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Resonance Raman Spectra of CN^--Bound Cytochrome Oxidase: Spectral Isolation of Cytochromes a^{2+}, a_3^{2+}, and $a_3^{2+}(CN^-)$

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ABSTRACT: Reduced cyanide-bound cytochrome oxidase in the absence of any oxygen gives a resonance Raman spectrum consistent with that expected for low-spin heme *a*. Thus, in contrast to prior reports, ligand binding of cytochrome a_3 to form a six-coordinate low-spin ferrous heme does not result in any unusual electronic structure, hydrogen bonding, environment, or conformation of the formyl group. It appears unlikely that there are any changes in this group in cytochrome a_3 that control the ligand affinity or redox potential in physiological forms of the ferrous enzyme. With the use of our difference spectrometer and by appropriately selecting the laser excitation frequency, we are able to isolate spectrally cytochromes a^{2+}, a_3^{2+}, and $a_3^{2+}(CN^-)$. The addition of a small amount of oxygen to a preparation of the cyanide-bound reduced enzyme results in a complex with the same Raman spectrum as that previously reported to originate from the cyanide-bound reduced complex. Any oxygen present in the sample leads to enzyme turnover resulting in a mixed valence state $[a^{2+}a_3^{3+}(CN^-)]$. The comparison between the data on the cyanide-bound reduced enzyme and the data on the CO-bound reduced enzyme illustrates that cyanide binding affects only the modes that respond to the spin state of the ferrous iron, while CO binding affects vibrational modes that respond to a π-electron density change as well.

Cytochrome oxidase is the terminal enzyme in the electron-transport chain in mitochondria. It reduces dioxygen to water and generates a proton gradient across the mitochondrial membrane. This enzyme has four redox centers, which consist of two heme *a* chromophores referred to as cytochromes *a* and a_3 and two Cu centers. The iron atom in cytochrome *a* is six-coordinate and low spin in both the 2+ and 3+ oxidation states and does not bind any external ligands. The iron atom in cytochrome a_3 is five-coordinate and high spin in the ferrous state and six-coordinate and high spin in the ferric (resting) state. O_2 binds to ferrous cytochrome a_3 where it is reduced to water. [For a detailed review, see Wikström et al. (1981)].

In order to understand the molecular mechanism of this enzyme's function, it is necessary to characterize the structure of both types of cytochromes in the various intermediates following the binding of molecular oxygen. However, study of the enzyme bound to the physiologically important ligand, O_2, is difficult by conventional resonance Raman methods because of its rapid reduction to water. In addition, for the interpretation of the resonance Raman spectra of O_2-bound complexes, it is necessary to separate the contributions of cytochromes *a* and a_3 and then find the sensitivity of the Raman lines to oxidation-state changes and spin-state changes as well as other ligand-induced and protein-induced perturbations. An approach to such an understanding is to study the spectra of the enzyme with alternate ligands such as carbon monoxide, cyanide, azide, or sulfide bound to the cytochrome a_3. In other papers we examined the geometry of the CO ligand bound to cytochrome a_3 (Argade et al., 1984a) and the behavior of the porphyrin modes upon CO binding (Argade et al., 1986). In this paper we focus on CN^- binding. CN^- inhibits the function of this enzyme; hence, the study of the CN^--bound complex has physiological importance as well.

Resonance Raman spectra of CN^--bound reduced cytochrome oxidase have been reported in the past, and the results were quite surprising. The mode associated with the C=O stretching vibration of the formyl group from cytochrome a_3, which is found at 1671 cm^{-1} for the oxidized enzyme and 1664 cm^{-1} for the reduced enzyme, was reported to disappear in the CN^--bound reduced enzyme (Salmeen et al., 1978) and in cyanide-bound mitochondria (Adar & Erecinska, 1979). In contrast, this mode is observed at 1660 cm^{-1} in reduced high-spin (2-methylimidazole) heme *a* model compounds and at 1644 cm^{-1} in the reduced low-spin [bis(imidazole)] heme

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a model compounds (Van Steelandt-Frentrup et al., 1981). This difference implied a special role played by the protein upon cyanide binding. From the disappearance of the formyl stretching mode in the CN^--bound reduced enzyme, it was concluded that the binding of O_2 to the heme iron would lead to a change in the formyl group geometry (presumably move it out of the porphyrin plane), which might be an important factor in both ligand affinity and redox potentials (Babcock & Salmeen, 1979; Babcock & Chang, 1979). However, the finding of the disappearance of the formyl group in the low-spin cytochrome a_3 is inconsistent with the behavior of the above-mentioned model compounds and the behavior of the low-spin CO-bound reduced enzyme (Argade et al., 1986). In the latter case, the formyl group was found to shift from 1664 up to 1666 cm^{-1}. The difference between the behavior of the CO-bound enzyme and the model compounds was rationalized as resulting from a compensating effect of the spin-state change and an electronic perturbation of the porphyrin π-orbitals due to the presence of the CO.

In view of the inconsistency between the reported results on the cyanide-bound enzyme and those in these other systems, we have reinvestigated CN^- binding to cytochrome oxidase. Our CN^- binding studies show that the samples used in the previously reported spectra (Salmeen et al., 1978) of the CN^--bound reduced enzyme may be contaminated with oxygen which caused the enzyme to turn over and leave the cytochrome a_3(CN) clamped in the ferric oxidation state. Thus, the reported spectra were in fact those of CN^- bound to the mixed-valence enzyme. We find that the behavior of the formyl group in the CN^--bound reduced enzyme is consistent with that found in the low-spin heme *a* model compound. Furthermore, we introduce a method for the spectral isolation of cytochromes a^{2+}, a_3^{2+}, and $a_3^{2+}(CN^-)$.

Materials and Methods

Mammalian (beef heart) cytochrome oxidase was separated by the method described by Babcock et al. (1976) and frozen under liquid N_2 until ready for use. The buffer–detergent system used was 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) at pH 7.4 with 1% dodecyl β-D-maltoside. For optical absorption measurements, the enzyme concentration was 10 μM, and for the resonance Raman measurements, it was 50 μM (for 441.6-nm excitation) or 80 μM (for 413.1-nm excitation). The fully reduced preparation was made by dithionite reduction of the resting enzyme under nitrogen. The cyanide was prepared at 500 mM, neutralized to pH 7.9, and stored in a sealed container. This stock solution was added to the oxidase preparation (pH 7.4) to give a final cyanide concentration of about 20 mM. Mixed-valence cyanide-bound preparations [$a^{2+}a_3^{3+}(CN^-)$] were made by adding dithionite or ascorbate–TMPD (tetramethyl-phenylenediamine) to an aerobic preparation of the CN^--bound resting enzyme. The samples were placed in either a one- or a two-compartment spinning cell and sealed under nitrogen for the resonance Raman measurements. The resonance Raman apparatus has been described before (Rousseau, 1981). In the Raman data presented here, a linear base-line correction was made on the spectra, which were then faithfully traced without any smoothing. The spectral resolution is approximately 4 cm^{-1}, and the peak positions assigned are correct within ± 1 cm^{-1}.

