Resonance Raman Spectra of Cytochrome *c* Oxidase. Excitation in the 600-nm Region†

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ABSTRACT: The resonance Raman (RR) spectra of oxidized, reduced, and oxidized cyanide-bound cytochrome *c* oxidase with excitation at several wavelengths in the 600-nm region are presented. No evidence is found for laser-induced photoreduction of the oxidized protein with irradiation at $\lambda \sim 600$ nm at 195 K, in contrast to the predominance of this process upon irradiation in the Soret region at this temperature. The Raman spectra of all three protein species are very similar, and there are no Raman bands which are readily assignable to either cytochrome *a* or cytochrome a_3, exclusively. The Raman spectra of the three protein species do, however, exhibit a number of bands not observed in the RR spectra of other hemoproteins upon excitation in their visible absorption bands. In particular, strong Raman bands are observed in the low-frequency region of the RR spectra (<500 cm^{-1}). The frequencies of these bands are similar to those of the copper–ligand vibrations observed in the RR spectra of type 1 copper proteins upon excitation in the 600-nm absorption band characteristic of these proteins. In cytochrome *c* oxidase, these bands do not disappear upon reduction of the protein and, therefore, cannot be attributed to copper–ligand vibrations. Thus, all the observed RR bands are associated with the two heme A moieties in the enzyme.

Membrane-bound mitochondrial cytochrome *c* oxidase catalyzes the four-electron reduction of molecular oxygen to water. The functioning enzyme contains two copper and two iron atoms per protein molecule, the latter in the form of heme A. All four metal centers have been shown to be spectroscopically and functionally nonequivalent (Malmström, 1973; Nicholls & Chance, 1974; Babcock et al., 1976; Falk et al., 1977). The detailed role of the four metal centers in cytochrome *c* oxidase in the electron transfer process is currently being actively pursued. One way to ascertain the pathway of electrons through the protein is to follow the transfer in the time domain. It is now becoming evident that it is necessary to improve the characterization of the optical spectrum of the protein before time-resolved methods can be exploited. The optical spectrum of the enzyme is dominated by the two cytochromes and exhibits many of the overt features which are typical of the spectra of other hemoproteins (Smith & Williams, 1970). There are some distinct differences, however, arising principally from the conjugation of the 8-formyl substituent, which is characteristic of heme A, into the π-electron system of the porphyrin. The conjugation of the unsaturated substituent results in a shift to lower energy of both the Soret (420 nm) and visible [α (600 nm) and β (560 nm)] bands of cytochromes *a* and a_3, as well as an increase in the intensity of the visible bands (especially the α band) relative to that of the Soret band. The relative effects of the formyl-group conjugation on the spectra of the two chromophores cannot be readily ascertained, however, since the severe overlap of the cytochrome *a* and a_3 bands precludes a straightforward interpretation of the spectrum.

An additional complication in the assignment of the 600-nm absorption band of cytochrome *c* oxidase is the possible contribution in this region of ligand–metal charge transfer transitions associated with one or both of the copper centers in the enzyme. It has been suggested (Gibson & Greenwood, 1965) that there is an absorption due to Cu_a buried under the α bands of cytochromes *a* and a_3 and that this copper transition undergoes changes in intensity upon changes in the redox state of the protein. Recently, it has also been suggested that Cu_{a_3} in cytochrome *c* oxidase might be a type 1 copper center (Powers et al., 1979). If so, it would be expected to make a significant contribution to the absorption spectrum of cytochrome *c* oxidase in the 600-nm region, since type 1 copper proteins are characterized by an intense absorption band at this wavelength (Solomon et al., 1976).

It is evident that the potential contribution of all four metal centers in cytochrome *c* oxidase to the 600-nm region of the absorption spectrum makes a detailed assignment of this region exceedingly difficult. However, it is possible to obtain a less ambiguous assignment of the visible absorption bands of cytochrome *c* oxidase with RR[1] spectroscopy. Resonance-enhanced Raman scattering has been used extensively for the study of other hemoproteins and porphyrin systems, and the observed spectra have been successfully interpreted in terms of the interaction of the normal vibrations of the porphyrin ring with its π-electron system (Spiro, 1975; Spiro & Gaber, 1977; Warshel, 1977). RR spectroscopy has also been used to study the metal centers of various nonheme metalloprotein systems (Spiro & Gaber, 1977). In particular, the RR spectra of a number of type 1 copper proteins have been obtained by excitation of the intense 600-nm absorption band characteristic of these systems (Siiman et al., 1976; Miskowski et al., 1975). Most of the resonance-enhanced vibrational modes observed in the RR spectra of the type 1 copper proteins occur at low frequencies (<500 cm^{-1}) and have been attributed to copper–ligand vibrations. It is known that the 600-nm absorption band of the type 1 copper proteins disappears upon reduction of the protein with a concomitant disappearance of the low-frequency resonance-enhanced vibrational modes. Thus,

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[1] Abbreviations used: RR, resonance Raman; EPR, electron paramagnetic resonance.

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comparison of the RR spectra of oxidized and reduced cytochrome *c* oxidase should allow an assessment of the contribution of copper-associated absorption bands to the 600-nm region of the system.

