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Photodissociated Cytochrome *c* Oxidase: Cryotrapped Metastable Intermediates

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ABSTRACT: By freezing CO-bound cytochrome *c* oxidase at cryogenic temperatures, we have been able to cryotrap metastable intermediates of photodissociation. The differences in the resonance Raman spectrum between these intermediates and ligand-free reduced cytochrome oxidase at cryogenic temperatures are the same as those between the phototransient and the fully reduced preparation detected with 10-ns excitation at room temperature. The largest difference occurs in the iron–histidine stretching mode of cytochrome a_3, which shifts by up to 8 cm^{-1} to higher frequency in the photoproduct. At 4 K the iron–histidine mode displays two unrelaxed frequencies in the photoproduct, which we attribute to two different unrelaxed structures of the heme pocket. The frequencies and intensities of the lines in the resonance Raman spectrum are sensitive to the incident laser power density in both the ligand-free fully reduced preparation and the photoproduct even at 4 K. At 77 K the carbonyl stretching mode of the formyl group in cytochrome a_3^{2+} is especially sensitive to laser power, displaying two frequencies—1666 cm^{-1} at low-flux density and 1674 cm^{-1} at high-flux density. These frequencies may reflect a change in conformation of the formyl group or a change in its interaction with the protein such as in hydrogen bonding to the carbonyl of the formyl group. The absence of immediate relaxation of the CO photoproduct must be considered when one studies the structure and kinetics of the O_2 intermediates that are formed in triple trapping and flow-flash experiments following photodissociation of the CO-bound enzyme.

In order to determine the molecular basis for the function of heme proteins that bind oxygen, it is necessary to understand the interactions between the heme and the protein and also how these interactions change when oxygen is bound or released. The static interactions may be studied by using techniques that probe equilibrium states, but to follow the evolution of changes in interactions upon going from one state of ligand binding to another, techniques must be used in which metastable intermediates may be isolated. Ultimately, this should lead to an understanding of how protein structure controls and regulates active-site properties. Although oxygen is the physiological ligand of many heme proteins, it is often not a good candidate for studies of metastable intermediates because it is reduced by the active site. This reduction is slow in oxygen-transport and -storage proteins such as hemoglobin and myoglobin (Antonini & Brunori, 1971) but is very rapid in enzymes such as cytochrome oxidase (Hill & Greenwood, 1984). Carbon monoxide (CO) therefore has been used successfully as a model for oxygen since it is the same size, binds very strongly, and is relatively unreactive. Another advantage realized in studies that utilize CO is its very high photolability (Sawicki & Gibson, 1976). Thus, after the CO-bound protein is formed, generation of the ligand-free form of the protein may be initiated on the subpicosecond time scale by optical photodissociation (Martin et al., 1983). The ensuing relaxation may be followed by using time-resolved spectroscopy to study the structure and kinetics of the intermediates in the pathway from the ligand-bound to the ligand-free state. Resonance Raman scattering has been found to be a particularly useful spectroscopic technique with which to monitor the time evolution of heme changes following photodissociation and to map out the relaxation pathway (Friedman et al., 1982; Friedman, 1985; Rousseau & Friedman, 1987).

Cryogenic techniques have also been used successfully to cryotrap metastable intermediates by first freezing the CO-bound protein at cryogenic temperatures and then photodissociating the CO (Ondrias et al., 1983b; Rousseau & Argade, 1986; Sassaroli et al., 1986; Rousseau & Friedman, 1987). It was demonstrated that in hemoglobin the spectroscopic differences between the equilibrium deoxy preparation and the CO-photodissociated preparation were the same at cryogenic temperatures (80 K) as at room temperature on the nanosecond time scale (Ondrias et al., 1983a). This was a satisfying result because it suggested that spectroscopic studies at cryogenic temperatures could be used to infer properties of the protein under physiological conditions.

The comparison between room temperature time-resolved intermediates and cryotrapped intermediates of photodisso-

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ciated myoglobin was also made, but, unlike hemoglobin, the results of the time-resolved measurements were very different from those obtained with the cryotrapped samples. At room temperature no differences are detected in the spectrum between the CO photoproduct and equilibrium deoxymyoglobin at times longer than ~400 ps (Findsen et al., 1985; Rousseau & Argade, 1986; Rousseau & Friedman, 1987). The only difference in the 30–400-ps range was found in the core-size marker line of the resonance Raman spectrum (Dasgupta et al., 1985). Other Raman modes in the photoproduct, e.g., the iron–proximal histidine (Fe–His) stretching mode, appeared fully relaxed to their deoxy values within 30 ps (Findsen et al., 1985). If the same correspondence between the 10-ns photoproduct and the 80 K intermediate was to hold in myoglobin as was seen in hemoglobin, no differences between the photoproduct and the ligand-free preparation would be expected at 80 K. Thus, the relatively large differences detected in the comparison between equilibrium deoxymyoglobin and the CO photoproduct in the cryotrapped preparation, which indicates an unrelaxed heme in the photoproduct, came as a surprise (Rousseau & Argade, 1986; Sassaroli et al., 1986; Rousseau & Friedman, 1987). The similarities in the structure of myoglobin and the hemoglobin β-subunits are many, and yet the room temperature relaxation rates of the photoproducts of these two proteins differ by many orders of magnitude and the room temperature/cryogenic temperature differences also remained unexplained. It must be concluded that the correspondence between the properties of intermediates generated at room temperature and at cryogenic temperatures in hemoglobin cannot be generalized to other heme proteins.