Results

In Figure 1 a series of absorption spectra are presented. The change in the visible band of the reduced enzyme (Figure 1A) upon binding CN^- (Figure 1B) is consistent with that reported previously (Van Buuren et al., 1972). Upon briefly exposing the sample used in Figure 1B to air, the spectrum shown in Figure 1C resulted with a clear doublet in the Soret region. This spectrum is the same as the CN^--bound mixed-valence enzyme displayed in Figure 1D. These data show that completely anaerobic conditions are essential in order to prepare the CN^--bound reduced enzyme. The changes in the Soret and visible band of the resting enzyme (Figure 1F) upon

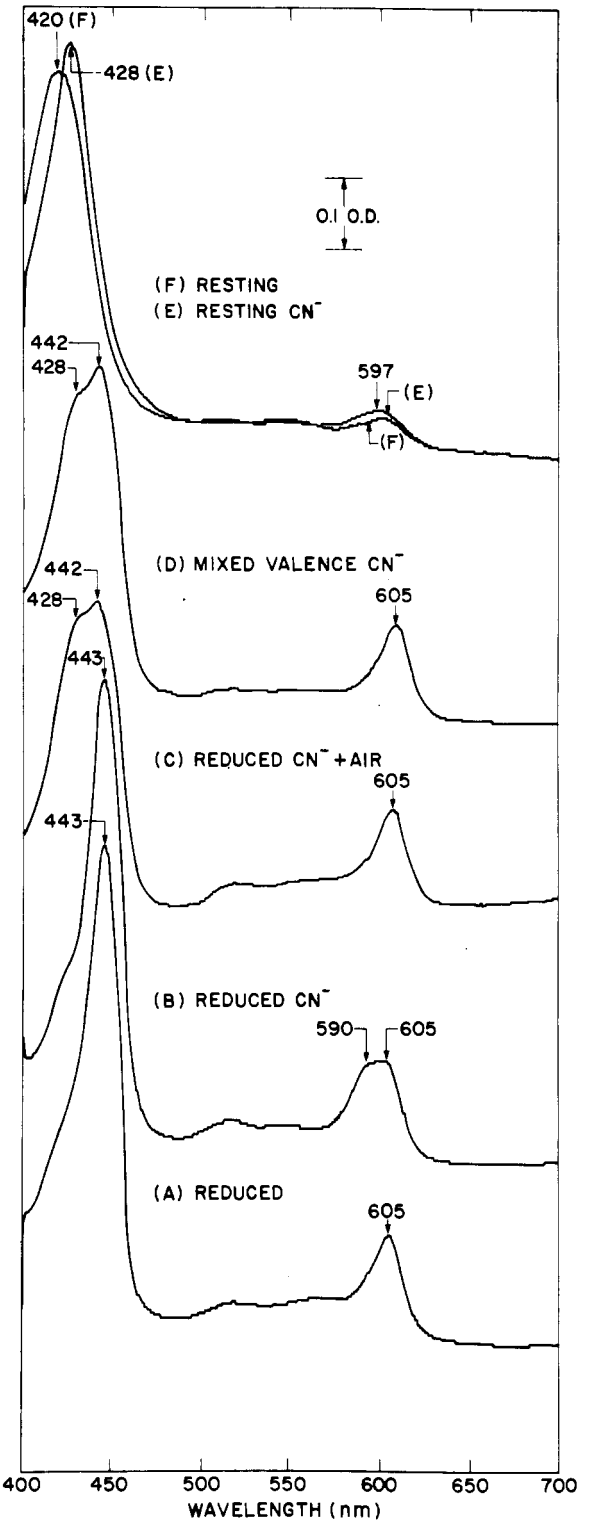

FIGURE 1: Absorption spectra of various forms of cytochrome oxidase: (A) dithionite-reduced enzyme; (B) dithionite-reduced enzyme + 20 mM anaerobic CN^-; (C) sample used in (B) briefly exposed to air; (D) resting enzyme + 20 mM CN^- + dithionite; (E) resting enzyme + 20 mM CN^-; (F) resting enzyme. The concentration of the enzyme was 10 μM in 50 mM Hepes, pH 7.4, and 1% dodecyl β-D-maltoside.

