Raman Spectra of Heme a, Cytochrome Oxidase–Ligand Complexes, and Alkaline Denatured Oxidase†

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ABSTRACT: We report 441.6 nm excitation resonance Raman spectra of oxidized and reduced monomeric heme a–imidazole, cytochrome oxidase–exogenous ligand complexes in various redox states, and alkaline denatured oxidase. These data show that, in reduced oxidase, the cytochrome a_3 Raman spectrum has bands at 215, 364, 1230, and 1670 cm^{-1} not observed in the cytochrome *a* spectrum. The appearance of these bands in the reduced cytochrome a_3 spectrum is due to interactions between the heme a of cytochrome a_3 and its protein environment and not to intrinsic properties of heme a. These interactions are pH sensitive and strongly influence the vibrational spectra of both heme a groups. We assign the 1670-cm^{-1} band to the heme a formyl substituent and propose that the intensity of the 1670 cm^{-1} is high for reduced cytochrome a_3 because the C=O lies in the porphyrin plane and is very weak for oxidized and reduced cytochrome *a*, oxidized cytochrome a_3, and oxidized and reduced heme a–imidazole because the C=O lies out of the plane. We suggest that movement of the C=O in and out of the plane explains the ligand induced spectral shift in the optical absorption spectrum of reduced cytochrome a_3. Finally, we confirm the observation of Adar & Yonetani (private communication) that, under laser illumination, resting oxidase is photoreactive.

Mitochondrial cytochrome *c* oxidase contains 2 mol of heme a and 2 mol of copper per mol of enzyme and catalyzes the four-electron reduction of oxygen to water. Although chemical extraction of the porphyrin yields a single type of heme a (Caughey et al., 1975), the protein ligand binding properties, optical absorption, EPR,[1] and MCD spectra, redox titrations, and magnetic susceptibility data establish that the two hemes in the oxidase molecule are functionally and spectroscopically distinguishable. Many of the same experiments also establish that in the protein each of the two copper atoms has distinguishing properties. These experiments, summarized most recently by Malmstrom (1973) and Nichols & Chance (1974), show the following. In the oxidized enzyme, the iron of one of the hemes yields a $g = 3$ EPR signal characteristic of a six-coordinated low spin ferric ion. This iron does not bind exogenous ligands and, in the nomenclature of Keilin & Hartree (1939), corresponds to cytochrome *a*. The iron of the second heme a, belonging to cytochrome a_3, binds exogenous ligands in both oxidized and reduced states. Magnetic circular dichroism spectra (Babcock et al., 1976) and magnetic susceptibility data (Falk et al., 1978) indicate that cytochrome a_3 has high spin iron in both redox states, consistent with its ligand binding property, but the expected characteristic $g = 6$ high spin ferric EPR signal has not been observed for the resting enzyme. Furthermore quantitation of the copper EPR spectrum of the oxidized enzyme accounts for only about one cupric ion per molecule. Using the conclusion from MCD data that cytochrome a_3 has high spin iron, together with the EPR spectra and an analysis of magnetic susceptibility data, Palmer et al. (1976), following the original suggestion of Van Gelder & Beinert (1969), showed that the magnetic properties of the oxidized enzyme can be accounted for by the following model. The EPR signals are attributed to one of the cupric ions along with the low spin ferric ion of cytochrome *a*. The other cupric ion ($S = 1/2$) and the high spin ($S = 5/2$) ferric ion of cytochrome a_3 are proposed to be antiferromagnetically coupled, via a bridging imidazole from a histidine residue, resulting in a $S = 2$ ground state which is not expected to show an EPR spectrum with standard instrumentation.

Although the magnetic data can be explained reasonably well, other observations remain unexplained. For example, unlike other hemeproteins, when reduced cytochrome oxidase binds carbon monoxide, its optical absorption spectrum shifts toward shorter wavelengths (Lemberg, 1969); the optical absorption band frequencies and relative intensities for oxidase are unusual compared with those for other hemeproteins (Caughey et al., 1976; Malmstrom, 1973); changes in the redox state or ligand at one heme are thought to alter the redox properties of the other heme (Wikstrom et al., 1976). While the simplest interpretation of the magnetic data precludes any strong coupling between the two heme *a* groups (as in a heme–heme dimer) (Palmer et al., 1976), data revealing the detailed molecular basis for the proposed interactions have yet to be obtained.

One source of new information is the heme vibrational spectrum obtained by resonance enhanced Raman scattering (Warshel, 1977). Raman band frequencies are interpretable in terms of the atomic displacements corresponding to the normal vibrations of the hemes, and the intensity distribution within the spectrum and its dependence on excitation wavelengths reflect the interaction between the π-electron system and these atomic displacements. Thus resonance Raman data provide information different from that obtained by other techniques such as magnetic measurements and optical absorption spectroscopy. In a preliminary note (Salmeen et al., 1973), we reported that cytochrome oxidase resonance Raman spectra, obtained with 441.6-nm excitation, had unusual features when compared with the spectra of other hemeproteins (Yamamoto et al., 1973; Yamamoto, 1973). All other oxidized hemeprotein spectra had a strong polarized band between 1368 and 1375 cm^{-1} which shifted by about 10 cm^{-1} to lower fre-

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[1] Abbreviations used: MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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quency upon dithionite reduction. In contrast the resting (oxidized) oxidase spectrum had a strong polarized band at 1360 cm^{-1} with a prominent shoulder at about 1375 cm^{-1}. This band shape was independent of power between 3 and 25 mW. The spectrum of dithionite reduced oxidase showed a strong band at 1360 cm^{-1}, typical of that observed for other reduced hemeproteins, and bands at 215 and 1670 cm^{-1} having no counterpart in any of the other hemeprotein spectra. These preliminary results suggested that further investigation might reveal the structural basis for some of the oxidase properties described above.