In this report, we present the results of RR studies of oxidized, reduced, and oxidized cyanide-bound cytochrome *c* oxidase with excitation wavelengths ranging from 568.2 to 615 nm and at 457.9 nm. RR spectra of cytochrome *c* oxidase have been previously reported (Nafie et al., 1973; Salmeen et al., 1973, 1978; Adar & Yonetani, 1978), but each of these studies was performed with excitation in the Soret region and only at a single excitation wavelength. We first present the RR spectra obtained for the various states of the protein and discuss the differences in these spectra. We confirm the observation of previous investigators that laser irradiation of cytochrome *c* oxidase in the Soret region results in photoreduction of the enzyme (Adar & Yonetani, 1978; Salmeen et al., 1978). However, EPR studies undertaken in conjunction with our RR experiments demonstrate that the photoreduction mechanism is suppressed when the wavelength of irradiation is in the 600-nm region, particularly at low temperatures. Finally, we discuss the specific assignment of individual Raman bands observed with excitation in the 600-nm region.

Materials and Methods

Beef heart cytochrome *c* oxidase was isolated by the procedures of Yu et al. (1975) or Hartzell & Beinert (1974). The protein was solubilized in 0.5% cholate–50 mM phosphate buffer (pH 7.4) and stored at 190 K until use. The preparations contained 9–11 nmol of heme A per mg of protein as measured by the pyridine hemochromagen assay (Takemori & King, 1965). The protein concentration used for all the RR experiments was 38 mg/mL as determined by the method of Lowry et al. (1951).

Reduced cytochrome *c* oxidase was prepared by adding an excess of sodium dithionite dissolved in 5–10 μL of buffer to the protein sample contained in the 1-mm path length Raman cuvette. Prior to the addition of the sodium dithionite, the sample was flushed with argon and allowed to equilibrate for 20 min at 4 °C in a glove bag containing an argon atmosphere. The protein sample was then transferred to the Raman cuvette (within the glove bag), and the dithionite was added. The Raman cuvettes were fitted with ground glass stoppers to prevent the reentry of oxygen upon removal of the sample from the glove bag. The cyanide complex of oxidized cytochrome *c* oxidase was prepared by adding an excess of potassium cyanide dissolved in 5–10 μL of buffer to the protein sample contained in the Raman cuvette. For both the reduced and cyanide-bound samples, the protein was allowed to equilibrate at 4 °C for 20 min after the addition of the dithionite or cyanide, before the Raman experiments were initiated. After all of the RR spectra had been obtained for the reduced protein, the sample was removed from the spectrometer and diluted, and the optical absorption spectrum was recorded on a Cary 14 spectrophotometer. This spectrum confirmed that the protein was reduced. In the case of the oxidized cyanide-bound protein, an optical absorption spectrum obtained after the completion of the RR experiments on this sample also confirmed the ligation state of the protein.

RR spectra were obtained on a custom built Raman spectrometer in the laboratory of Professor Aaron Lewis at Cornell University. The details of the construction of the spectrometer are described elsewhere (Perreault et al., 1976). The excitation wavelengths between 580 and 615 nm were obtained with a Coherent Radiation Model 490 tunable dye laser with Rhodamine 6 G as the dye. The dye was pumped with a Coherent Radiation Model CR-12 argon ion laser operating in the all-lines mode. The wavelength of the dye laser radiation was determined to the nearest 0.1 nm by using a prototype Tropel digital wavemeter. Excitation at 457.9 nm was also obtained with the Model CR-12 argon ion laser while excitation at 568.2 nm was obtained with a Coherent Radiation Model CR-2 krypton ion laser. The incident laser power at the sample was 100–240 mW for all spectra recorded by using the krypton ion or dye lasers and approximately 50 mW for the spectra recorded with excitation at 457.9 nm. The exact laser powers used in obtaining spectra of the samples at various wavelengths are given in the legends of the appropriate figures.

RR spectra at all excitation wavelengths were obtained with the optics in a backscattering (180°) configuration where the incident beam was polarized perpendicular to the scattering plane. The scattered radiation was collected with no polarization analyzer in the scattered beam unless otherwise stated. RR spectra recorded with excitation wavelengths between 580 and 615 nm and at 568.2 nm were obtained by using photon counting at 2-cm^{-1} intervals with a nominal counting time of 30 s/channel. For the RR spectra recorded with 457.9-nm excitation, the counting time was reduced to 10 s/channel, while the counting interval was maintained at 2 cm^{-1}. The spectral slit width for all RR spectra was approximately 3 cm^{-1}.

The long counting times necessary to obtain most of the spectra resulted in a 6–8-h total data acquisition time for each spectrum (200–1650 cm^{-1}). Because of these long times, provisions were necessary to ensure a constant flux of incident radiation per channel and to maintain the integrity of the protein samples. A constant flux of incident radiation was maintained by means of a recycling integration system (G. Perreault, unpublished experiments) which monitored the laser radiation incident on the sample and adjusted the counting times such that the incident flux was constant for each 2-cm^{-1} frequency interval. To ensure the integrity of the protein samples over the long period of time necessary to acquire the data, all RR spectra were recorded at a temperature of 120 K. This temperature was maintained to within ±1 K during the recording of the spectra by means of a Lake Shore Cryotronics Spectrim Model 21 cryostat. In order to determine whether any sample deterioration occurred during the recording of the spectra, selected spectra were rerecorded without changing the focal point of the incident laser beam. Each of these spectra was found to be identical with that previously obtained.