Cytochrome *c* oxidase is the terminal enzyme in the electron-transport chain (Wikstrom et al., 1981). It catalyzes the transfer of four electrons from cytochrome *c* to O_2 to form water. The enzyme has four redox centers—two iron atoms and two copper atoms. The iron atoms are complexed by porphyrin rings of the heme *a* type. One of the hemes, cytochrome *a*, is six coordinate with histidine occupying the fifth and sixth coordination positions. It, along with one of the two copper atoms, Cu_A, appears to be the immediate recipient of the electrons from the cytochrome *c*. The other heme, labeled cytochrome a_3, has histidine as its fifth coordinating ligand and serves as the O_2-binding site. The second copper atom, Cu_B, is closely associated with cytochrome a_3 as evidenced by their strong antiferromagnetic coupling.

The resonance Raman spectrum of photodissociated CO-bound cytochrome oxidase with 10-ns excitation has been reported (Findsen & Ondrias, 1984). It was found that there are differences between its spectrum and that of the fully reduced ligand-free enzyme. The largest difference was detected in the iron–histidine stretching mode. In this paper we compare the spectra of intermediates generated at room temperature by photodissociation using time-resolved techniques to those generated at low temperature with the cryotrapping techniques. We find small changes in the spectrum of the fully reduced enzyme upon freezing to cryogenic temperatures and a dependence of the spectrum on the incident laser power. In addition, we observe an equivalence between the spectral properties of the photoproducts generated at cryogenic temperatures and those generated at room temperature with 10-ns pulses.

Experimental Procedures

Mammalian (beef heart) cytochrome *c* oxidase was prepared both by the method described by Babcock et al. (1976) and by the method of Yonetani (1960) and stored under liquid nitrogen until ready for use. The enzyme was solubilized in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid at pH 7.4 with 1% dodecyl β-D-maltoside and reduced with minimal sodium dithionite. The heme concentration was typically about 200 μM.

Resonance Raman data on cryotrapped preparations were obtained on samples that were sealed in a holder with transparent quartz windows and immersed into liquid nitrogen or liquid helium. A helium–cadmium laser (441.6 nm) was used as the Raman excitation source for all of the low-temperature data. For spectra at high-power density the ~15-mW laser beam was focused with a spherical lens on the sample surface, yielding a spot of about 50 μm in diameter (power density ~5–10 W/mm^2). For spectra at low-power density a cylindrical lens was used, yielding a power reduction of about ×25. The photoproduct was prepared by freezing a CO-bound preparation of the cytochrome *c* oxidase and photodissociating it with the helium–cadmium laser. At the laser intensities and temperatures (4 and 77 K) used in these experiments there was no evidence of any CO-bound species in the resonance Raman spectra with 441.6-nm excitation. The continuous wave spectra were obtained on conventional Raman instrumentation with photomultiplier detection. The 10-ns data were obtained by using a Nd-YAG (second harmonic) pumped hydrogen Raman shifter with an output at 436.5 nm. The pulse repetition rate was 29 Hz, and the intensity was about 0.5 mJ/pulse. Samples were placed in a rotating cell such that each pulse was impingent on fresh sample. Following dispersion, the spectrum was detected by a photodiode array.

Results

Upon freezing reduced cytochrome oxidase at cryogenic temperatures, small changes are detected in the frequencies of several Raman active modes as compared to their room temperature values. In Figures 1 and 2 we show the spectra of the low- and high-frequency regions of the spectrum, respectively, comparing spectra obtained at room temperature to those obtained at 77 K. At the cryogenic temperature we report two sets of data, one obtained at high-power density (tight focus) and one obtained at low-power density (focus with cylindrical lens).

The main differences between the room temperature (300 K) and low-temperature (77 K) data occur in the lines at 213, 340, 1491, 1519, 1568, 1584, and 1664 cm^{-1}. At 77 K at low laser power these lines appear at 216, 344, 1495, 1524, 1571, 1588, and 1667 cm^{-1}, respectively. In addition, new lines appear at 327 and 371 cm^{-1}. Some other lines sharpen, change intensity, and shift by 1–2 cm^{-1}, but no larger changes are detected under conditions of low laser power density. It should be noted that two of the lines in which frequency differences are detected are those that have been previously assigned as the iron–proximal histidine stretching mode (213 cm^{-1}) (Ogura et al., 1983; Argade et al., 1984) and the carbonyl stretching mode of the formyl group (1664 cm^{-1}) (Salmeen et al., 1973, 1978).

As the laser-power density is increased, changes in the Raman spectrum take place. The most prominent changes include a 2-cm^{-1} shift in the Fe–His stretching mode from 216 to 214 cm^{-1}, the appearance of a strong line at 323 cm^{-1}, and a splitting of the carbonyl mode into components at 1666 and 1674 cm^{-1}. No differences due to laser-power density are detected in those high-frequency modes that are sensitive to the heme electron density or the iron spin equilibrium (Choi et al., 1983).