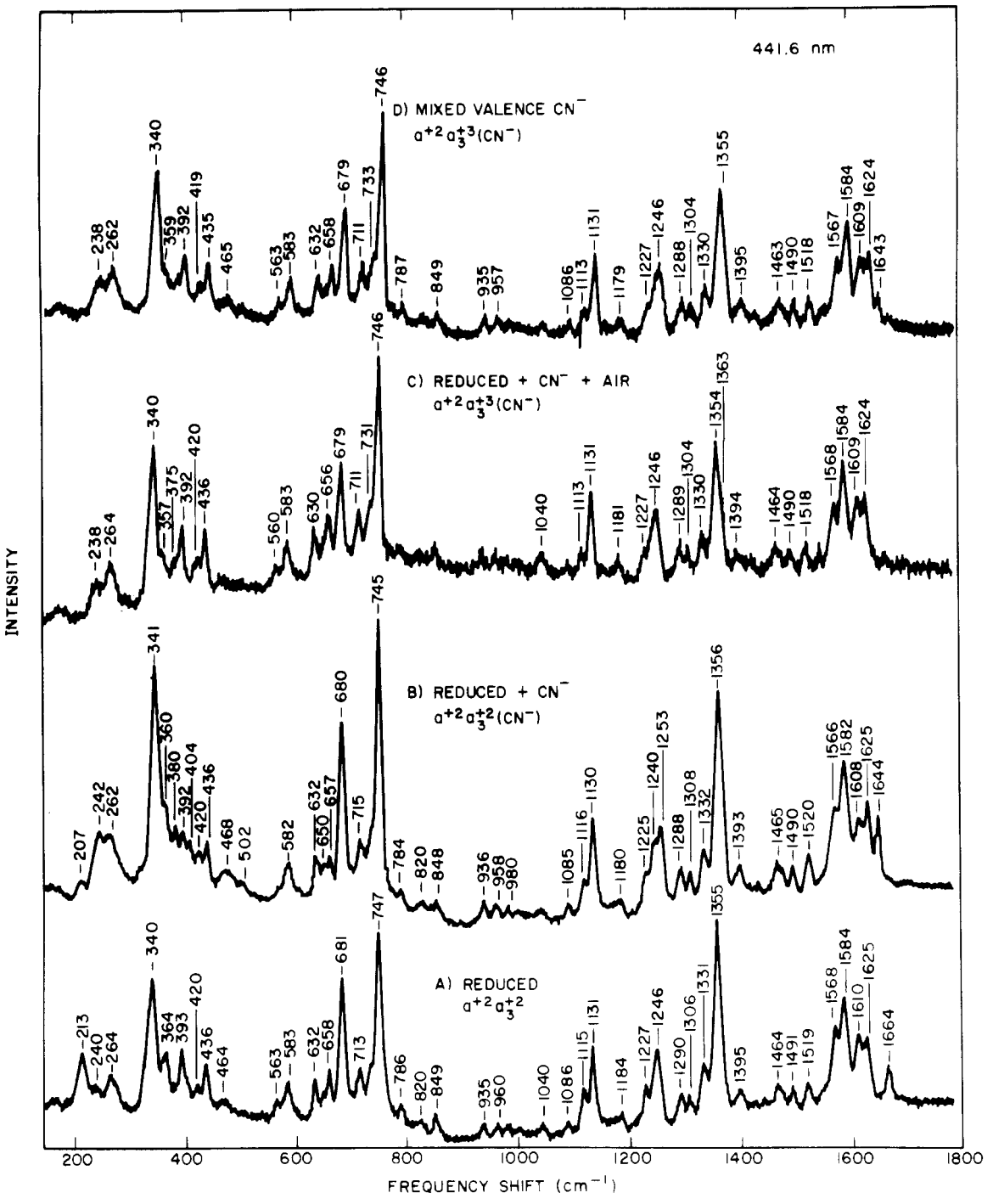

FIGURE 2: Resonance Raman spectra of (A) dithionite-reduced enzyme, (B) dithionite-reduced enzyme + 20 mM anaerobic CN^-, (C) the sample used in (B) briefly exposed to air, and (D) resting enzyme + 20 mM CN^- + dithionite. The concentration of the enzyme was 50 μM; exciting laser wavelength was 441.6 nm with 20-mW power; each spectrum was averaged for ~8 h.

binding CN^- (Figure 1E) are also consistent with those reported previously (Van Buuren et al., 1972).

The resonance Raman spectra of several cytochrome oxidase complexes obtained with 441.6- and 413.1-nm excitation are shown in Figures 2 and 3, respectively. Figure 2A and 3A show the resonance Raman spectra of the fully reduced enzyme, which are in agreement with the previously reported spectra (Babcock et al., 1981) with the exception of the reduced enzyme from thermophilic PS_3 bacteria where changes in the low-frequency region are detected and are attributed to a different a_3 heme environment (Ogura et al., 1984). Spectra in Figures 2B and 3B are those of the cyanide-bound reduced enzyme. Evidence for six-coordination may be seen by the disappearance of the five-coordinate iron–histidine stretching mode at 213 cm^{-1} (see Discussion) in the 441.6-nm excitation spectrum. Other prominent changes include an increase in intensity at 242 cm^{-1}, changes in the 350–450-cm^{-1} region, the decrease in intensity of the line at 1227 cm^{-1} and the appearance of a new line at 1253 cm^{-1}, the decrease in intensity of the lines at 1567 and 1610 cm^{-1}, and the shift in frequency of the line at 1664 to 1644 cm^{-1}.

When a small amount of air is admitted to a cyanide-bound reduced enzyme, the spectra shown in Figures 2C and 3C resulted. Again, in the 441.6-nm spectrum (Figure 2C), the line at 213 cm^{-1} is observed to disappear. However, the spectrum is very different from that of the CN^--bound reduced enzyme (Figure 2B). In particular, note the structure in the 350–450-cm^{-1} region, the intensity of the strong pair of lines

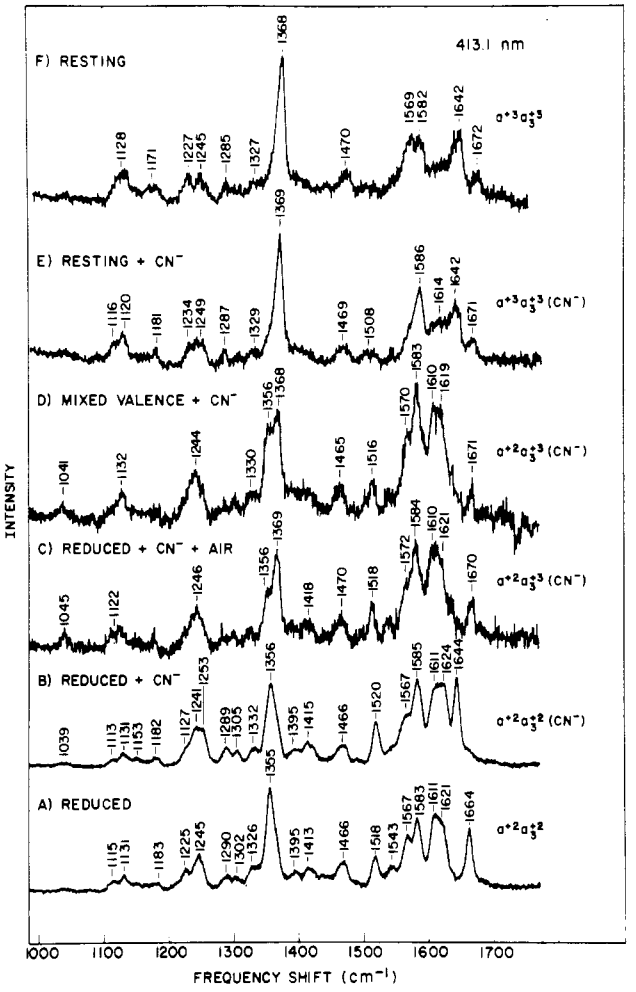

FIGURE 3: Resonance Raman spectra of (A) dithionite-reduced enzyme, (B) dithionite-reduced enzyme + 20 mM anaerobic CN^-, (C) sample used in (B) briefly exposed to air, (D) resting enzyme + 20 mM CN^- + dithionite, (E) resting enzyme + 20 mM CN^-, and (F) resting enzyme. The concentration of the enzyme was 80 μM; the exciting laser wavelength was 413.1 nm with 30-mW power; each spectrum was averaged for ~4 h.

at 679 and 746 cm^{-1} and the absence of any line at 1253 cm^{-1}, the presence of a high-frequency shoulder on the 1354-cm^{-1} line in the 441.6-nm excitation spectrum (Figure 2C) and a clear doublet at 1356 and 1369 cm^{-1} in the 413.1-nm spectrum (Figure 3C), and the disappearance, with 441.6-nm excitation, of the line at 1644-cm^{-1} and the appearance of a line at 1670 cm^{-1} with 413.1-nm excitation. Spectra similar to those in Figures 2C and 3C are obtained if the enzyme is reduced with ascorbate–TMPD rather than dithionite, but with ascorbate–TMPD the additional exposure to air is unnecessary. Unlike reduction with dithionite, which eliminates all the oxygen, with the ascorbate–TMPD reduction the oxygen is not completely excluded.