In order to further investigate the possible deterioration of the protein in the laser beam through processes such as photoreduction, which are known to occur in cytochrome *c* oxidase (Adar & Yonetani, 1978; Salmeen et al., 1978), we obtained EPR spectra of the protein after laser irradiation. These studies were performed with irradiation at both 457.9 and 590 nm and at the temperatures 195 and 277 K. In all cases, the laser power densities, sample path lengths, and protein concentrations were identical with those used in the RR studies. The protein samples, contained in flat 1-mm path length quartz EPR tubes, were irradiated while immersed in either an ice water (277 K) or dry ice–acetone (195 K) bath. At various times, the sample was removed from the bath and immediately frozen at 77 K. Within 10 min after removal from the laser beam, the EPR spectrum of the sample was recorded at 12 K on a Varian E-line Century Series X-band spectrometer equipped with an Air Products Heli-Trans low-temperature system. After the EPR spectrum was recorded, the sample was returned to the immersion Dewar and irradiation was continued.

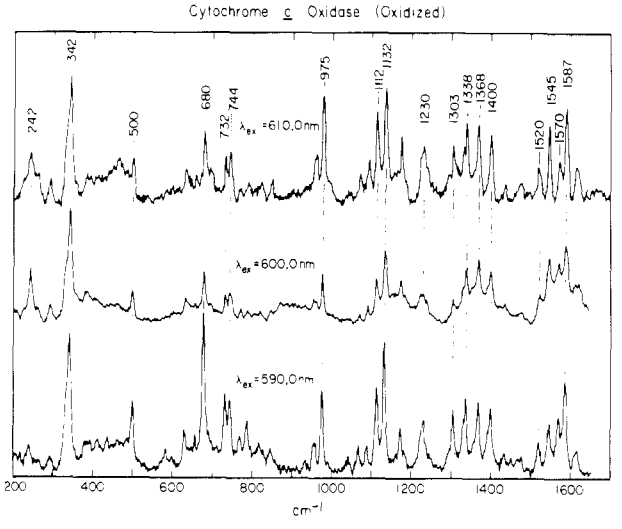

FIGURE 1: Resonance Raman spectra of oxidized cytochrome *c* oxidase obtained with excitation in the visible region of the absorption spectrum. The laser power at the sample was approximately 150 mW at λ_{ex} = 610.0 and 600.0 nm and 220 mW at λ_{ex} = 590.0 nm. The base lines of the Raman spectra of the oxidized protein shown in this figure, as well as those of the Raman spectra of the reduced (Figure 2) and cyanide-bound (Figure 3) species, have been corrected for background fluorescence. Spectral accumulation conditions used in acquiring the Raman spectra shown in Figures 1–3 are given in the text.

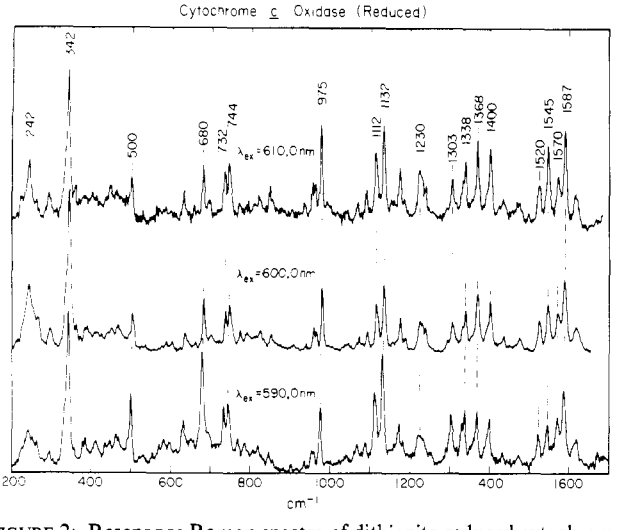

FIGURE 2: Resonance Raman spectra of dithionite-reduced cytochrome *c* oxidase obtained with excitation in the visible region of the absorption spectrum. The laser power at the sample was approximately 130 mW at λ_{ex} = 610 nm, 150 mW at λ_{ex} = 600.0 nm, and 170 mW at λ_{ex} = 590.0 nm.

The extent of photoreduction of the protein sample was determined by comparing the intensity of the low-spin cytochrome *a* EPR signal of the irradiated sample to that of the sample prior to irradiation. The cytochrome *a* EPR signal was integrated by the method of Aasa & Vänngård (1975) using the g = 3.0 component to determine the total area. During the course of photoreduction, a g = 6 EPR signal, characteristic of the partially reduced protein, appears. This signal was integrated by the method of Aasa et al. (1976), and its relative intensity was determined with respect to the low-spin cytochrome *a* signal of the nonirradiated sample.