The spectra of the cryotrapped photoproducts were generated by freezing CO-bound cytochrome oxidase to cryogenic temperatures and then photodissociating the CO by directing

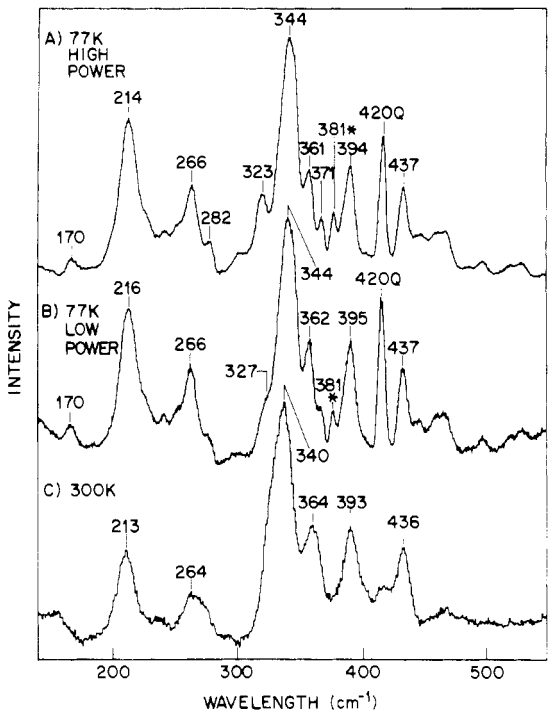

FIGURE 1: Low-frequency region of fully reduced ligand-free cytochrome *c* oxidase. The bottom spectrum (C) was obtained on a solution sample at room temperature. Spectra A and B were obtained on samples frozen at 77 K. In spectrum B, the laser power at the sample was about 15 mW at 441.6 nm, and it was focused with a cylindrical lens. In spectrum A the same laser power was focused with a spherical lens, yielding a much higher power density. The line at 381 cm^{-1} marked with an asterisk is a laser fluorescence line, and that at 420 cm^{-1} marked with a Q originates from the quartz windows on our sample cell.

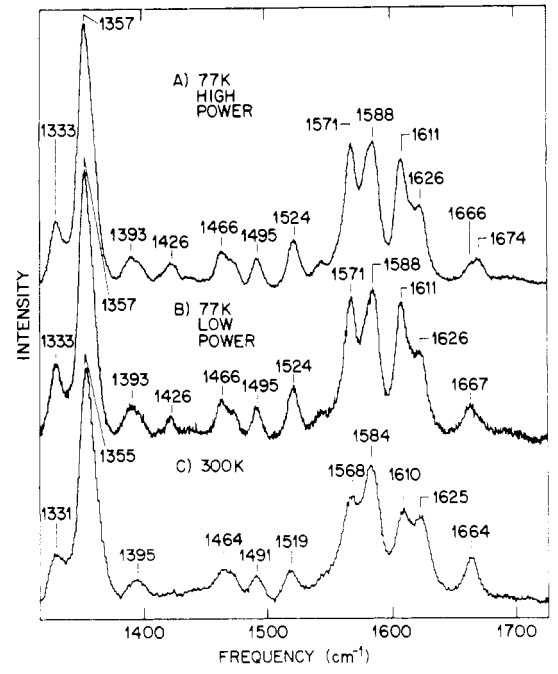

FIGURE 2: High-frequency region of fully reduced ligand-free cytochrome *c* oxidase comparing the effect of the laser power density on the frozen samples. The conditions are the same as in Figure 1.

the 441.6-nm laser beam on the sample. The spectra of the cryotrapped photoproduct are compared to those of the reduced enzyme in Figures 3 and 4. The absence of any splitting in the electron density marker line indicates that in our spectrum there are no significant contributions from unpho-

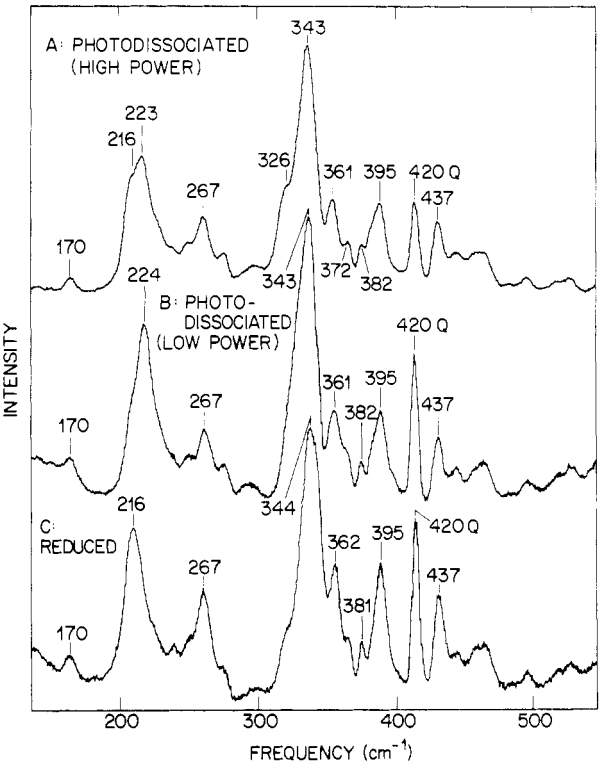

FIGURE 3: Comparison of spectra of photodissociated CO-bound cytochrome *c* oxidase at high (spectrum A) and low (spectrum B) laser power densities and the ligand-free fully reduced preparation (spectrum C) at 77 K in the low-frequency region. The 441.6-nm laser (power ~15 mW) was focused on the samples with a cylindrical lens for the low-power spectra (spectra B and C) and with a spherical lens for the high-power spectra. The line at 381 cm^{-1} is a laser fluorescence line, and the one at 420 cm^{-1} originates from the windows of the quartz sample cell.