We report here resonance Raman spectra (441.6-nm excitation) of oxidized and reduced monomeric heme a-imidazole complex, of ligand bound cytochrome oxidase in various redox states, and of alkaline denatured oxidase. Resonance Raman spectra of heme a-imidazole were recently reported by Kitagawa et al. (1977) using 514.5- and 488.0-nm excitations. However, their heme a spectra cannot be compared with our cytochrome oxidase spectra because their excitation wavelengths were different from ours. Furthermore, the optical absorption spectrum of the reduced heme a-imidazole samples used by Kitagawa et al. had a Soret maximum at 453 nm, indicative of aggregate formation. We show here that aggregate formation is reflected in the heme a Raman spectrum and that the Raman spectra of aggregated and monomeric heme a differ from each other and from those of cytochrome oxidase.

Contrary to our earlier observation, Adar & Yonetani (private communication) recently observed that, with 413-nm excitation, the intensity of the 1360 cm^{-1} band relative to that of the 1375-cm^{-1} band in the spectrum of oxidized cytochrome oxidase depends on illuminating laser power, indicating photoreactivity of the enzyme. We report here that after modifying the illumination optics of our spectrometer to reduce the power density at the sample, we confirm the observation of Adar & Yonetani. The simplest interpretation for the appearance of the 1360-cm^{-1} band in the spectrum of resting oxidase is laser induced photoreduction because the spectra of other (chemically) reduced hemeproteins show a strong band near 1360 cm^{-1}. But under our experimental conditions, the spectrum of resting enzyme, while showing a prominent 1360-cm^{-1} band, does not show bands at 215 and 1670 cm^{-1} characteristic of dithionite reduced oxidase, thus suggesting that the photoreaction does not lead to complete reduction of the enzyme.

The conclusions from the data presented here are: the 215- and 1670-cm^{-1} bands in the reduced protein spectrum are due to cytochrome a_3; the appearance of these bands is due to interactions between heme a and its protein environment rather than to intrinsic properties of heme a; and the interactions are pH sensitive and strongly influence the vibrational spectra of both heme a groups. Finally we propose a mechanism explaining the intensity of the 1670-cm^{-1} band in the reduced protein spectrum which also rationalizes the ligand-induced spectral shift in the optical absorption spectrum of reduced cytochrome a_3.

Materials and Methods

Solubilized cytochrome oxidase, prepared as described previously (Babcock et al., 1976) by the method of Hartzell & Beinert (1975), was suspended in 0.5% recrystallized cholate or 0.5% Tween 20 and 50 mM Hepes buffer (pH 7.4). The spectroscopic ratios for solubilized product agreed with those cited by Lemberg (1969). Dithionite reduced enzyme was prepared by adding a few crystals of solid dithionite (Virginia Smelting) after replacing the ambient air over the sample with argon. The 5 mm × 10 mm rectangular Raman cuvettes were fitted with ground-glass stoppers and the cuvette entrance was continuously flushed with argon during dithionite addition to minimize reentry of oxygen.

The formate and cyanide complexes of oxidized enzyme and the partially reduced cyanide and formate complexes in aerobic steady state were obtained as described previously (Babcock et al., 1976). The optical absorption spectrum of each sample was recorded before and after obtaining the Raman spectrum to confirm the state of the protein and to ensure that the samples did not change during the recording of the Raman spectrum. However, these optical spectra do not rule out reversible photochemical reactions occurring in the laser illuminated sample volume.

The cyanide complex of the fully reduced protein was prepared as follows. Cytochrome oxidase was anaerobically reduced with dithionite and the sample capped with a rubber septum. A neutralized 1 M KCN solution was made anaerobic by addition of dithionite and a volume, sufficient to adjust the oxidase solution to 10 mM, was added through the septum using a needle and syringe. The optical spectrum, recorded before and after obtaining the Raman spectrum, demonstrated that the cyanide complex of the fully reduced enzyme was stable during the experiment.

The alkaline pH titration of cytochrome oxidase was carried out using methods similar to those described by Lemberg & Pilger (1964). Solutions of cytochrome oxidase (50 μM heme a) in 0.5% cholate, 0.1 M potassium phosphate buffer were adjusted to the desired pH using 1.0 N NaOH. Dithionite was added anaerobically and the absorbance in the 600-nm region was monitored. The time course for the absorbance changes and shifts in absorbance maxima at the various pH values were similar to those described by Lemberg and occurred in two pH dependent steps. In the first, the α and Soret band maxima for the native reduced enzyme shifted from 604 and 443 nm to 595 and 435 nm, respectively, when the pH was made moderately alkaline. The apparent pK of these changes was between 9.3 and 9.5. In the second, the α band maximum shifted from 595 nm at moderately alkaline pH to 574 nm at pH 12.8, indicating Schiff's base formation at the higher pH. This change showed an isosbestic point at 584 nm. The Soret maximum for the Schiff's base form of the protein occurred at 428 nm. The oxidized form of the pH 11.4 sample was prepared by addition of a crystal of ferricyanide to the dithionite reduced protein. In all experiments in which pH induced spectral changes were monitored, the sample pH was also measured after the Raman spectrum was recorded.