Results

Our RR spectra of oxidized, reduced, and oxidized cyanide-bound cytochrome *c* oxidase with excitation at 590, 600, and 610 nm are shown in Figures 1–3, respectively. For the

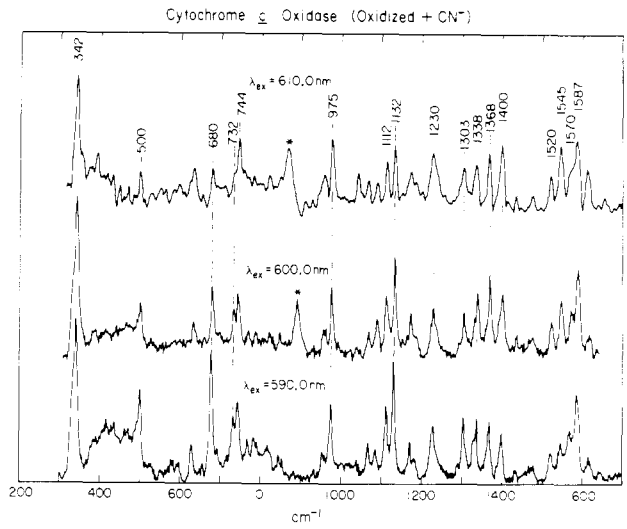

FIGURE 3: Resonance Raman spectra of oxidized cyanide-bound cytochrome *c* oxidase. The laser power at the sample was approximately 220 mW at λ_{ex} = 610.0 nm, 165 mW at λ_{ex} = 600.0 nm, and 240 mW at λ_{ex} = 590.0 nm. The peaks labeled by asterisks in the Raman spectra obtained with λ_{ex} = 610.0 and 600.0 nm were not reproducible and are probably not associated with Raman scattering from cytochrome *c* oxidase.

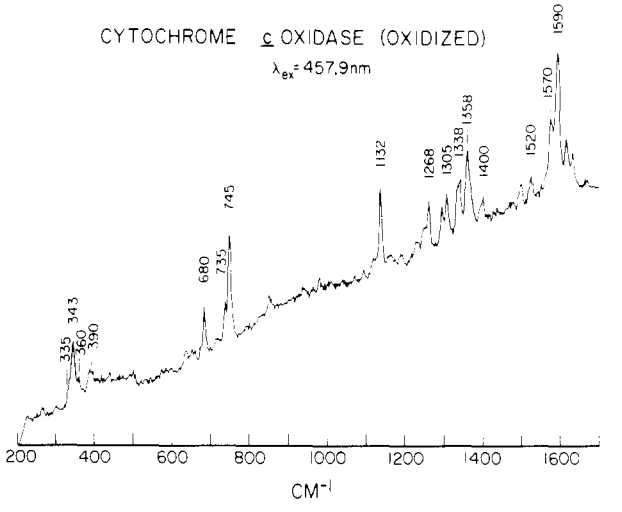

FIGURE 4: Resonance Raman spectrum of "oxidized" cytochrome *c* oxidase obtained with excitation in the Soret region of the absorption spectrum (λ_{ex} = 457.9 nm). The power at the sample was approximately 50 mW. Spectral accumulation conditions used in acquiring the spectrum are given in the text.

oxidized and oxidized cyanide-bound protein, RR spectra were also obtained at several additional excitation wavelengths in this region, but these spectra are similar to those shown in Figures 1 and 3 and are not reproduced here. For comparison, the RR spectrum of oxidized cytochrome *c* oxidase obtained with excitation at 457.9 nm is shown in Figure 4.

General Features of the RR Spectra Observed with Excitation in the 600-nm Region. The RR spectra observed with excitation in the 600-nm region of oxidized, reduced, and oxidized cyanide-bound cytochrome *c* oxidase from 600 to 1650 cm^{-1} are, in general, quite similar to those observed for other hemoproteins upon excitation in their visible bands (Strekas & Spiro, 1972a,b, 1973; Spiro & Strekas, 1972; Brunner et al., 1972; Spiro, 1975). The cytochrome *c* oxidase RR spectra do, however, exhibit several bands which are not observed in the spectra of other hemoproteins. For example, other hemoproteins, and metalloporphyrins in general, have a single Raman band at around 750 cm^{-1}, whereas the cytochrome *c* oxidase spectra contain a doublet at 732 and 744

cm^{-1}. There are also bands at 975, 1112, and 1520 cm^{-1} which are not observed in other hemoprotein RR spectra, while bands at 1570, 1545, 1400, 1172, and 960 cm^{-1} appear to undergo much larger resonance enhancements than do the analogous bands in other hemoprotein RR spectra.

The RR spectra of cytochrome *c* oxidase between 200 and 600 cm^{-1} differ dramatically from those of other hemoproteins excited in their visible bands. There are bands at 242 and 342 cm^{-1} in the cytochrome *c* oxidase RR spectra, which exhibit extremely large resonance enhancements as well as a relatively intense band at 500 cm^{-1}. Bands attributed to iron–nitrogen stretching modes have been observed in the RR spectra of other hemoproteins in the region between 200 and 400 cm^{-1} (Brunner & Sussner, 1973), but these bands do not exhibit nearly as large resonance enhancements as do the bands in the RR spectra of cytochrome *c* oxidase. The band observed at 500 cm^{-1} is also highly unusual, having no analogue in the RR spectra of other hemoproteins or metalloporphyrins in general.