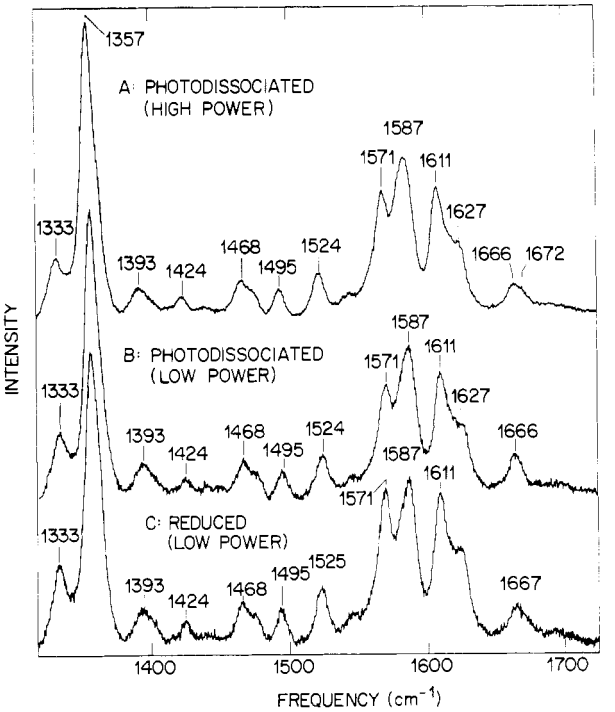

FIGURE 4: Comparison of spectra of photodissociated CO-bound cytochrome *c* oxidase at high (spectrum A) and low (spectrum B) laser power densities and the ligand-free fully reduced preparation (spectrum C) at 77 K in the high-frequency region. The conditions are the same as in Figure 3.

tolyzed material. At low-power densities (spectra 3B and 4B), the Fe–His stretching mode is substantially stronger in the

photoproduct than in the chemically reduced preparation and is shifted from 216 to 224 cm^{-1}. The carbonyl stretching mode is at about the same frequency (1666–1667 cm^{-1}). Some changes in intensity, some small changes in frequency, and differences in line width are also observed in many lines of the spectrum.

Just as in the reduced preparations, we were concerned that the incident laser intensity would also have an effect on the spectral properties of the photoproduct. Therefore, we examined the effect of the laser-power density on the spectra of the photoproducts. These results are also shown in Figures 3 and 4 for the low- and high-frequency regions, respectively. Upon increasing the laser-power density, a spectrum is generated that is qualitatively similar to that of the reduced enzyme under high-power-density conditions. In particular the Fe–His stretching mode shifts down to 216 cm^{-1}, a prominent shoulder becomes evident at 326 cm^{-1}, and the carbonyl mode splits into components at 1666 and 1673 cm^{-1}.

Alben et al. (1981) and Fiamingo et al. (1982) have studied the infrared absorption spectra of the C–O stretching frequency at cryogenic temperatures in the Fe–CO-bound complex of cytochrome oxidase as well as in the Cu–CO complex of the photodissociated frequencies. They reported a temperature dependence in the frequencies in the 15–80 K range. To determine if differences also could be detected in the heme modes in this temperature range, we carried out a series of measurements at 4 K. Qualitatively, the results we obtained at 4 K were the same as those we obtained at 77 K, although there were some significant quantitative differences.

The main differences we find are illustrated in Figure 5 in which the low-frequency region of the 4 K cryotrapped ligand-free reduced enzyme is compared to two spectra of the photoproduct obtained with different power densities. In the spectrum of the ligand-free reduced enzyme, just as at 77 K, the Fe–His stretching mode shifts to lower frequency at higher laser power (from 218 to 216 cm^{-1}). Unlike the data at 77 K there is a substantial power dependence in the intensity of the mode in the 360–362-cm^{-1} region. At 4 K with low laser power this mode, which originates from cytochrome a_3, is substantially more intense than it is at 77 K. This may be seen by comparing Figure 5 to Figures 1 and 3. Moreover, when the laser-power density at 4 K is increased, the spectrum reverts to the same as that of the low power density spectrum obtained at 77 K. The data from the photoproduct at 4 K indicate that it does not exhibit the high intensity for the mode at 362 cm^{-1}.

The Fe–His stretching mode is located at 224–225 cm^{-1} in the photoproduct at 4 K just as it is at 77 K. However, we have found that with elevated laser power or continued exposure the line shifts down by 2–3 cm^{-1} and a shoulder develops at low frequency. This is different from the behavior we detected at 77 K. In that case we were unable to detect a shift in the peak frequency, only the development of the shoulder at ~216 cm^{-1}. Although small, by repeating the measurement several times we feel confident in the shift in the peak frequency of the line at the high-laser power in the 4 K data. Thus, at the higher temperature (77 K) there appear to be only two positions for the line, 224 and 216 cm^{-1}, whereas at 4 K the line shifts down to an intermediate value.