The spectra in Figures 2D and 3D are those of the cyanide-bound mixed-valence enzyme [$a^{2+}a_3^{3+}(CN^-)$]. The near identity of these spectra to the spectra generated by the addition of a small amount of air to the CN^--bound reduced sample is self-evident. The small differences between these two spectra that we can detect (the ratio of the lines at 1356 and 1368 cm^{-1} and a weak line at 1643 cm^{-1} in the spectrum of Figure 2D) we attribute to some reduction of the cytochrome a_3 chromophore in the mixed-valence preparation.

Spectra of Figure 3E,F show the resting and CN^--bound oxidized preparations, respectively, at 413.1-nm excitation. These are in agreement with those reported by others (Ondrias & Babcock, 1980; Babcock et al., 1981). The primary changes that are detectable upon cyanide binding to the resting enzyme involve a change in the doublet at 1227 and 1245 cm^{-1}, a shift in the line at 1569 and 1586 cm^{-1}, and the appearance of additional intensity near 1614 cm^{-1}. The formyl mode of cytochrome a_3 at 1671 cm^{-1} is unaffected by cyanide binding in the oxidized state.

Discussion

The addition of cyanide to the fully reduced cytochrome oxidase preparation leads to a six-coordinate species. MCD studies of CN^--bound reduced cytochrome oxidase have shown that the iron atom in this six-coordinate ferrous enzyme is low spin (Babcock et al., 1976). The coordination state change is confirmed in our data by the disappearance of the line at 213 cm^{-1} (see Figure 2B). This line has been assigned as the iron–histidine stretching mode in high-spin ferrous heme *a* model compounds and in cytochrome oxidase from thermophilic bacteria (Ogura et al., 1983), consistent with the assignment of a line in the 200–230-cm^{-1} region in proteins with heme *b* as the prosthetic group (e.g., Hb and Mb) as well as in various heme *b* model compounds (Kitagawa et al., 1979; Hori & Kitagawa, 1980; Nagai et al., 1980; Argade et al., 1984b). This mode has not been found in the Raman spectrum of heme proteins when the iron is six-coordinated (Rousseau et al., 1984).

Reduced and Mixed-Valence CN^--Bound Oxidase. The spectrum we obtain for the cyanide-bound reduced enzyme is significantly different from that reported by other workers on the reduced enzyme (Salmeen et al., 1978) and on intact mitochondria (Adar & Erecinska, 1979). Most striking is the absence of the formyl mode in the previously reported spectra with 441.6-nm excitation in contrast to our data (Figure 2B) in which we find a line at 1644 cm^{-1}, which we assign as originating from the carbonyl stretching mode of the formyl group. The spectra of the CN^--bound reduced cytochrome oxidase reported by others is the same as that we get when a small amount of air is added to the CN^--bound reduced enzyme preparation (Figure 2C). Moreover, the absorption and resonance Raman spectra of the CN^--bound reduced enzyme to which air was added are the same as the corresponding spectra of the CN^--bound mixed-valence enzyme [$a^{2+}a_3^{3+}(CN^-)$]. We therefore conclude that in previously reported spectra the samples were contaminated with a small amount of oxygen. We postulate that the presence of oxygen in a preparation containing dithionite causes the enzyme to turnover. When it is in the oxidized state, the cyanide forms a tightly bound complex with the cytochrome a_3^{3+}. The binding affinity of the enzyme for CN^- is 2 orders of magnitude higher in the oxidized enzyme than in the fully reduced enzyme (Van Buuren et al., 1972; Naqui et al., 1984). The dithionite maintains the cytochrome *a* in the 2+ valence state, resulting in a mixed-valence form of the enzyme since the CN^- blocks reduction of the a_3^{3+} heme. We point out that from the studies by Adar & Erecinska (1979) this form of the enzyme appears to occur in mitochondria when cyanide is present as a respiratory inhibitor.

The reported absence of any line in the formyl mode region of cytochrome a_3 upon the addition of cyanide to reduced cytochrome oxidase by other workers can now be accounted for by the variation in the resonance enhancement upon formation of the mixed-valence state. For the CN^--bound mixed-valence enzyme, the Soret absorption maximum of the oxidized cyanide-bound cytochrome a_3^{3+} heme is blue shifted at 428 nm whereas that of reduced cytochrome a^{2+} remains unshifted at 442 nm (Figure 1C,D). Thus, with 441.6-nm

excitation the enhancements of the modes from cytochrome a_3^{3+} are weak, and hence, the formyl stretching is not observed. However, when we go to the deeper blue excitation (413.1 nm), a mode at 1671 cm^{-1} appears (Figure 3C,D), the frequency obtained for the formyl mode line in the CN^--bound cytochrome a_3 in the 3+ oxidation state (Figure 3E).

Spectral Isolation of Each Cytochrome. The frequency splitting of the Soret absorption of cytochrome oxidase when ligands are added to cytochrome a_3 leads to the possibility of isolating the Raman spectra of cytochrome *a* and cytochrome a_3 through proper use of the excitation frequency. This has been pointed out in the past, and certain lines from each heme have been identified (Salmeen et al., 1978; Babcock et al., 1981; Woodruff et al., 1981). The actual separation of the Raman spectra that maybe obtained is difficult to predict since the Raman scattering intensity need not follow the absorption intensity and the intensity profile is not the same for all the modes (Champion & Albrecht, 1979). However, with some prior knowledge of the behavior of certain modes under various conditions (e.g., oxidation state, effect of ligand binding, etc.) the degree of separation may be determined empirically. We adopt that approach here and show that excellent separation may be obtained in some cases. The specific mechanism of the excitation-frequency dependence of the Raman intensity is discussed in a separate paper (Argade et al., 1986).