Photoreduction of Cytochrome c Oxidase. In general, certain bands are observed in the RR spectra of hemoproteins which are sensitive to the oxidation state of the heme iron (Spiro, 1975). With excitation in the Soret, oxidized hemoproteins typically exhibit a strong, *polarized* band between 1375 and 1368 cm^{-1}, which characteristically shifts to around 1360 cm^{-1} upon reduction of the heme moiety. With excitation in the visible region, the only reliable oxidation-state marker is a Raman band observed around 1640 cm^{-1}, which shifts to approximately 1620 cm^{-1} upon reduction of the heme.

In two recent RR studies of cytochrome *c* oxidase obtained with excitation in the Soret region (Salmeen et al., 1978; Adar & Yonetani, 1978), it was reported that a doublet is observed in the spectrum of the oxidized protein at 1375 and 1360 cm^{-1}, with the relative intensity of these two bands being a function of the power of the incident laser radiation. At very low powers, the 1375-cm^{-1} component dominates, while at high powers, the 1360-cm^{-1} band is the more intense member of the doublet. Upon reduction of the protein with dithionite, only the 1360-cm^{-1} band is observed. On the basis of the observed power dependence of the relative intensities of the two bands, both Adar & Yonetani (1978) and Salmeen et al. (1978) have concluded that laser-induced photoreduction is occurring in cytochrome *c* oxidase with excitation in the Soret region.

The observation of a 1358-cm^{-1} band in our Soret-region RR spectrum of "oxidized" cytochrome *c* oxidase confirms that photoreduction is occurring in our sample upon irradiation at 457.9 nm with ~50 mW of incident laser power, even at the low temperature (120 K) at which our experiments were performed. This 1358-cm^{-1} band, however, exhibits shoulders at *both* 1368 and 1373 cm^{-1}. Upon dithionite reduction of the protein, the shoulder at 1373 cm^{-1} disappears, while the 1368-cm^{-1} shoulder remains (Figure 5). These observations indicate that the 1373-cm^{-1} band is characteristic of the oxidized protein, whereas the 1368-cm^{-1} band has a different origin from the 1358- and 1373-cm^{-1} oxidation state marker bands.

Our RR spectra of cytochrome *c* oxidase obtained with 600-nm excitation exhibit a single strong band at 1368 cm^{-1}. In contrast to the 1358- and 1373-cm^{-1} oxidation state marker bands which are *polarized* (Spiro, 1975), our 1368-cm^{-1} band is *depolarized* and does not shift upon reduction of the protein (compare Figures 1 and 2). Thus, the 1368-cm^{-1} band which we observe with 600-nm excitation is not an oxidation state marker and in all probability corresponds to the weak shoulder

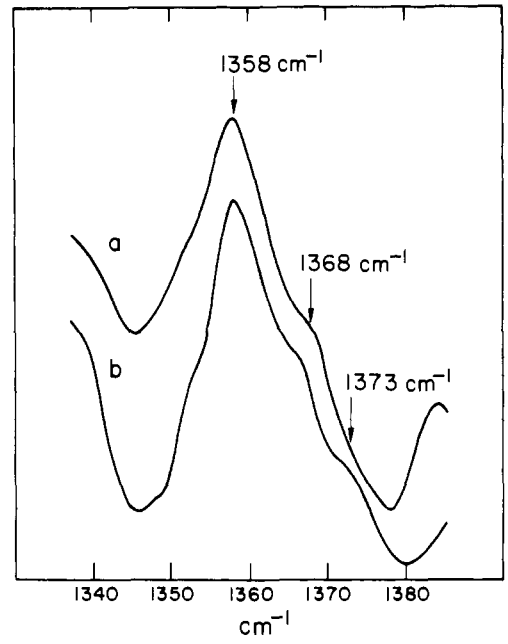

FIGURE 5: Resonance Raman spectra from 1340 to 1380 cm^{-1} of (a) dithionite-reduced and (b) "oxidized" cytochrome *c* oxidase obtained with λ_{ex} = 457.9 nm. The Raman spectrum of the "oxidized" protein (b) is an expansion of the 1340–1380-cm^{-1} region of the spectrum shown in Figure 4. The Raman spectrum of the reduced protein (a) was obtained with conditions identical with those used to obtain spectrum b. The intensity of the 1358-cm^{-1} band relative to the 1373-cm^{-1} band in the spectrum of the "oxidized" protein indicates that extensive photoreduction of the sample has occurred with irradiation at λ_{ex} = 457.9 nm. The occurrence of the shoulder at 1368 cm^{-1} in the spectrum of both the "oxidized" and reduced protein suggests that this band has a different origin from the 1373- and 1358-cm^{-1} bands (see text for additional discussion).

on the 1358-cm^{-1} band observed with Soret region excitation. Indeed, the RR spectra of cytochrome *c* obtained with excitation in the visible region exhibit a *depolarized* band at 1367 cm^{-1} which differs in origin from the *polarized* 1375- and 1360-cm^{-1} bands observed with excitation in the Soret region (Friedman & Hochstrasser, 1973). This 1367-cm^{-1} band of cytochrome *c* is also insensitive to the oxidation state of the protein.