In the high-frequency region the most noteworthy change with temperature occurs in the relative intensity of the 1571/1587 cm^{-1} pair (data not shown). In the 4 K photoproduct these modes have the same intensity as they do in the reduced preparation, whereas at 77 K the ratio is quite different, as may be seen in Figure 4. The final difference noted

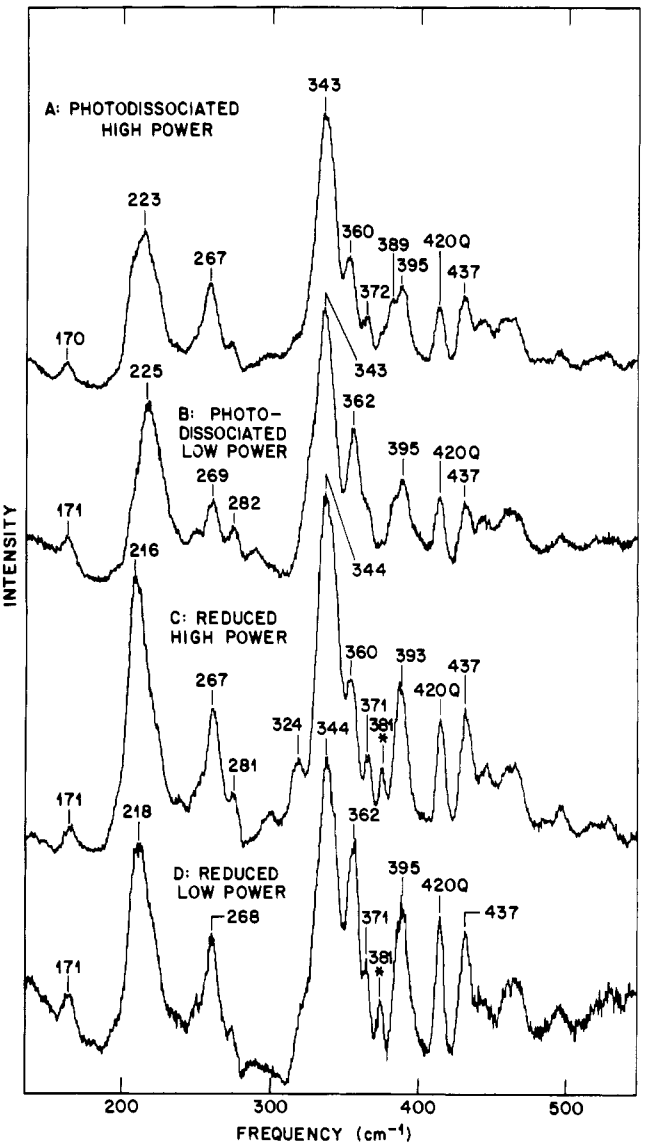

FIGURE 5: Comparison of the spectra of photodissociated CO-bound cytochrome *c* oxidase (spectra A and B) and the fully reduced preparation (spectra C and D) at 4 K. The low-power spectra (spectra B and D) were obtained with 5–15 mW of power at 441.6 nm focused with a cylindrical lens, whereas a spherical lens was used to focus the laser to obtain the high power density spectra (spectra A and C). The line at 381 cm^{-1} is a laser fluorescence line, and the line at 420 cm^{-1} originates from the windows of the quartz sample cell.

in the comparison between the 4 K and the 77 K data is in the formyl region in which at 77 K in the photoproduct higher laser power favored the formation of the higher frequency component (1672 cm^{-1}). However, at 4 K even at higher laser power only the lower frequency component (1666 cm^{-1}) is present in the spectrum.

A spectrum of the photoproduct generated at room temperature with 10-ns pulses has been reported by Findsen and Ondrias (1984). The Fe–His mode was observed to shift to higher frequency by 9 cm^{-1} (213–222 cm^{-1}). Other than that, the changes in the spectrum were very small. We have also obtained data on the photoproduct at room temperature with 10-ns pulses (Figure 6) and detect a 6-cm^{-1} shift in the position of the Fe–His mode in the photoproduct (213–219 cm^{-1}). Other than this, only very small differences could be reproducibly detected in the spectrum. Thus, the differences between the photoproduct within 10 ns of photolysis and its fully reduced counterpart at room temperature correspond to those in cryotrapped samples at low laser power density, namely,