Examination of the spectra of the CN^--bound mixed-valence enzyme with 413.1-nm excitation (Figure 3C,D) shows that the contributions of both cytochromes $a_3^{3+}(CN^-)$ and a^{2+} are comparable. This is inferred from the intensity of the respective electron-density marker lines at 1368 and 1356 cm^{-1}. On the contrary, the spectrum with 441.6-nm excitation (Figure 2C,D) is dominated by cytochrome a^{2+} with only a minor contribution from cytochrome $a_3^{3+}(CN^-)$. This is clear from the weakness of the shoulder at 1363 cm^{-1} and the weakness of the formyl mode (1671 cm^{-1}). We esimate that the contribution of cytochrome $a_3^{3+}(CN^-)$ to the spectrum in Figure 2C is less than 10% of the cytochrome a^{2+} contribution. Hence, within this limitation the spectrum in Figure 2C is that of reduced cytochrome *a*. A similar situation arises in the case of the CO-bound reduced enzyme where the Soret absorption maximum of CO-bound cytochrome a_3^{2+} shifts to 430 nm whereas that of cytochrome a^{2+} remains unshifted at 443 nm. Thus, with 441.6-nm excitation, the resonance Raman spectrum is dominated by cytochrome *a*, and with 413.1-nm excitation, it is dominated by CO-bound cytochrome a_3^{2+} although the separation is not as good as in the case reported here (Argade et al., 1986). Since it is possible to get the spectrum of cytochrome a^{2+} with different oxidation and ligation state of cytochrome a_3, the magnitude of the heme–heme interaction in cytochrome oxidase is now being assessed by resonance Raman spectroscopy (Argade et al., 1985).

With 441.6-nm excitation, the spectrum of the fully reduced enzyme (Figure 2A) is a mixture of cytochromes a^{2+} and a_3^{2+}. Thus, by subtraction of the spectrum of cytochrome a^{2+} obtained above (Figure 2C and 4A) from the fully reduced enzyme (Figure 2A), a spectrum of cytochrome a_3^{2+} can be obtained. Such a difference spectrum is shown in Figure 4B. In order to overcome the spectrometer resettability problem, the spectra of the reduced enzyme and the CN^--bound mixed-valence enzyme were recorded simultaneously in a two-compartment spinning cell (Rousseau, 1981). As in the ligand-free enzyme, with 441.6-nm excitation, the spectrum of the CN^--bound reduced enzyme (Figure 2B) is a mixture of cytochromes a^{2+} and $a_3^{2+}(CN^-)$. Thus, by subtraction of the spectrum of cytochrome a^{2+} (Figures 2C and 4A) from the spectrum of the cyanide-bound reduced enzyme, a spectrum of cytochrome $a_3^{2+}(CN^-)$ can be obtained, as shown in Figure 4C.

In generating spectra of specific intermediates by subtraction of a spectrum of cytochrome *a* from the composite spectrum, several assumptions must be made. We discuss these in detail in a separate paper (Argade et al., 1986), but we reiterate them here also. (1) We have quantitatively subtracted out from the composite spectrum the spectrum of cytochrome *a*. (2) The contribution of CN^--bound cytochrome a_3^{3+} is negligible in the spectrum of the mixed-valence enzyme $[a^{2+}a_3^{3+}(CN^-)]$ with 441.6-nm excitation. (3) There is no spectral heme–heme interaction (modification of cytochrome *a* Raman modes due to binding and/or changes in oxidation of cytochrome a_3). (4) There is no direct interaction between CN^- and cytochrome *a*. (5) There are no contributions in the spectrum from the Cu chromophores. We address these assumption below.

First, in the data reported here we find that, by varying the magnitude of cytochrome *a* that we subtract, some relative intensities change but frequencies do not. Thus, this parameter does not introduce serious errors into the data. Second, from the near complete absence of any scattering intensity at 1368 cm^{-1} in the spectrum of cytochrome $a^{2+}a_3^{3+}(CN^-)$ with 441.6-nm excitation, it is apparent that the contribution of cytochrome $a_3^{3+}(CN^-)$ to this spectrum is very small (<10%). Third, there are no reports of significant heme–heme interaction in the spectral properties of cytochrome *a* upon binding CN^- or, forth, of a direct interaction between CN^- and cytochrome *a*, although these absences could result from lack of sensitivity of other techniques. A way of addressing the question of spectral heme–heme interaction is to compare the spectra of cytochrome *a* obtained in the mixed-valence cyanide-bound preparation reported here with those obtained in preparations with other ligands. Such a comparison with cytochrome *a* obtained from a CO-bound preparation reveals no significant differences, implying either identical heme–heme interactions or the absence of any spectal heme–heme interaction in these two cases (Argade et al., 1985). Finally, the possible contributions from Cu centers to our observed spectra cannot be addressed from our present results. We should point out that Woodruff et al. have argued that there are possible contributions in the spectra from Cu centers although other workers (Choi et al., 1983) have questioned this interpretation. Within these potential limitations we may discuss the spectra of the components of the cytochrome oxidase that we have been able to isolate.

Analysis of Isolated Spectra. Comparison of the spectra of cytochromes a^{2+} and a_3^{2+} in Figure 4A,B shows that their structure is different. In the ensuing discussion we show that these results are consistent with cytochrome *a* being six-coordinate low spin and cytochrome a_3 being five-coordinate high spin. The Fe–His stretching mode at 213 cm^{-1} is present in only the spectrum of cytochrome a_3^{2+} as discussed before. Similarly, the formyl mode is present at 1664 cm^{-1} in the spectrum of cytochrome a_3^{2+}, but there is no line above 1625 cm^{-1} in the spectrum of cytochrome a^{2+}. It has been proposed that the formyl line in cytochrome a^{2+} may be so strongly hydrogen bonded that the formyl stretching vibration is shifted down to 1610 cm^{-1} (Callahan & Babcock, 1983). We discuss this mode in greater detail below. The electron-density marker line for both cytochrome a^{2+} and a_3^{2+} is at 1355 ± 1 cm^{-1}, even though the former is six-coordinate and latter is five-coordinate. This line is sensitive to the perturbation of the porphyrin π-orbitals and is found to shift to 1368 cm^{-1} in CO-bound cytochrome a_3^{2+}. Thus, the same position of this