The question, of course, remains as to whether the photoreductive process also occurs with excitation in the 600-nm region. While the 1375- and 1360-cm^{-1} bands are a reliable measure of the oxidation state of hemoproteins with excitation in the Soret region, these Raman bands are not resonance enhanced with excitation in the visible region. As was previously noted, the only reliable oxidation state marker band for hemoproteins excited in the visible region is a Raman band observed around 1640 cm^{-1}, which shifts to approximately 1620 cm^{-1} upon reduction of the heme moiety (Spiro, 1975). However, in our RR spectra of cytochrome *c* oxidase obtained with excitation in the 600-nm region (Figures 1–3), *no* Raman bands are observed between 1612 and 1660 cm^{-1}. Thus, the oxidation state of this protein cannot be determined reliably from the RR spectra.

Since the RR spectra of cytochrome *c* oxidase obtained with excitation in the 600-nm region do not appear to be sensitive to the oxidation state of the heme moieties, we have used EPR spectroscopy to monitor the redox state of the protein after laser irradiation. The results of these EPR studies are shown in Figure 6. When cytochrome *c* oxidase is irradiated at 590 nm at a temperature of 195 K, there is neither a reduction in the intensity of the cytochrome *a* (g = 3) or Cu_a (g = 2; not shown in Figure 6) EPR signals nor the appearance of an

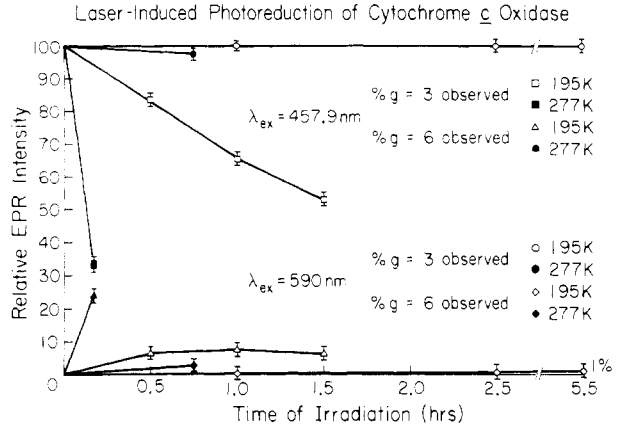

FIGURE 6: Change in the cytochrome a ($g = 3$) and appearance of high-spin heme ($g = 6$) EPR signals upon laser irradiation of cytochrome c oxidase. The sample concentration, path length, and laser power density were identical with those in the RR experiments. The EPR spectra were recorded at 12 K with a microwave frequency of 9.2 GHz, a microwave power of 0.2 mW, and a modulation amplitude of 16 G.

appreciable amount of signal at $g = 6$, characteristic of the partially reduced protein. These results suggest that maximally only 1–2% of the protein molecules are photoreduced, even after 6 h of irradiation at 590 nm (which is comparable to the time required for the acquisition of our RR spectra using 600-nm region excitation). In contrast to the absence of photoreduction induced by irradiation at 590 nm, irradiation at 457.9 nm at a temperature of 195 K results in a steady decrease with time of the intensity of the cytochrome a and Cu_a (not shown in Figure 6) EPR signals. Concomitant with the loss of intensity of these EPR signals, a new signal appears at $g = 6$, which maximally accounts for 8% of one heme. In addition, a signal characteristic of a free radical appears at $g = 2$. These observations indicate that appreciable photoreduction of the protein occurs with irradiation at 457.9 nm, which is consistent with our RR results.

When the temperature is raised to 277 K and the protein is irradiated at 457.9 nm, photoreduction of the heme moieties occurs much more rapidly (Figure 6). After 10 min in the laser beam, approximately 70% of the intensity of the cytochrome a and Cu_a (not shown in Figure 6) EPR signals is lost, and a signal accounting for 25% of one heme appears at $g = 6$. With irradiation at 590 nm at a temperature of 277 K, there is also evidence for the occurrence of photoreduction but to a much lesser extent than with irradiation at 457.9 nm. After 45 min of irradiation at 590 nm, approximately 3% of the intensity of the cytochrome a EPR signal is lost, and a small signal appears at $g = 6$.

In order to determine whether the photoreduced species would decay with time, we maintained the sample irradiated at 457.9 nm at 195 K for several hours after the completion of irradiation. The EPR spectrum of this sample was found to be identical with that obtained immediately after removal from the laser beam, thus indicating that the photoreduced species accumulated after 1.5 h of irradiation does not decay rapidly at 195 K in the absence of further irradiation.

Specific Features of the RR Spectra Observed with Excitation in the 600-nm Region. (1) Lack of Evidence for Copper-Associated Vibrations. It has been suggested that one or both of the copper centers in cytochrome c oxidase might be type 1 in character (Gibson & Greenwood, 1965; Powers et al., 1979). Type 1 copper proteins exhibit intense resonance-enhanced Raman bands between 200 and 500 cm^{-1}, when the exciting radiation falls within the 600-nm absorption band which is characteristic of these proteins (Siiman et al., 1976; Miskowski et al., 1975). The reduction of type 1 copper proteins results in the disappearance of the 600-nm absorption band, with a concomitant disappearance of the low-frequency Raman bands. However, in the case of cytochrome c oxidase, the low-frequency bands observed in the RR spectrum do not disappear upon reduction of the enzyme (compare Figures 1 and 2). This observation leads us to conclude that all of the bands observed in the RR spectrum of cytochrome c oxidase with excitation at 600 nm are associated with the heme A moieties. Comparison of the RR spectra of the oxidized and reduced protein at other excitation wavelengths in the 600-nm region likewise fails to reveal any bands which can be attributed to copper–ligand vibrations.