Heme a was isolated as described by Takemori & King (1965). $NaDodSO_4$ solutions of heme a-imidazole complexes were prepared by the method of Vanderkooi & Stotz (1966) with the minor modifications described previously (Babcock et al., 1976). The Soret peak for the oxidized heme a-imidazole in $NaDodSO_4$ was at 422 nm and at 435 nm for reduced complex, indicating that heme a aggregates did not form in any measurable concentration. In one sample of reduced heme a-imidazole, the $NaDodSO_4$ concentration was lowered and aggregates formed, as judged by the Soret peak at 453 nm with the shoulder at 430 nm. This sample was used to determine the effect of aggregates on the Raman spectrum.

Raman spectra were obtained with the 441.6-nm output of a He-Cd laser (Spectra Physics Model 185, nominal power 50 mW, or RCA Model LD2186, nominal power 15 mW). Spectra were recorded with the beam focused into and transmitted through the samples. The $1/e^2$ radius, ρ, of the approximately Gaussian intensity distribution of the laser output is 0.4 and 0.75 mm for the RCA and Spectra Physics lasers, respectively. The $1/e^2$ radius in the focused spot Gaussian intensity distribution is $\lambda f/\pi\rho$, where f is the lens focal length,

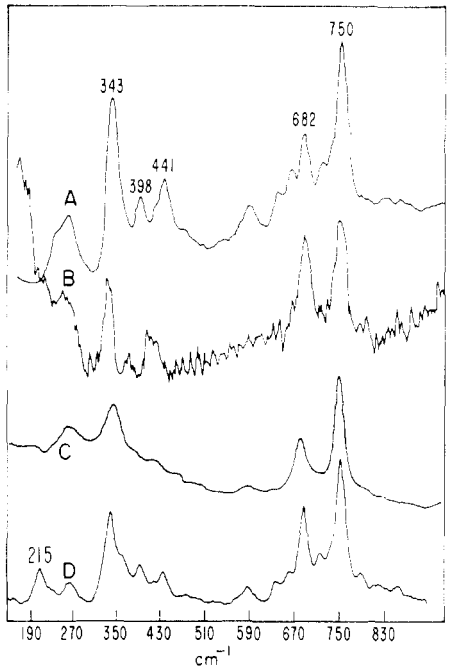

FIGURE 1: Raman spectra in the range 150 to 910 cm^{-1}. (A) Resting (oxidized) cytochrome oxidase (50 μM in heme a) in cholate-Hepes buffer. Laser power (10 mW) corresponded to focused flux of 6.5×10^6 mW/cm^2. (B) Oxidized monomeric heme a-imidazole (50 μM) in $NaDodSO_4$. Laser power as in A. (C) Dithionite reduced monomeric heme a-imidazole in $NaDodSO_4$. Laser power as in A. (D) Dithionite reduced cytochrome oxidase. Laser power as in A.

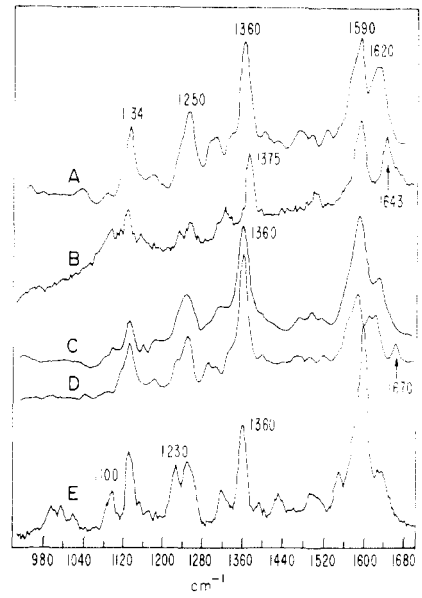

FIGURE 2: Raman spectra in the range 910–1700 cm^{-1}. Traces A–D are the same samples and conditions as the correspondingly labeled traces of Figure 1. (E) Aggregated dithionite reduced heme a-imidazole obtained by lowering $NaDodSO_4$ concentration of sample of Figure 1D.

and λ the laser wavelength. Our previously published cytochrome oxidase spectra were obtained using a 20-mm focal length lens and the Spectra Physics laser with power ranging from 3 to 25 mW incident on the lens, corresponding to focused radiation flux from 7×10^6 to 58×10^6 mW/cm^2, respectively. Most of the spectra displayed here, except those showing the power dependence of the 1360–1375-cm^{-1} bands in the spectrum of the oxidized protein, were obtained using the 20-mm lens and the RCA laser with a maximum power of 10 mW on the lens corresponding to 6.5×10^6 mW/cm^2 at the focus. The power dependent data were obtained using a 60-mm lens and the Spectra Physics laser with powers incident on the lens ranging from 0.4 to 34 mW corresponding to 0.1×10^6 to 8.8×10^6 mW/cm^2.