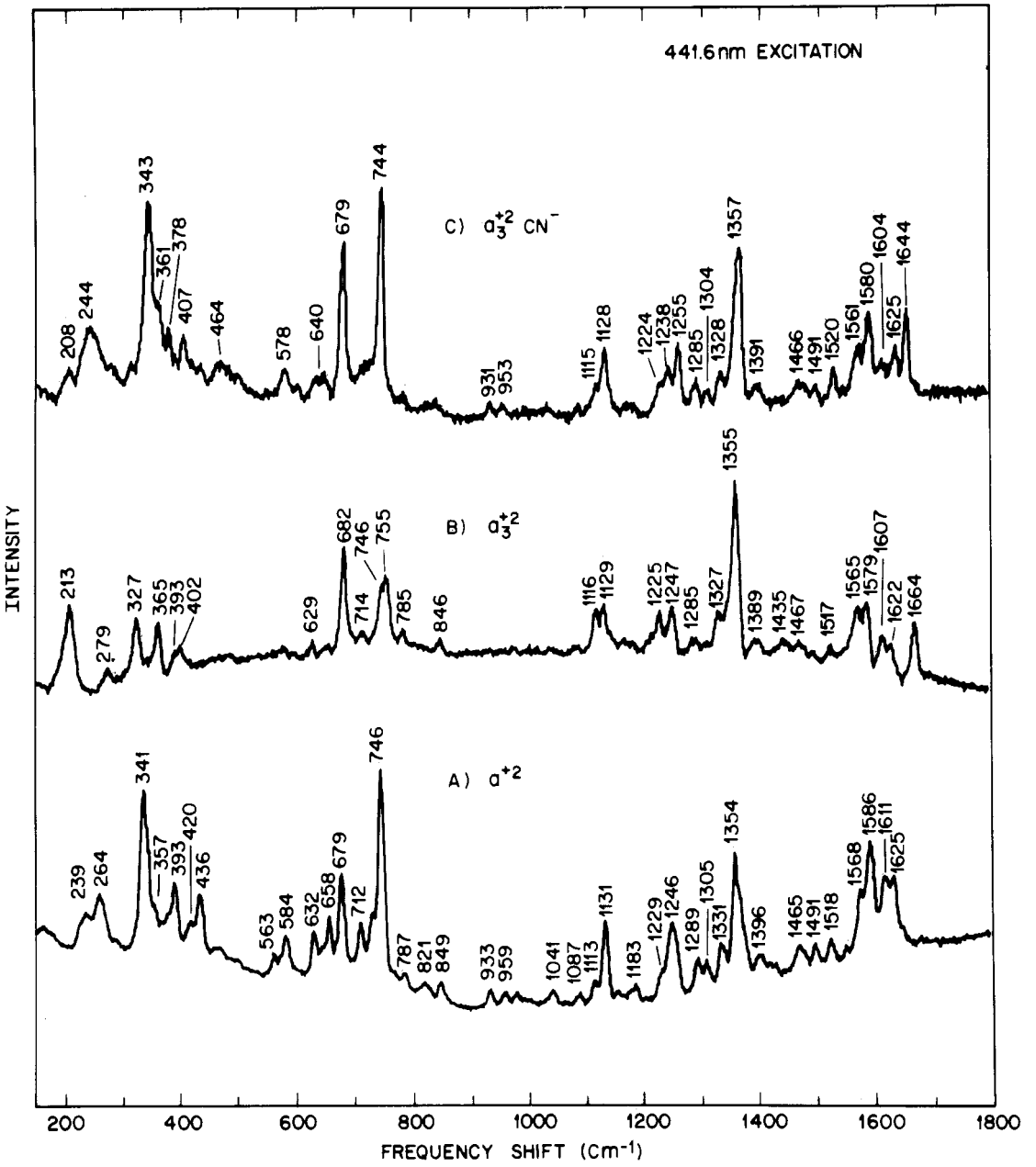

FIGURE 4: Resonance Raman spectra obtained with 441.6-nm excitation. (A) CN^--bound mixed-valence enzyme. This spectrum is predominantly from cytochrome a^{2+}. The spectra of the reduced enzyme and the CN^--bound mixed-valence enzyme were recorded simultaneously in a two-compartment spinning cell. The latter spectrum (i.e., the spectrum of cytochrome a^{2+}) was subtracted from the former to just avoid getting negative peaks, resulting in a spectrum of cytochrome a_3^{2+} shown in (B). Spectra of the CN^--bound reduced enzyme and CN^--bound mixed-valence enzyme were recorded simultaneously in a two-compartment spinning cell. The latter spectrum (i.e., the spectrum of cytochrome a^{2+}) was subtracted from the former to just avoid getting negative peaks, resulting in a spectrum of cytochrome $a_3^{2+}(CN^-)$ shown in (C).

line for both cytochromes a^{2+} and a_3^{2+} indicates that the sixth ligand of cytochrome a does not electronically perturb the porphyrin π-orbitals. This conclusion is also supported by the closeness of the Soret absorption band for the two chromophores and is consistent with model compound data in which there is no significant difference in the electron-density marker line in five- and six-coordinate heme a compounds with imidazole ligands (Van Stellandt-Frentrup et al., 1981).

A line at 327 cm^{-1} is clearly seen in the spectrum of cytochrome a_3^{2+}, which is masked by a strong line from cytochrome a^{2+} at 340 cm^{-1} in the spectrum of the reduced oxidase. In the spectrum of the reduced oxidase from thermophilic bacteria PS_3, a shoulder at 328 cm^{-1} on the 340-cm^{-1} line is clearly seen (Ogura et al., 1984). This indicates that the 327-cm^{-1} line in the spectrum of cytochrome a_3^{2+} is real and our procedure to generate a spectrum of a_3^{2+} is also reliable. The higher intensity of the 328-cm^{-1} line in PS_3 oxidase may indicate a different interaction of the peripheral groups of the heme with the protein as has been proposed before (Ogura et al., 1984).

Heme a has a formyl group at position 8 of the porphyrin periphery. This group has been proposed to modulate the redox potential and to influence the optical absorption spectrum by moving in and out of plane (Seybert et al., 1977; Callahan & Babcock, 1983). In addition, it was suggested that proton translocation may be controlled by hydrogen bonding of the formyl group in cytochrome a (Babcock & Callahan, 1983). Recently, Ogura et al. (1984) have shown that the intensity of the carbonyl stretching mode of the formyl group in cytochrome a_3 is correlated with enzyme activity. Thus, since the formyl group has strong functional implications, an unambiguous understanding of its properties is essential.

Table I: Proposed Assignments of the Carbonyl Stretching Mode of the Formyl Group in Cytochrome Oxidase and Model Compounds

heme	coordination no. and spin state[a]	frequency (cm^{-1})
cytochrome *a*		
Fe^{2+}	6, ls	1610[b]
Fe^{3+}	6, ls	1650[b]
cytochrome a_3		
Fe^{2+}	5, hs	1664[c]
$Fe^{2+}(CO)$	6, ls	1666[d]
$Fe^{2+}(CN^-)$	6, ls	1644
Fe^{3+}	6, hs	1671
$Fe^{3+}(CN^-)$	6, ls	1671
model compounds		
heme a^{2+}(2-MeIm)		
CH_2Cl_2	5, hs	1660[e]
H_2O	5, hs	1640[e]
heme a^{2+}(*N*-MeIm)$_2$		
CH_2Cl_2	6, ls	1645[b]
H_2O	6, ls	1633[b]
heme $a^{3+}(Me_2SO)_2$	6, hs	1672[f]
heme a^{3+}(*N*-MeIm)$_2$	6, ls	1670[b]

[a] hs, high spin; ls, low spin. [b] Babcock & Callahan, 1983. [c] Babcock et al., 1981. [d] Argade et al., 1986. [e] VanStellandt-Frentrup et al., 1981. [f] Choi et al., 1983.