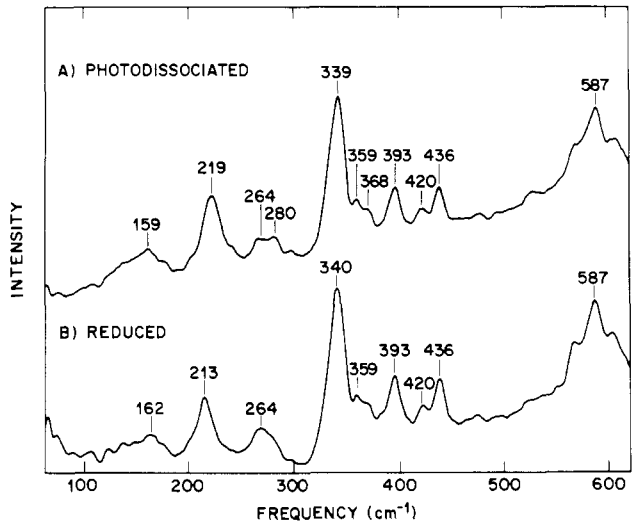

FIGURE 6: Comparison of the spectra of photodissociated CO-bound cytochrome *c* oxidase (spectrum A) and the fully reduced preparation (spectrum B) at room temperature with 10-ns pulses at 436.5 nm.

the largest difference is in the Fe–His stretching mode in both cases.

Discussion

Effects of Freezing. When ligand-free fully reduced cytochrome *c* oxidase is cryotrapped (4 and 77 K), there are some large changes in the vibrational frequencies. It is quite apparent that these frequency differences are present in both cytochromes *a* and a_3. Modes such as the Fe–His stretching frequency and the carbonyl stretching frequency of the formyl group, both of which shift to higher frequency in the frozen state by about 3 cm^{-1}, demonstrate the sensitivity of cytochrome a_3 to freezing. However, it is also quite noteworthy that the magnitude of the shift seen in the Fe–His stretching mode is substantially smaller than that found in hemoglobin or myoglobin, both of which also have five-coordinate hemes. The shifts of the Fe–His stretching mode in those latter two proteins are 18 and 8 cm^{-1}, respectively (Ondrias et al., 1983b; Sassaroli et al., 1986). The changes in frequency of the Fe–His stretching mode in the hemoglobin and myoglobin data were attributed to modifications in the repulsive part of the nonbonded interactions between the histidine and the heme that occur upon freezing these proteins.

It was argued that upon freezing hemoglobin and myoglobin a reduction in the repulsive forces due to lower thermal factors of vibrational modes involving the heme and the proximal histidine allowed the iron–histidine bond length to attain a more favorable distance and hence a stronger bond (Ondrias et al., 1983b; Sassaroli et al., 1986). If this explanation of the data is correct, it is difficult to understand why such an effect does not also occur in cytochrome *c* oxidase. Thus, it appears that a conformational change is also involved. In hemoglobin and myoglobin some relaxation of the histidine may occur upon freezing, allowing it to take on a more favorable orientation resulting in a stronger Fe–His bond. This relaxation appears to be absent in cytochrome *c* oxidase, as evidenced by the very small change in the Fe–His stretching frequency upon freezing.

The lines assignable as originating from cytochrome *a* (Argade et al., 1986) also display significant shifts upon freezing. The lines at 1519(ν_{11}), 1568(ν_{37}?), and 1586(ν_2) cm^{-1} have been shown to be core-size sensitive on the basis of their behavior in other proteins and model compounds (Choi et al., 1983). The line at 1519 cm^{-1} originates from cytochrome a^{2+}, while in the region of the other two lines there are also contributions from cytochrome a_3^{2+} [1569(ν_{37}?) and 1579(ν_2?) cm^{-1}]. Primarily on the basis of the 1519-cm^{-1} line, we conclude that upon freezing cytochrome *c* oxidase the cytochrome *a* core contracts. The magnitude of this contraction based on the core-size correlation (Choi et al., 1983) is ~0.01 Å. (The core size is defined as the distance between the heme center and the pyrrole nitrogen atoms.) At present it is not known if the heme core contraction of cytochrome *a* is a consequence of specific interactions in cytochrome oxidase or is a general phenomenon for six-coordinate bis(imidazole) hemes. There are clear intensity differences between the room temperature and cryotrapped samples in the high-frequency region (1550–1650 cm^{-1}). Since both hemes have contributions to the spectra throughout this region, it is difficult to assign the differences. However, cytochrome a_3 has lines at 1565 and 1579 cm^{-1} at room temperature (Ching et al., 1985; Argade et al., 1986). The relative intensity of these two lines (possibly ν_{37} and ν_2) may depend on the degree of heme distortion. If the distortion changes upon freezing, the resulting changes in the relative intensities could account for our observed data. Spectral separation in the cryotrapped samples, as has been carried out in room temperature samples (Ching et al., 1985; Argade et al., 1986), could resolve this point.

Laser-Induced Changes. Several Raman active modes were found to change frequency when the laser intensity was increased by bringing a tighter focal spot on the cryotrapped sample at 77 K. Clear changes were detected only in lines assignable to cytochrome a_3. For example, laser-induced changes were detected in the lines at 216 cm^{-1} (Fe–His stretching mode of cytochrome a_3) and in the line at 1666 cm^{-1} (the C–O stretching mode of the formyl group). (This mode in cytochrome *a* is either silent in the reduced form or is at lower frequency.) The lines from cytochrome *a* that were sensitive to freezing did not change when the laser-power density at the sample was increased.