(2) The Question of Specific Assignments of the Raman Bands. The direct comparison of the RR spectra of oxidized, reduced, and oxidized cyanide-bound cytochrome oxidase shown in Figures 1–3 does not allow the obvious assignment of any band to either cytochrome a or cytochrome a_3, exclusively. With the exception of the 732- and 744-cm^{-1} bands, no bands clearly exist as doublets. Although there are some bands observed in the cytochrome c oxidase RR spectra not seen in the RR spectra of other hemoproteins, most of the cytochrome c oxidase bands have analogues in the RR spectra of these other proteins. On the basis of previous RR studies of other hemoproteins, which have demonstrated that the frequencies of certain Raman bands are sensitive to the spin state of the iron (Rakshit & Spiro, 1974; Spiro, 1975), one might have expected that these spin state marker bands could be used to provide a partial assignment and an estimate of the relative contribution of low-spin cytochrome a and high-spin cytochrome a_3 to the RR spectrum. In particular, the observation of bands at 1640, 1590, and 1500 cm^{-1} has been shown to be indicative of a low-spin heme moiety, with a conversion to the high-spin form resulting in shifts of these bands to 1610, 1555, and 1475 cm^{-1}, respectively. The 1500- and 1475-cm^{-1} bands are only observed with excitation in the Soret region, however, and, consistent with this previous observation, our RR spectra of cytochrome c oxidase obtained with visible region excitation do not exhibit any significant Raman bands at 1500 or 1475 cm^{-1}. Our RR spectra of oxidized cytochrome c oxidase do exhibit a band at 1612 cm^{-1}, which would be assigned to high-spin cytochrome a_3 by the above criterion, and a band at 1587 cm^{-1}, which would be assigned to low-spin cytochrome a. However, no band is observed at 1640 cm^{-1} to accompany the 1587-cm^{-1} band, and no band is observed at 1555 cm^{-1} in conjunction with the band at 1612 cm^{-1}. Upon the addition of cyanide to the oxidized protein, which converts cytochrome a_3 from high to low spin, a weak band does appear near 1640 cm^{-1}, but without any significant change in the intensity of the 1612-cm^{-1} band. These inconsistencies between the RR spectra of cytochrome c oxidase and those of other hemoproteins make any assignment of the 1587- and 1612-cm^{-1} bands to the individual cytochromes dubious at best.

(3) Other Spectral Changes Observed upon Reduction or the Addition of Cyanide. Comparison of the RR spectra shown in Figures 1–3 reveals that reduction or the binding of cyanide to the oxidized protein does not lead to large frequency shifts or to the appearance of any significant new bands in the RR spectrum. This is to be contrasted to the RR spectra of oxidized and reduced cytochrome c oxidase recorded with excitation in the Soret region, where reduction of the protein results in the appearance of additional bands at 215, 364, 1230, and 1670 cm^{-1} (Salmeen et al., 1973, 1978). The 1670-cm^{-1}

band has been assigned to the C=O stretching vibration of the formyl group on reduced cytochrome a_3. There is no reason, however, to expect that our RR spectra obtained with excitation in the 600-nm region should exhibit the same spectral changes upon reduction of the protein as those observed in the RR spectra obtained with excitation in the Soret region, since the mechanism by which the vibrational modes of the porphyrin gain resonance enhancement is different for excitation in the two regions of the absorption spectrum.

Although there do not appear to be large frequency shifts or additional resonance enhancements upon protein reduction or cyanide binding, there are significant changes in the relative intensities of many of the bands relative to their intensities in the spectrum of the oxidized protein at the same excitation wavelength. For example, the 342-cm^{-1} band of the reduced protein is nearly 50% more intense than that of the oxidized protein compared to the 1132-cm^{-1} band when the excitation wavelength is 600 nm. The bands at 975, 1303, and 1520 cm^{-1} are also much more intense in the 600-nm spectrum of the reduced protein, as is the doublet at 732 and 744 cm^{-1}, with the 744-cm^{-1} band exhibiting a larger intensity gain than its 732-cm^{-1} partner. The intensities of the various RR bands of the oxidized cyanide-bound protein do not differ quite as visibly from those of the oxidized species as do those in the reduced protein, although there are clearly observable differences. For example, with excitation at 600 nm, the intensity of the 744-cm^{-1} band relative to the 732-cm^{-1} band is much larger in the RR spectrum of the oxidized cyanide-bound protein than in that of either the oxidized or reduced species, as are the bands at 1070 and 1090 cm^{-1}.

Discussion

In this investigation, we did not observe any bands in the RR spectrum of cytochrome *c* oxidase which can be attributed to copper–ligand vibrations. The fact that the resonance enhancement of copper–ligand vibrations is insufficient for their observation under the conditions of our RR experiments implies that either (1) cytochrome *c* oxidase does not exhibit a copper-associated absorption band near 600 nm or (2) there is a copper-associated absorption band near 600 nm (characteristic of a type 1 copper) but that the resonance enhancements which occur upon excitation of this transition are much smaller than those which occur upon excitation of the visible absorption bands of the heme moieties. In view of the ease of obtaining RR spectra of type 1 copper proteins (Miskowski et al., 1975; Siiman et al., 1976) compared to cytochrome *c* oxidase, we feel that this latter possibility is unlikely.