The carbonyl stretching mode, $\nu_{C=O}$, of the formyl group appears in the Raman spectrum in the 1600–1700-cm^{-1} region. (See Table I for a listing of these frequencies.) We consider first the behavior of this mode for cytochrome a_3. In the fully reduced enzyme it is at 1664 cm^{-1}, and it shifts to 1644 cm^{-1} upon binding CN^-. This change is consistent with that observed in heme *a* model compounds. The $\nu_{C=O}$ at 1660 cm^{-1} in high-spin 2-methylimidazole heme *a* model compounds shifts to 1644 cm^{-1} in the low-spin bis(imidazole) complex (Van Steelandt-Frentrup et al., 1981). This shows that the behavior of $\nu_{C=O}$ in the CN^--bound reduced cytochrome oxidase is consistent with a high to low spin state change. Thus, no unusual electronic structure, hydrogen bonding, or out-of-plane rotation needs to be invoked in order to account for the behavior of the formyl mode upon binding CN^-. We contrast this behavior of $\nu_{C=O}$ with that in the CO-bound reduced enzyme where $\nu_{C=O}$ moves *up* 2 cm^{-1} to 1666 cm^{-1} upon ligand binding (Argade et al., 1986), although there is a high spin to low spin state change in this case as well. In a separate paper (Argade et al., 1986), we show that this results from a change in π-electron density of the porphyrin macrocycle upon binding CO, which contributes a frequency increase in $\nu_{C=O}$ compensating for the decrease in the frequency of this line due to the spin-state change. The dependence of $\nu_{C=O}$ on the π-electron density of the porphyrin macrocycle is further supported by the observation that $\nu_{C=O}$ at 1672 cm^{-1} for the oxidized enzyme (Figure 3F) shifts to 1664 cm^{-1} when reduced (Figure 3A) and there is no spin-state change (Babcock et al., 1981). From the above discussion it is clear that the formyl group is sensitive to both the spin state and the oxidation state of the iron. However, changes in this mode along with those in the electron-density marker line and the Soret absorption can be interpreted to obtain formation about the spin-state change in the ferrous heme.

The carbonyl stretching mode of the formyl line in cytochrome *a* in the fully reduced enzyme has been assigned at 1610 cm^{-1} by Callahan & Babcock (1983). They assigned this line on the basis of the absence of lines at this frequency for low-spin ferrous heme compounds, the presence of a line at 1650 cm^{-1} in the ferric enzyme (presumably originating from this mode) and for the reduced enzyme its disappearance at high pH (pH 10) with the concomitant appearance of a line at 1633 cm^{-1}. The anomalously low frequency (1610 cm^{-1}) of this mode in the reduced enzyme has been attributed to a strong hydrogen-bonding interaction that becomes weaker upon oxidation of the heme iron (Babcock & Callahan, 1983). This valence-dependent change in hydrogen-bond strength has been proposed as a mechanism by which valence-state changes are communicated to the protein and as a mechanism for proton translocation (Babcock & Callahan, 1983). However, this assignment has been questioned by Choi et al. (1983) and by Ogura et al. (1984).

Choi et al. (1983) argued that the 1610-cm^{-1} line resulted from ν_{10} of the cytochrome a_3 heme. This is the expected frequency range for ν_{10} to appear in high-spin five-coordinate hemes. Ogura et al. (1984) pointed out that in spectra of ferrous model compounds there is a line at 1608 cm^{-1} that could be ν_{10} although we note that this line is present in the spectra of the high-spin heme *a* model compounds but absent in the spectrum of the low-spin model compounds (Van Steelandt-Frentrup et al., 1981).

The data reported here cannot fully settle the issue of this assignment but we do address some of these proposals. First, it should be noted that in spectra of the ferrous heme there is a line at 1611 cm^{-1} in cytochrome a^{2+}, a line at 1607 cm^{-1} in cytochrome $a_3{}^{2+}$, and a line at 1604 cm^{-1} in cytochrome $a_3{}^{2+}(CN^-)$. Thus, we may immediately rule out the proposal of Choi et al. (1983) that the line observed at 1611 cm^{-1} results from ν_{10} of cytochrome $a_3{}^{2+}$. Instead, our data illustrate that the line at 1611 cm^{-1} does indeed originate from cytochrome a^{2+}. We assign the line at 1607 cm^{-1} in cytochrome $a_3{}^{2+}$ as ν_{10} for the high-spin ferrous heme. This is in agreement with the frequency of 1607 cm^{-1} in the 2-methylimidazole heme *a* model compound spectrum (Van Steelandt-Frentrup et al., 1981). In the spectrum of cytochrome $a_3{}^{2+}(CN^-)$ there are lines at 1604 and 1625 cm^{-1}. We assign the line at 1625 cm^{-1} to the vinyl stretching mode. The line at 1604 cm^{-1} cannot be due to the formyl group of cytochrome $a_3{}^{2+}(CN^-)$ since that mode is at 1644 cm^{-1}. Although, it is tempting to attribute it to ν_{10} in the low-spin six-coordinate heme, it is too weak to make that assignment with confidence especially since it generally occurs at higher frequency in low-spin hemes (Choi et al., 1983). However, it does illustrate that modes from the heme *a* chromophore may occur in this region. Thus, the assignment of the 1611-cm^{-1} line in cytochrome a^{2+} is still unresolved. From the data reported here, we are now confident that a strong line at 1611 cm^{-1} does originate from cytochrome a^{2+} and that in another ferrous six-coordinate low-spin heme [$a_2{}^{2+}(CN^-)$] a Raman line not assigned to the carbonyl stretching mode of the formyl group is detected in this region. However, we cannot determine whether the line at 1611 cm^{-1} results from a skeletal mode of the porphyrin macrocycle of cytochrome *a* or the carbonyl stretching mode of its formyl group. If the latter mode is absent from the resonance Raman spectrum, we speculate its absence indicates that the formyl group is forced out of plane in cytochrome *a* in the reduced enzyme so as to be no larger conjugated with the heme, and consequently, it is not resonance enhanced (Babcock & Salmeen, 1979; Babcock & Chang, 1979).