The spectra shown here were obtained with the incident beam polarized perpendicularly to the scattering plane, but with no polarization analyzer in the scattered beam. As discussed elsewhere (Salmeen et al., 1973; Yamamoto, 1973), with excitation near the Soret absorption band, all of the strong Raman bands are polarized with depolarization ratios less than about 0.3. The depolarization ratios of some bands vary slightly among the various chemical species, but such variations are small and are not used as arguments in the discussion.

Scan rates were 1.0 or 0.5 cm^{-1}/s. The spectral slit width was 5 cm^{-1}. The uncertainty in absolute frequencies is ± 2 cm^{-1} and in frequency differences it is ± 0.4 cm^{-1}. The spectrometer and photon counting system have been described elsewhere (Rimai et al., 1973). The oxidase spectra were obtained at 4 °C with cuvette cooling achieved by blowing cold gas through a jacketed cuvette holder. Heme a-imidazole spectra were obtained at room temperature to avoid precipitation of the $NaDodSO_4$.

The sample concentrations were about 25 μM in protein (50 μM in heme a) except when lower concentrations resulted from sample manipulation. Heme a samples were 50 μM. Although higher protein concentrations would have yielded Raman spectra with better signal-to-noise ratios, the concentrations used enabled us to measure the optical absorption spectra directly in the Raman cuvettes using a standard Cary 14 or 17.

Results

The experimental results are shown in Figures 1–6. The Raman spectra are superimposed on a fluorescence background comparable in intensity to the strongest Raman bands. Buffers containing cholate were free from fluorescence; thus the background in spectra of oxidase in cholate buffer was caused either by extraneous material copurifying with the oxidase or by intrinsic heme a fluorescence, as suggested by Adar et al. (1976). The background spectrum differed substantially from that recorded for dilute FMN, a possible contaminant, but we did not investigate it in detail. The background intensity in the oxidized protein spectrum is about 30% greater than it is for the dithionite reduced protein. Tween buffer had a fluorescent impurity resulting in a background in the Raman spectra of oxidase in Tween two to three times greater than it was in the cholate samples.

Traces A and D of Figures 1 and 2 show spectra of cytochrome oxidase in cholate buffer similar to those reported previously (Salmeen et al., 1973). In that previous report, the absolute frequencies of the 1590- and 1670-cm^{-1} bands were labeled incorrectly as 1580 and 1660 cm^{-1}. A few other band frequencies are revised slightly as a result of measuring band positions in higher resolution scans.

As displayed in Figures 3 and 5, the oxidized protein spectrum may have bands at 1360 and 1375 cm^{-1}, whereas the spectra of other oxidized hemeproteins have only a single band in this range with a frequency between 1368 and 1375 cm^{-1}, depending on the protein. In our previous work, the unusual doublet in the oxidized oxidase spectrum was slightly variable among independent protein samples, but for a given sample its band shape did not change as the power was varied from 7×10^6 to 58×10^6 mW/cm^2. However, upon changing our illuminating optics thereby reducing the radiation flux by nearly 10 times, we now find that the intensity of the 1360-cm^{-1} band

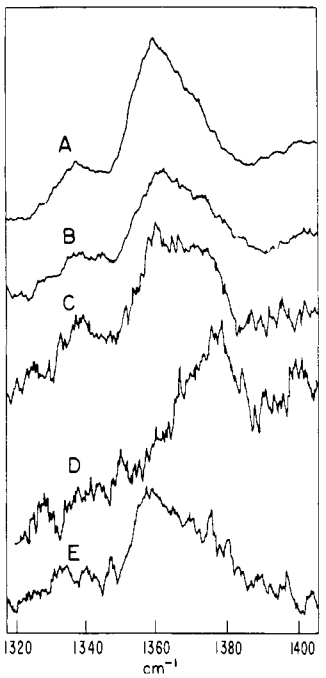

FIGURE 3: Power dependence of 1360–1375 cm^{-1} Raman bands for resting (oxidized) cytochrome oxidase (50 μM in heme a) in Tween–Hepes buffer. (A) Laser power (34 mW) corresponded to focused flux of 8.8×10^6 mW/cm^2 (one scan; no attenuation of the laser beam). (B) Same sample as in A, 7.8 mW (2.0×10^6 mW/cm^2), 5 scans, gain as in A. (C) Same sample as in A, 4.3 mW (1.1×10^6 mW/cm^2), 4 scans, gain 3.3 times that of A. (D) Same sample as in A, 0.4 mW (0.1×10^6 mW/cm^2), 70 scans, gain 2.5 times that of A. (E) Same sample as in A to which has been added 100 μM potassium ferricyanide, 4.3 mW (1.1×10^6 mW/cm^2), 4 scans, gain 2.5 times that of A.