To obtain a better understanding of the nature of the changes, we have computer resolved the carbonyl stretching mode of the formyl group into its component lines. The results of this fitting procedure are shown in Figure 7. For each sample we have normalized the intensity to the 1666-cm^{-1} line. In each case (for both reduced and photodissociated samples) when the laser-power density is increased, the intensity of a high-frequency component increases substantially. In the fully reduced enzyme the intensity of the higher frequency component is 25% that of the 1666-cm^{-1} line at low laser power, and it is not detected in the spectrum of the photodissociated species at low-power density.

There is no change in the peak position (1666–1667 cm^{-1}) of the carbonyl stretching mode of the formyl group upon photodissociation (Figures 4 and 7) at low laser power density, although there are large changes in the Fe–His stretching mode (Figure 3). Thus, these two modes do not appear to be coupled. Consequently, separate interactions must give rise to the shifts in these two cases. These results are consistent with those of Ogura et al. (1984), who found that the intensities of the Fe–His stretching mode and the carbonyl stretching mode of the formyl group had different dependencies on incubation temperature and pH.

The two contributions in the spectral region of the carbonyl stretching mode of the formyl group are separated by about 8 cm^{-1}. The range of values reported by Choi et al. (1983) for frequency shifts due to changes in H-bonding were 10–20 cm^{-1} for the full range of H-bonded to non-H-bonded formyl groups. Thus, our shift of 8 cm^{-1} is fully consistent with such

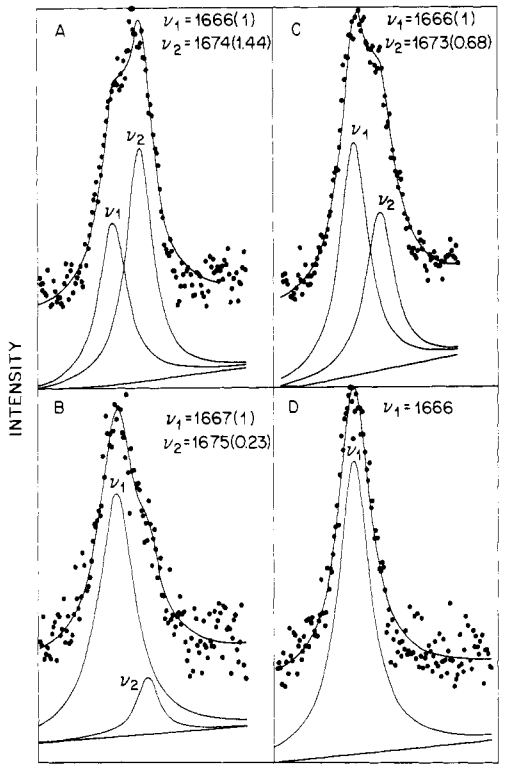

FIGURE 7: Data and spectral fits of the carbonyl stretching mode of the formyl group of reduced and photodissociated cytochrome *c* oxidase at 77 K. The fits were made with Lorentzian lines, but equivalent results were obtained with Gaussian lines. The results of the fits are listed near each spectrum. The frequency of each line (in cm^{-1}) is followed by its intensity in parentheses. In each case the line intensity is normalized to the intensity of the line at 1666 cm^{-1}. (A) Fully reduced ligand-free enzyme at high laser power density. (B) Fully reduced ligand-free enzyme at low power density. (C) Photodissociated CO-bound enzyme at high laser power. (D) Photodissociated CO-bound enzyme at low laser power.

a change. We assign the line at 1666 cm^{-1} as originating from a weakly H-bonded structure of cytochrome a_3. We speculate that the higher frequency component at about 1674 cm^{-1} corresponds to the non-H-bonded form. By inference, in the room temperature solution data, the line at 1664 cm^{-1} would then represent a weakly H-bonded carbonyl group of cytochrome a_3 in the reduced enzyme. Thermal effects at the heme due to elevated laser power could cause the H-bond to rupture, possibly due to orientational changes of the heme group. Although the hydrogen-bonding change is an attractive possibility to account for the frequency differences we detect, we cannot rule out a conformational change of the formyl group without a change in degree of H-bonding as the origin of these differences. Also, it cannot be determined from the present data if the multiple forms we detect are related to those reported in the infrared spectra by Fiamingo et al. (1982).

The other changes in the spectrum of reduced cytochrome a_3 in the 77 K cryotrapped samples at increased laser power appear to cause the vibrational modes to shift toward their fully reduced room temperature solution values. The same is true in the photodissociated samples. Thus, increased laser power may essentially anneal the heme and its surroundings such that the structure of the heme environment that was frozen-in when the sample was brought to cryogenic temperature relaxes. This makes it essential to carry out cryogenic temperature experiments at low laser power. Moreover, the experiments reported here were done in an immersion Dewar in which the sample was completely surrounded by either liquid nitrogen or liquid helium. However, this did not prevent thermal effects from occurring. Thus, at high laser power thermal heating can occur locally at the heme and modify its properties without affecting the bulk sample, which would be expected to maintain the cryogenic temperature due to the immersion. These effects are reduced at 4 K compared to those at 77 K, but thermal effects are clearly still present even at the lower temperature. The reduced magnitude of the effects at 4 K compared to those at 77 K is consistent with our thermal annealing hypothesis.

