frequencies and the Ct–N distances of heme *a*. If the polarized "band IV" of heme *a* is indeed the same molecular motion as the anomalously polarized band IV of other heme proteins, then Ct–N vs. band IV correlations for heme *a* should be colinear with those of other heme systems. If not, then the proportionality between band IV frequency and Ct–N distance may be different for heme *a* than for other hemes. The close correspondence of the low-spin heme frequencies (1588 cm^{-1} for both oxidized cytochrome *a* and cyanomethemoglobin; 1586 cm^{-1} for reduced cytochrome *a* vs. 1584 cm^{-1} for ferrocytochrome *c*) suggests the former situation. However, lack of correspondence of the high-spin frequencies (1572 cm^{-1} for cytochrome a_3 in resting oxidase vs. 1562 cm^{-1} for aquomethemogloin; 1569 cm^{-1} for cytochrome a_3 in reduced oxidase vs. 1556 cm^{-1} for deoxyhemoglobin) suggests the latter. The dilemma can be resolved in one of two ways, by supposing that the structures of high-spin cytochrome a_3 in both oxidation states are anomalous, with a Ct–N distance considerably smaller than that of representative heme proteins, or by supposing that the high-spin forms of cytochrome a_3 have normal structures and that the band IV frequency dependence upon Ct–N distance is steeper for heme *a* than for other hemes. The former case suggests a 5-coordinate geometry for the heme of cytochrome a_3 in resting oxidase, similar to the proposal (Spiro et al., 1979) for resting HRP and cytochrome *c'*. However, recent model heme *a* studies (G. T. Babcock, unpublished experiments) suggest that the latter alternative is correct. The data in this work do not allow choice between these two alternatives. The oxidation- and spin-state indicator frequencies of the resting enzyme do, however, appear inconsistent (Sievers et al., 1979) with the formulation of resting cytochrome a_3 as an iron(IV) heme species (Seiter & Angelos, 1980).

The 100–1000-cm^{-1} region of the RR spectrum of resting oxidase contains an impressive number of strong features (Figure 1). The 758- and 687-cm^{-1} peaks are observed at similar frequencies in all metalloporphyrin spectra. In cytochrome oxidase, the 758-cm^{-1} peak shifts in response to changes in oxidation state and spin state, suggesting that it is a cytochrome a_3 mode. The corresponding cytochrome *a* mode may be the shoulder at 740 cm^{-1} in resting oxidase. If so, this is one mode that differs in frequency between low-spin cytochrome a_3 and cytochrome *a* because the position of the somewhat broadened peak in the cyanide adduct is at 745 cm^{-1} rather than at 740 cm^{-1}.

The lower frequency region (<500 cm^{-1}) sits atop a broad base-line excursion which is due to the Suprasil cell window material of the observation cuvettes. With the exception of the peak at 265 cm^{-1}, none of the oxidized oxidase frequencies below 500 cm^{-1} were observed in previous reports with 600.0- (Bocian et al., 1979) or 441.6-nm (Babcock & Salmeen, 1979) excitation. The enhancement of these modes is therefore unique for our present excitation condition. From this, we may conclude from RR intensity arguments that the modes below 500 cm^{-1} are vibrations of, or are coupled to, the cytochrome a_3 chromophore.