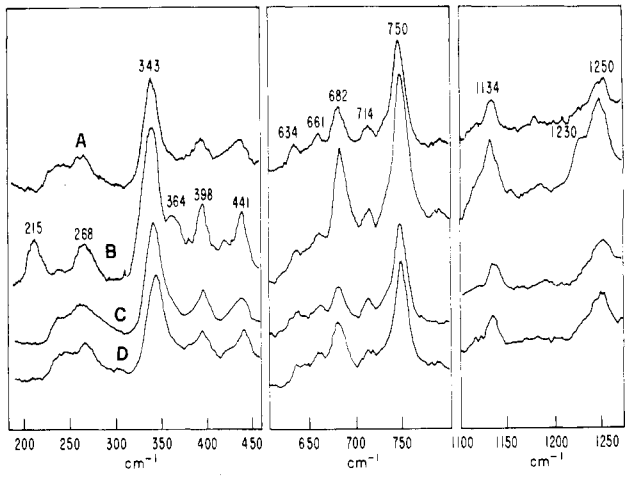

FIGURE 4: Higher resolution Raman spectra. (A) Resting (oxidized) cytochrome oxidase (50 μM) in cholate–Hepes buffer, 34 mW (8.8×10^6 mW/cm^2). (B) Dithionite reduced cytochrome oxidase. Same sample and conditions as in Figure 1D. (C) Cyanide complex of oxidized cytochrome oxidase, 10 mW (6.5×10^6 mW/cm^2). (D) Same sample as in trace C, partially reduced by TMPD–ascorbate; same power as in C.

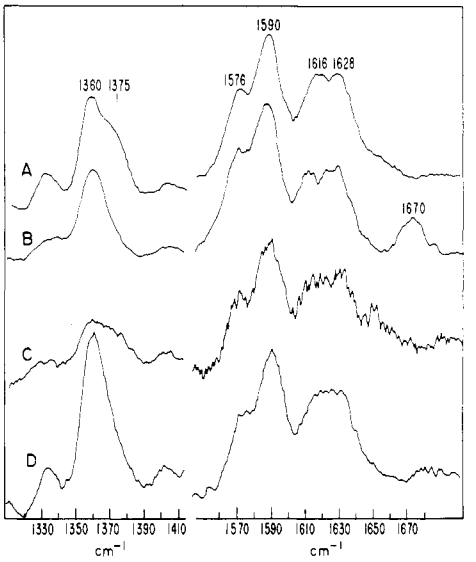

FIGURE 5: Higher resolution Raman spectra. Each scan is for same samples and conditions as correspondingly labeled scans in Figure 4.

relative to that of the 1375-cm^{-1} band is power dependent. Figure 3 shows this result for oxidized protein suspended in Tween buffer. For unknown reasons the photoreactivity is apparently less when the protein is suspended in cholate buffer. For example, in the spectrum of the same concentration protein in cholate, the 1375-cm^{-1} band is prominent even at the highest powers available to us (compare Figure 3A with Figure 5A). Figure 3E shows that ferricyanide in concentration equal to the heme concentration causes only a small change in the ratio of the two band intensities. Because the signal-to-noise ratio of the spectrum recorded with low power is correspondingly low (for example, the spectrum shown in Figure 3D required about 2 h of data accumulation), we did not attempt to record the low power spectrum over the entire 1600-cm^{-1} range shown in Figures 1 and 2. Consequently, the data for oxidized protein are an unknown superposition of spectra from oxidized and photoreacted protein. Nevertheless, even in the spectrum recorded with the highest power available, the characteristic reduced state bands at 215 and 1670 cm^{-1} are not observable, indicating that the photoreaction under these conditions does not result in fully reduced protein.

Traces B and C of Figures 1 and 2 show spectra of non-aggregated heme a–imidazole in the oxidized and reduced states, respectively. Trace 2E corresponds to aggregated reduced heme a–imidazole as indicated by a Soret peak in the optical absorption spectrum at 453 nm and shoulder at 432 nm. Aggregation is clearly reflected in the Raman spectrum by the appearance of bands at 1100 and 1230 cm^{-1} and the large relative intensity of the 1590-cm^{-1} band. These aggregated samples were not characterized in detail, except to note that the Raman spectrum does reflect the presence of aggregates. In contrast to the spectrum of reduced oxidase, those for reduced monomeric and aggregated heme a–imidazole do not show bands at 215, 364, 1230, and 1670 cm^{-1}.

Traces A and B of Figures 4 and 5 show high resolution scans through the major bands of oxidized and reduced oxidase spectra, illustrating in greater detail the differences between the spectra of photoreacted oxidized and dithionite reduced oxidase. Traces 4C and 5C show the spectra of the cyanide complex of oxidized protein recorded at the same power level as that of traces 4A and 5A. The addition of cyanide has no readily discernible effect on the photoreaction as evidenced by the 1360- and 1375-cm^{-1} bands. The spectrum obtained with formate as an added ligand was similar to that of the cyanide complex.

The spectrum of the partially reduced cyanide complex in aerobic steady state is shown in Figures 4D and 5D. The bands at 215, 364, 1230, and 1670 cm^{-1}, observed for the dithionite reduced oxidase, are unobservable for the partially reduced cyanide complex. The same result was obtained for partially reduced formate complex in aerobic steady state. However, the formate–oxidase sample eventually reduced all of the