Changes upon Photodissociation. The only large change upon photodissociation occurs in the Fe–His stretching mode. At low laser power, this line moves from 216 cm^{-1} in the fully reduced enzyme to 224 cm^{-1} in the photodissociated enzyme. This 8-cm^{-1} change is consistent with, albeit a bit larger than, the change we detect in the transient spectrum (10 ns) at room temperature where the line shifts from 213 to 219 cm^{-1}. We invoke the same mechanism for this frequency shift as has been proposed for similar changes in hemoglobin and myoglobin (Ondrias et al., 1983b; Sassaroli et al., 1986). Namely, the CO-bound heme has an iron atom that is mainly in-plane, thus forcing the histidine to assume an orientation perpendicular to the heme plane. Upon photodissociation, the iron can move out of the heme plane. However, the histidine maintains an orientation perpendicular to the plane rather than tilting to its position in the fully reduced enzyme. This latter motion requires a large movement of the polypeptide chain to which the histidine is bound and thereby does not occur at low temperature or on a time scale as short as 10 ns. The similarity of this behavior in both the room temperature and the cryogenic temperature samples demonstrates that freezing does not influence the degrees of freedom giving rise to these changes. In this sense, cytochrome *c* oxidase behaves like hemoglobin, which also displays an identity of behavior at room temperature and at cryogenic temperature, but dissimilar from myoglobin, in which no frequency changes are detected in the Fe–His stretching mode at room temperature on time scales as short as 30 ps (Findsen et al., 1985), whereas frequency differences are detected in cryotrapped samples (Sassaroli et al., 1986).

The only other change upon photodissociation that can be readily identified in the spectra occurs in the relative intensity of the line at 1571 cm^{-1}. This line appears much weaker in the spectrum of the photoproduct than in the spectrum of the ligand-free fully reduced enzyme in the 77 K cryotrapped samples. In contrast, the intensity of this mode in the photoproduct at 4 K is increased such that it is the same as that in the fully reduced spectrum. This region of the spectrum has a strong overlap between cytochromes *a* and a_3 (Ching et al., 1986), and therefore we cannot make definite assignments of the origins of these differences.

The Fe–His stretching mode of the photoproduct behaves qualitatively different at 4 K than it does at 77 K. At the higher temperatures we find that there are two well-defined frequencies—one at 224 cm^{-1} and one at 216 cm^{-1}. Extended exposure leads to greater formation of the lower frequency component. At the lower temperature (4 K) extended laser exposure causes the line to shift down by 2–3 cm^{-1}. This behavior is similar to that seen in room temperature time-resolved studies. Findsen and Ondrias (1984) reported that the Fe–His stretching mode shifted by $\sim$10 cm^{-1} in the photoproduct to higher frequency (214–224 cm^{-1}). Argade and Rousseau (unpublished results) could only detect a shift of 6 cm^{-1} with 10-ns pulses (Figure 6). Although the origin of this difference in behavior was never identified, Findsen and Ondrias (1984) reported that low laser power was necessary to detect the larger frequency shift and that high laser power yielded a smaller shift.

The time-resolved results coupled with the results reported here at 4 K indicate that there may be two different metastable intermediates that are generated in the CO photoproduct. In the first the Fe–His shift from the ligand-free reduced state is greatest. The second state is somehow annealed by the laser. Thus, at low laser power where thermal effects are held to a minimum, an intermediate is formed that can be partially annealed at increased laser power density. From the present data, the precise forms of these intermediates cannot be determined. The simplest explanation for these data would be various degrees of relaxation of the heme pocket. Partial stages of relaxation have been detected, for example, in hemoglobin (Friedman, 1985) and myoglobin (Rousseau & Argade, 1986; Sassaroli et al., 1986). Another possible source of partially relaxed states could be the presence or absence of CO in the heme pocket following photodissociation. This has also been cited as responsible for hemoglobin relaxation (Hofrichter et al., 1983, 1985). In cytochrome *c* oxidase Alben et al. (1981) and Fiamingo et al. (1982) have reported that CO binds to the Cu_B at cryogenic temperatures. Additional experiments in both the cryogenic and time-resolved regimes should clarify the origin of these intermediate states and under what conditions CO remains in the heme pocket after photodissociation as well as its possible influence on heme relaxation.

The observation that the photoproduct of cytochrome *c* oxidase differs from the fully reduced enzyme has implications concerning functional studies of this enzyme. Flow-flash measurements, in which a mixture of CO-bound cytochrome *c* oxidase is mixed with an O_2-saturated solution followed by photolysis of the CO for subsequent O_2 binding, have been used extensively in the past to study the enzyme structure, function, and kinetics (Gibson & Greenwood, 1963; Antonini & Brunori, 1977; Hill & Greenwood, 1984). If the O_2 binds to a heme that has not fully relaxed, then the properties of that state may differ from those that result from binding to a fully relaxed state. Similarly, the use of photolysis of CO at low temperature to form reaction intermediates is taken advantage of in the triple-trapping technique (Chance et al., 1975; Clore & Chance, 1978; Blair et al., 1985). One must be concerned with whether or not the oxygen binds to an unrelaxed form of the enzyme in these experiments. If so, the structural and kinetic properties of such an intermediate may not be the same as those that would result from binding to a fully relaxed form of the enzyme. Time-dependent studies on the enzyme at both room temperature and cryogenic temperature should resolve these points.

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