In contrast to their intensity in resting oxidase, the low-frequency peaks essentially disappear in reduced oxidase and have sharply reduced intensity in the cyanide adduct. This effect might be radionalized with respect to the resting → cyanide transition by presuming that the peaks in question represent out-of-plane normal modes which have appreciable Raman intensity in nonplanar, high-spin heme *a* but are weak or Raman inactive in the planar, low-spin case. This does not, however, explain the behavior upon reduction because the a_3 heme is high spin and nonplanar (as evidenced by the band IV positions) in both oxidized and reduced oxidase. Moreover, calculations based upon eq 2 predict reduction in intensity of low-frequency cytochrome a_3 modes from oxidized to reduced oxidase, but only a factor of three rather than the observed factor of at least ten. Note that, as predicted by eq 2, high-frequency heme modes are equally intense in oxidized and reduced oxidase. It is unexpected that heme modes in a given frequency range follow eq 2 while other heme modes differing only in frequency do not. A possible explanation is that the low-frequency modes in resting oxidase are not heme modes, but instead are associated with axial groups bound to the iron. If this is the case, the observed modes may include the bridge between the a_3 heme and Cu_u^{2+}. The low intensity of the low-frequency modes of the cyanide adduct and their disappearance upon reduction may then be related to the weaker axial electronic interactions (which are reflected in the small antiferromagnetic coupling constant for the a_3^{3+}–Cu_u^{2+} interaction) in low-spin cytochrome a_3 (Tweedle et al., 1978), and the absence of such interactions in the reduced enzyme.

The RR data at present do not allow a unique choice between failure of the single-state resonance expression (eq 2) and enhancement of nonheme (e.g., Cu_u^{2+} associated) modes as an explanation of the low-frequency intensity patterns. It is noteworthy that the low-frequency region of the oxidized cytochrome oxidase RR spectrum (Figure 1) bears a striking qualitative resemblance to the spectra of simple blue (type I) copper proteins such as azurin and stellacyanin, and multicopper oxidases such as laccase (Ferris et al., 1979; Siiman et al., 1976; Miskowski et al., 1975). However, some similarity also exists between the cytochrome oxidase spectra and those of other heme proteins excited at 413.1 nm (unpublished data).

We have performed isotope labeling experiments in an effort to identify axial cytochrome a_3 vibrations. Dissolution of a sedimented resting oxidase preparation in 90% $H_2{}^{18}O$ followed by 16-h incubation at 0 °C produced no detectable changes in RR frequencies. (The $H_2{}^{18}O$ and $H_2{}^{16}O$ spectra were compared by digital subtraction of the spectra by using a microprocessor-controlled recorder.) This result is consistent with (but does not prove) the absence of a $a_3{}^{3+}$–O^{2-}–$Cu_u{}^{2+}$ bridge. Formation of the cyanide adduct with $^{13}CN^-$ likewise failed to reveal an isotope shift which would allow us to identify the Fe–C stretching vibration.

Under our spectral acquisition conditions, we observe minimal (<10%) photoreduction of cytochrome oxidase, using the oxidation-state marker as our criterion. Therefore, the extensive photoreduction observed by previous workers is a preparation-dependent phenomenon. Our comparative studies of resting, reduced, and liganded cytochrome oxidase preparations have allowed identification of RR peaks analogous to four of the five principal indicator bands that have previously been identified as having particular sensitivity to heme core size and oxidation or spin state of the iron atom in all heme proteins so far examined. A sixth indicator band that may be unique to *a*-type hemes has been identified. The indicator band positions of cytochrome a_3 in resting oxidase are more similar to resting horseradish peroxidase and oxidized cytochrome *c'* than to aquomethemoglobin or -myoglobin. The analogy to HRP and cytochrome *c'* is not complete, however, when detailed indicator band positions and shifts are examined. On balance, cytochrome oxidase hemes, especially the a_3 heme, appear at present unique in their high-frequency RR spectrum. In the low-frequency region, resting oxidase exhibits peak positions and intensity patterns which have not been reported in any other heme protein to date (including resting cytochrome oxidase itself under RR excitation at 441.6 nm or longer wavelengths). This unusual low-frequency spectrum may be due to the direct resonance with the $a_3{}^{2+}$ heme which is provided by the 413.1-nm krypton laser line, or it may be that vibrations of nonheme axial structures perhaps involving the iron–copper bridge and the $Cu_u{}^{2+}$ site are observed. Wavelength-dependence studies aimed at clarifying these possibilities are in progress in our laboratories, as are studies of stable intermediate oxidation states of cytochrome oxidase and transients in the cytochrome oxidase electron-transfer sequence.

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