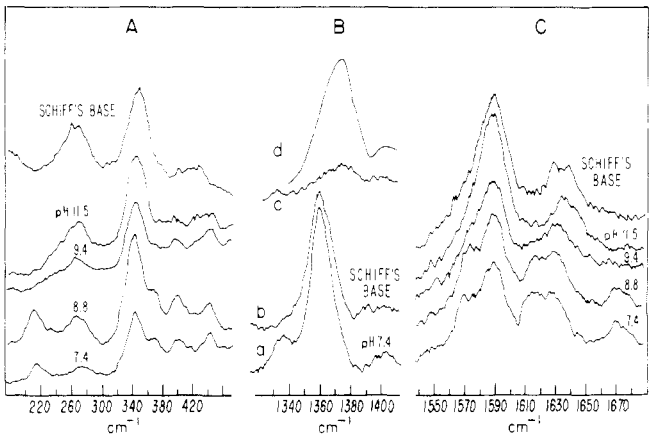

FIGURE 6: Major Raman bands of reduced cytochrome oxidase as a function of pH. Spectra under A and C labeled according to pH. Traces a and b under B are reduced protein at two values of pH. Trace c is experimental data obtained after adding 100 μM potassium ferricyanide to reduced sample at pH 11.4. Trace d is a manual replot of trace c normalized to peak intensity of trace a.

oxygen in solution, the residual excess ascorbate then fully reduced the protein, and the Raman spectrum of the sample became finally indistinguishable from that of the dithionite reduced protein.

Upon addition of cyanide to the *dithionite reduced enzyme*, the bands at 215, 364, 1230, and 1670 cm^{-1} became unobservable. The rest of the spectrum was indistinguishable from that of the reduced enzyme except that the ratio of the 1360-cm^{-1} band intensity to that of the 1590-cm^{-1} band became 1.2 instead of 1.6 as it is in the reduced protein spectrum.

Figure 6 shows the effects of an alkaline pH titration of the reduced protein. Major spectral changes occur in the ranges 200–500 cm^{-1} (in Figure 6A compare the bands at 215, 364, and 398 cm^{-1}) and 1500–1700 cm^{-1} (in Figure 6C compare the bands at 1576 and 1670 cm^{-1} and those between 1616 and 1628 cm^{-1}). Only minor changes occur in ranges not displayed. The relative intensity changes occurring between pH 7.5 and pH 11.5 parallel the pH dependence of the optical spectral shift from 605 nm to 595 nm with an apparent p*K* around 9.5. The entire spectrum of reduced oxidase at pH 11.5 is strikingly similar to that of reduced monomeric heme a. At pH 12.8 the optical absorption spectrum indicated the formation of the Schiff's base complex. The corresponding Raman spectrum indicates the formation of the Schiff's base by the appearance of a band at 1631 cm^{-1} (a typical C=N stretching frequency) and by small spectral changes between 364 and 375 cm^{-1}, both occurring between pH 11.5 and 12.8. Traces 6Ba and 6Bb show that the band at 1360 cm^{-1} remains unchanged throughout the pH range. Trace 6Bc is the experimental data for the pH 11.4 sample after oxidation with ferricyanide, and 6Bd is the same data of 6Bc normalized to the peak intensity of 6Ba. Note that, whereas the spectrum of oxidized oxidase at pH 7.4 recorded with 6×10^6 mW/cm^2 has a peak at 1360 cm^{-1} and a shoulder at 1375 cm^{-1}, the spectrum of the same concentration of oxidized protein at high pH and recorded with the same illuminating flux has only a single band at 1375 cm^{-1}.

Discussion

Two conclusions can be drawn directly from comparisons among these spectra.

(1) Since the bands at 215, 364, 1230, and 1670 cm^{-1} in the spectrum of dithionite reduced oxidase are not observed in the heme a–imidazole spectra, these bands can be attributed to the interaction between the heme a and the protein and not to intrinsic properties of heme a. Furthermore, the protein–heme a interactions responsible for the unique oxidase spectral bands disappear at high pH, as indicated by the similarities between the heme a–imidazole and high pH oxidase spectra. Although Kitagawa et al. (1977) also reported the absence of the 1670-cm^{-1} band from spectra of aggregated oxidized and reduced heme a–imidazole, this conclusion could not be reached by comparing their data with these oxidase spectra because their excitation wavelengths were different from ours. In addition, with their longer excitation wavelengths the bands below 600 cm^{-1} were not observed.

(2) The bands at 215, 364, 1230, and 1670 cm^{-1} in the fully reduced protein spectrum belong to cytochrome a_3. This conclusion rests on the ligand exchange experiments which show that the partially reduced cyanide and formate complexes in aerobic steady state consist of ligated oxidized cytochrome a_3 and reduced cytochrome *a*. Assuming that chemical changes in one heme do not cause spectral changes in the other, the bands for the fully reduced protein that are not observed for the partially reduced species (see Figures 4 and 5) must belong to reduced cytochrome a_3. Thus we conclude that the two hemes in reduced oxidase are structurally inequivalent as indicated by their vibrational spectra. This conclusion complements the conclusion from magnetic circular dichroism and magnetic susceptibility data that the reduced cytochromes *a* and a_3 have low and high spin iron, respectively (Palmer et al., 1976).

By analogy with the Raman spectra of other (chemically) reduced hemeproteins which show a strong band near 1360 cm^{-1}, the appearance of the 1360-cm^{-1} band in the resting oxidase spectrum can be attributed to laser induced photoreduction. However, the absence of the 215- and 1670-cm^{-1} bands and the conclusion that these bands belong to reduced cytochrome a_3 imply that, under our experimental conditions, only cytochrome *a* is photoreduced. While this is the simplest interpretation, we have not explored the possibility that the species obtained in the photoreaction may differ from those obtained by partial reduction of the cyanide and formate bound oxidase and from the dithionite reduced oxidase.