Our RR studies of cytochrome *c* oxidase also indicate that no bands are clearly resolved which are assignable to cytochrome *a* or a_3, exclusively. This result suggests that either only one of these chromophores is contributing significantly to the 600-nm absorption region, and therefore to the RR spectra, or both cytochromes contribute to the absorption and RR bands, but their vibrational frequencies are nearly identical and not resolvable. In order to determine which of these two interpretations of the RR spectra is most probable for the three protein species studied, we will present in a subsequent paper an analysis of the excitation profiles of a number of the Raman bands constructed from the data reported here (Bocian et al., 1979).

Photoreduction of Cytochrome c Oxidase. Our EPR studies of laser-induced photoreduction of cytochrome *c* oxidase demonstrate that there is both a marked temperature and a marked wavelength dependence on the rate of photoreduction. With irradiation at 457.9 nm, extremely rapid photoreduction occurs in liquid samples at 277 K. While freezing the samples at 195 K reduces the rate of photoreduction, the process still occurs readily at this temperature with 457.9-nm irradiation. The appearance of the 1358-cm^{-1} band in our RR spectra obtained with 457.9-nm excitation at 120 K further demonstrates that the photoreductive process is operative at very low temperatures. In contrast to the extensive photoreduction which occurs with irradiation at 457.9 nm, our EPR studies indicate that 590-nm radiation induces essentially no photoreduction in solid samples at 195 K and only small amounts of photoreduced species in liquid samples at 277 K. However, our EPR studies cannot rule out the possible generation in the laser beam of a transient population of photoreduced species which rapidly reoxidize upon the cessation of irradiation. We feel that this is unlikely in view of the fact that we have found that the state of reduction of the enzyme is maintained for long periods of time at 195 K. We, therefore, conclude that little, if any, photoreduction occurred during our RR studies performed at 120 K by using excitation in the 600-nm region.

The dependence of the rate of photoreduction on the wavelength of irradiation and the temperature is consistent with the mechanism, proposed by Adar & Yonetani (1978), in which reducing equivalents are provided catalytically through the photooxidation of flavin impurities in the sample. Since flavins have a strong absorption band near 450 nm and only weak absorptions in the 600-nm region, photooxidation of the flavin should occur much more rapidly with irradiation at 457.9 nm than at 590 nm. Furthermore, the redistribution of reducing equivalents should be dependent on the diffusion rates of the various species in the sample; thus, the rate of photoreduction of cytochrome *c* oxidase should be reduced substantially in frozen samples, as is observed. Finally, the generation of photooxidized flavin molecules is consistent with the observation of a free-radical species in the EPR spectrum of the samples irradiated at 457.9 nm.

Additional Comments on the Vibrational Spectra. As we have previously noted, the RR spectra obtained for cytochrome *c* oxidase upon excitation in the 600-nm region exhibit a number of Raman bands not observed in the RR spectra of other hemoproteins upon excitation in their visible absorption bands. However, it does not appear that any of these additional Raman bands can be readily assigned to either cytochrome *a* or cytochrome a_3, exclusively. One possible explanation for the additional bands which occur in the cytochrome *c* oxidase RR spectrum is that the substituents which are present on the heme A moiety, notably the formyl group, cause a significant reduction in the vibrational symmetry of the porphyrin ring. In the D_{4h} model for metalloporphyrins, there are 18 in-plane vibrations of E_u symmetry, which are not Raman allowed (Ogoshi et al., 1972). If the symmetry is less than D_{2h}, these vibrations transform into irreducible representations which could become resonance enhanced upon excitation of the heme A visible bands. Such an effect might be the origin of the unusual doublet structure at 732 and 744 cm^{-1}. Inasmuch as there is no apparent splitting in the doubly degenerate electronic transitions of either of the two cytochromes, the removal of the vibrational degeneracy must occur through kinematic terms in the vibrational Hamiltonian which do not significantly affect the π-electronic distribution and, hence, the potential energy.

The low-frequency regions (<500 cm^{-1}) of the RR spectra of cytochrome *c* oxidase are quite unusual, with certain bands displaying very large resonance enhancements. These resonance enhancements could be a reflection of unusual vibronic mixing of the Soret and α bands which could occur because

of the nature of the substituents on the heme A moiety. The cytochrome *c* oxidase spectra also contain a band at 500 cm^{-1} which has no analogue in the RR spectra of other hemoproteins or metalloporphyrins. Although it is possible that this band becomes resonance enhanced as a result of reductions in the vibrational symmetry of the ring, it is interesting to speculate whether it might be directly associated with a formyl group vibration. With excitation in the Soret region, the C=O stretch of the formyl group is observed at 1670 cm^{-1} in reduced cytochrome *c* oxidase (Salmeen et al., 1973, 1978). Although the Raman intensity of the C=O stretch does not appear to be strongly resonance enhanced with excitation in the 600-nm region, it is possible that the in-plane C—C—O bend of the carbonyl group does become resonance enhanced. This band would be expected at a frequency near 500 cm^{-1} (Cossee & Schachtschneider, 1966).

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