When other hemeproteins are excited by 441.6-nm radiation, an intense Raman band characteristic of the oxidized state is observed near 1375 cm^{-1}. Accordingly, the Raman spectra of the partially reduced formate and cyanide complexes should show a band near 1375 cm^{-1} arising from oxidized cytochrome a_3–ligand; such a band was not detected. Considering the shape of the observed 1360 cm^{-1} band and the signal-to-noise ratios, the 1375-cm^{-1} band would not be discernible if its intensity were less than about 10% that of the 1360-cm^{-1} band. Excitation profiles have not been measured for cytochrome oxidase. Nevertheless, relative intensities can be estimated as follows. The dominant term in the resonance Raman scattering intensity with 441.6-nm excitation involves the Soret band excited state. Thus interference terms causing oscillations in the excitation profiles, such as those observed when the excitation frequency is varied near the α band, can be neglected (Shelnutt et al., 1977), and the Raman intensity, *R*, is approximately proportional to $\epsilon^2/((\nu-\nu_0)^2 + \Gamma^2)$, where ϵ is the Soret band peak extinction coefficient, ν and ν_0 are the Soret band peak and laser frequencies, respectively, and Γ is the Soret band half-width at half-maximum. The Soret band maximum frequencies (cm^{-1}), line widths (cm^{-1}), and extinction coefficients (mM^{-1} cm^{-1}) are (Vanneste, 1966): oxidized cytochrome a_3 (24 155, 850, 81); oxidized cytochrome a_3–CN (23 474, 550, 60); oxidized cytochrome *a* (23 474, 800, 120); reduced cytochrome a_3 (22 573, 400, 125); reduced cyto-

chrome a_3-CN (22 676, 400, 96); and reduced cytochrome *a* (22 522, 550, 113). Thus, R(reduced cytochrome *a*)/R(oxidized cytochrome a_3) = 19 and R(reduced cytochrome *a*)/R(oxidized cytochrome a_3-CN) = 11, and the absence of the 1375-cm^{-1} band from spectra of partially reduced complexes is consistent with these relative intensity estimates.

Generally, assignments of spectral bands to specific bond vibrations require a normal coordinate analysis. However, as noted previously (Salmeen et al., 1973), experimental data from other conjugated molecules suggest that the 1670-cm^{-1} band is due to the heme a formyl group. This frequency is well above those assigned to C=C or C=N stretches in the spectra of any conjugated system (Rimai et al., 1973). Resonance enhancement of a C=O stretching vibration at 1670 cm^{-1} has been observed for retinal isomers (Rimai et al., 1971) and for other carbonyl containing carotenoids (Rimai et al., 1973). Resonance Raman spectra, obtained with 441.6-nm excitation, of chlorophylls a and b also show bands in the frequency range 1600–1700 cm^{-1} attributable to vibrations of keto and formyl carbonyls of peripheral substituents (Lutz, 1977). Furthermore, calculated classical normal modes of a D_{4h} symmetric porphyrin (Sunder & Bernstein, 1976; Rimai, unpublished) do not show such a high frequency band with any physically realistic set of force constants which at the same time even remotely yield the observed frequencies of the other major bands. Thus we affirm our earlier assignment of the 1670-cm^{-1} band to the C=O of the formyl group, with the added knowledge described above, that it is the formyl of reduced cytochrome a_3. The apparent absence of the 1670-cm^{-1} band from spectra of heme a-imidazole, oxidized protein, and reduced cytochrome *a* can be explained by the following argument.

The intensity of the C=O band depends on the coupling strength between the formyl group and the π-electron system. The presence of such coupling is indicated by the longer wavelengths of the heme a absorption bands relative to those of hemes not having a formyl group (Lemberg, 1969). Furthermore, the coupling between C=O and the π-electron system for a series of formyl containing porphyrins substituted into apomyoglobin was also found to be sensitive to heme-protein interactions (Sono & Asakura, 1975). The difference between the C=O band intensity in the spectrum of reduced oxidase and its intensity (i.e., less than the noise) in all the other spectra reported here implies corresponding differences between the coupling of the carbonyl into the π-electron system. The highest 1670-cm^{-1} band intensity (as in reduced cytochrome a_3) corresponds to the strongest coupling, which would occur when the C=O lies in the porphyrin plane. The low intensity (i.e., less than the noise) of the 1670-cm^{-1} band for all forms of the heme a chromophore except reduced cytochrome a_3 can be associated with weak coupling of the C=O such as would occur if the C=O were out of the heme plane.

The disappearance of the 1670-cm^{-1} band upon formation of the reduced cyanide complex requires that its intensity decrease by at least 10 times from that in the fully reduced spectrum. A small intensity decrease would accompany the cytochrome a_3 Soret band shift from 443 to 441 nm and the extinction coefficient decrease from 125 to 96 (Vanneste, 1966). Following the discussion above, R(reduced cytochrome *a*)/R(reduced cytochrome a_3) = 0.44 and R(reduced cytochrome *a*)/R(reduced cytochrome a_3-CN) = 0.77. Thus the intensity change of a Raman band attributable directly to changes in the electronic transitions is far too small to explain the experimentally observed intensity change. A plausible explanation is that the carbonyl moves out of the heme plane thereby decreasing the coupling to the π-electron system. This explanation is also consistent with the shorter wavelength optical bands of the reduced cyanide complex, since the decreased spatial extent of the π-electron system occurring when the C=O moves out of the porphyrin plane would shift absorption bands to shorter wavelengths.

The same mechanism may also explain the shift of the Soret band from 443 to 435 nm and the shift of the α band from 605 to 595 nm as the pH is increased to above 9.5 since the disappearance of the 1670-cm^{-1} band has the same pH dependence as the optical shift. However, as shown in Figure 6 other substantial Raman spectral changes occur during the alkaline pH titration, suggesting that structural changes in addition to the movement of the C=O also may occur.

The 215-cm^{-1} band occurs in the frequency region of many metal ligand vibrations. However, the two lowest frequency Fe-N stretching modes observed in all other hemeprotein spectra occur between 250 and 300 cm^{-1} (A_{1g}) and between 150 and 190 cm^{-1} (B_{1g}) (Sunder & Berstein, 1976; Rimai, unpublished). Corresponding bands are found in the oxidase spectra at about 260 and 160 cm^{-1}. Thus the 215-cm^{-1} band may not belong to a planar Fe-pyrrole nitrogen vibration, but possibly to an Fe-axial ligand vibration. Resonance enhancement of out-of-plane heme ligand vibrations can occur directly by excitation near charge transfer bands involving metal and ligand (Asher et al., 1977) or indirectly by vibronic mixing caused by nonplanarity in heme structure. This 215-cm^{-1} band does not obviously correlate with high-spin and low-spin iron states (for which the iron is presumably out of and in the plane, respectively) because it is absent in the spectra of high-spin (formate) and low-spin (cyanide) oxidized complexes and the low-spin cyanide reduced complex, but it is present in the spectrum of high-spin ligand-free dithionite reduced protein. Since the strength of the antiferromagnetic coupling between one copper ion and the iron of cytochrome a_3 requires proximity of the two metals with one bridging ligand, which we expect to be preserved upon reduction (Palmer et al., 1976), we can speculate that the 215-cm^{-1} band may relate to the iron-ligand-copper structure.

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Use of Immobilized Light-Harvesting Chlorophyll a/b Protein to Study the Stoichiometry of Its Self-Association†

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ABSTRACT: D. J. Davis & E. L. Gross (1976) *Biochim. Biophys. Acta 449,* 554-564 previously observed that the light-harvesting chlorophyll a/b protein or chlorophyll protein complex II self-associated as determined by ultracentrifugation. We have determined the stoichiometry of complex formation by immobilizing the monomer on ethylenediamine-Sepharose 4B and determining the ability of immobilized protein to bind the free protein. The amount of soluble protein bound to the immobilized protein increased as the concentration of soluble protein increased. The binding was maximal between pH 7 and 8. The maximum binding was three molecules bound per one molecule of protein immobilized. These results indicate that a tetramer is the intrinsic structural unit of the light-harvesting chlorophyll a/b protein in the chloroplast membrane. Upon complex formation, the chlorophyll fluorescence was decreased without any spectral change. The maximum binding was approximately doubled upon addition of 0.5 mM $CaCl_2$ whereas 5 mM NaCl had no effect. Addition of $CaCl_2$ had no effect on the fluorescence of the monomer. The light-harvesting chlorophyll a/b protein can be isolated from a sodium lauryl sulfate extract of chloroplasts by affinity chromatography using the immobilized light-harvesting chlorophyll a/b protein.

The light-harvesting chlorophyll a/b protein (chlorophyll protein complex II or CP II[1] of Thornber (1975)) is one of three chlorophyll protein complexes of the chloroplast membrane. The other two are the core complexes of the two photosystems (Vernon et al., 1971). This protein has an important structural role as it contains 40-60% of the chlorophyll and protein of the chloroplast membrane (Thornber, 1975). It also contains all of the chlorophyll b of the chloroplast. It has also been implicated in the regulation by cations of the spillover of excitation energy from photosystem II to photosystem I (Davis & Gross, 1976; Prochaka & Gross, 1977). Membrane structural changes involving the stacking of the grana membranes are thought to be responsible for the changes in spillover (Murakami & Packer, 1971; Murata, 1971; Gross & Prasher, 1974; Anderson, 1975). We think that both the changes in spillover and membrane stacking are due to changes in the interaction of this protein with itself and with the other core complexes (see Arntzen & Ditto, 1976).

Davis & Gross (1975, 1976) showed both a concentration dependent and divalent cation dependent association of CP II. However, the stoichiometry of association and the binding constants were not determined. To answer this question, we have studied the association of soluble CP II with immobilized CP II.

Materials and Methods

Preparation and Immobilization of CP II. CP II was isolated from spinach by the methods of Kung & Thornber (1971) as modified by Davis & Gross (1975) (i.e., CP II was isolated from a 1% $NaDodSO_4$ extract of spinach chloroplasts by means of batchwise hydroxylapatite chromatography followed by ammonium sulfate precipitation). CP II containing from 50 to 100 μg of chlorophyll was dialyzed against distilled water and added to 1 mL packed volume of activated Sepharose 4B which had been coupled to 0.1 M ethylenediamine according to the method of Liberatore et al. (1976). A water-soluble

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[1] Abbreviations used are: CP II, chlorophyll protein complex II or light-harvesting chlorophyll a/b protein; $NaDodSO_4$, sodium dodecyl sulfate; Chl, chlorophyll.

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