The binding of cyanide to the cytochrome a_3 chromophore does not lead to the type of electronic structure changes that occur on CO binding. Therefore, the electron-density marker line shifts only slightly from 1355 to 1357 cm^{-1}. Similarly, upon the formation of the low-spin bis(imidazole) heme *a* model compound there is very little change in the electron-density marker line compared to that in the high-spin 2-methylimidazole heme *a* model compound (Van Steelandt-

Frentrup et al., 1981). Moreover, since upon CN^- binding there is no significant π-electron density change, the optical absorption spectrum is nearly unchanged from that of the fully reduced enzyme (i.e., the peak of the Soret bands are at the same wavelength).

A mode at 1565 cm^{-1} in the spectrum of cytochrome a_3^{2+} (Figure 4B) shifts to 1561 cm^{-1} and decreases in intensity relative to the 1580-cm^{-1} band in cytochrome $a_3^{2+}(CN^-)$ (Figure 4C). A similar change is also observed in the CO-bound reduced enzyme (Argade et al., 1986). Thus, the 1565-cm^{-1} line seems to be sensitive to the high spin state to low spin state change. In the 1200-cm^{-1} region the 1225-cm^{-1} line becomes weaker on ligand binding, the 1247-cm^{-1} line moves to 1238 cm^{-1}, and a new line appears at 1255 cm^{-1}. A new line at 1253 cm^{-1} also appears in the spectrum of CO-bound reduced oxidase (Argade et al., 1986). Hence, this line also appears to be characteristic of the formation of a low-spin six-coordinate complex. The porphyrin–formyl group stretching mode has been proposed to lie in this region (Choi et al., 1983). However, the 1255-cm^{-1} mode is at the same place in both the CO- and CN^--bound reduced enzyme even though the behavior of the carbonyl stretching mode of the formyl group is quite different, as discussed above. As any oribital changes that change the force constant of the C=O bond are expected to change the porphyrin–formyl bond also, it seems likely that the 1255-cm^{-1} band is a porphyrin mode and is not associated with the formyl group.

The structure of the spectrum in the 200–500-cm^{-1} region is very different in cytochrome $a_3^{2+}(CN^-)$ relative to that in ligand-free cytochrome a_3^{2+}. Of these differences, the disappearance of the 213-cm^{-1} line has been discussed above. The spectrum of cytochrome a_3^{2+} does not have a line at 244 cm^{-1}, which appears in the spectrum of cytochrome $a_3^{2+}(CN^-)$. A line at 239 cm^{-1} is found for cytochrome a^{2+}, and the 244-cm^{-1} line may be a counterpart of this line, i.e., characteristic of formation of a six-coordinate low-spin complex. A similar explanation can be given for the origin of the 343-cm^{-1} in the cytochrome $a_3^{2+}(CN^-)$ spectrum, it being a counterpart of the 341-cm^{-1} line in cytochrome a^{2+}. The origin of lines at 378 and 407 cm^{-1} is not clear. One possibility is that they are counterparts of the 393- and 420-cm^{-1} lines in the spectrum of cytochrome a^{2+}. In any event, it is clear that qualitatively the spectrum of CN^--bound cytochrome a_3^{2+} (Figure 4C) resembles the spectrum of cytochrome a^{2+} (Figure 4A) in the low-frequency region, which is sensitive to the heme environment.

The spectra reported here bear directly on the pH dependence of cytochrome oxidase reported previously. In 441.6-nm excitation studies by Salmeen et al. (1978) and in 406.7-nm excitation work by Callahan & Babcock (1983) at high pH, the 213-cm^{-1} line was observed to disappear while ν_4 (1350–1380 cm^{-1}) was unchanged, but significant changes in the carbonyl stretching mode of the formyl group were detected. Specifically, the contribution at 1665 cm^{-1} disappeared, and a new line was detected in the 1633–1640-cm^{-1} region. These reported spectra are analogous to our spectrum of the reduced plus cyanide preparation. Consequently, the high-pH results are consistent with the conversion of cytochrome a_3^{2+} from a five- to a six-coordinate heme. Furthermore, the sixth ligand would appear to confer electronic properties on the heme equivalent to those of CN^-. In contrast, in the recently reported pH dependence of the Raman spectra of bacterial cytochrome oxidase (PS_3), the carbonyl stretching mode of the formyl group was unshifted at high pH. This suggests the possibility of the coordination of a different amino acid residue with different electronic properties in the bacterial enzyme than in the mammalian enzyme, although the data from the bacterial enzyme may be complicated by the presence of bound cytochrome *c*. Additional pH-dependence studies are needed to determine the origin of the apparent differences in the properties of these enzymes.

On the basis of the data reported here we conclude that the binding of cyanide to reduced cytochrome oxidase results in a six-coordinate low-spin cytochrome a_3. Ligand-specific differences lead to dissimilarities in some modes of the enzyme in the comparison between the CO-bound and CN^--bound preparations. For example, the electron-density marker line shifts only slighlty from its value in the fully reduced enzyme on binding cyanide but shifts significantly on binding CO. On binding cyanide, the line associated with the carbonyl stretching mode of the formyl group shifts by an amount consistent with the high- to low-spin change on the basis of model compound results. We conclude that there are no anomalies in the formyl group in this low-spin enzyme preparation. Our data show no evidence for unusual spin-dependent changes in the electronic structure of the formyl group, in possible hydrogen bonding to the protein, in its local environment, or in conformation with respect to the heme plane. Thus, from these data is appears unlikely that the formyl group in ferrous cytochrome a_3 undergoes any structural changes that would be needed for it to take part in proton translocation in the ligand binding step. The possibility of its activity in subsequent steps remains to be explored. With the spectral isolation of cytochromes a^{2+} and a_3^{2+} made possible in two independent systems (CO-bound reduced cytochrome a_3 and CN^--bound oxidized cytochrome a_3), the mechanism of heme–heme interaction can be addressed in the future.

Registry No. Cytochrome oxidase, 9001-16-5; cytochrome *a*, 9035-34-1; cytochrome a_3, 72841-18-0.

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Multiple Forms of the Glucocorticoid Receptor Steroid Binding Protein Identified by Affinity Labeling and High-Resolution Two-Dimensional Electrophoresis†

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ABSTRACT: Potential charge heterogeneity within the glucocorticoid binding protein (GBP) of the glucocorticoid receptor was examined by a combination of affinity labeling, immunopurification, and high-resolution two-dimensional (2D) gel electrophoresis. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of [^{3}H]dexamethasone 21-mesylate ([^{3}H]DM) labeled cytosol identified a major, competable, component of M_r ≃92 000 (92K). This component was recognized by anti-human glucocorticoid receptor antibodies but not by nonimmune serum, indicating that the 92K component was the reduced denatured GBP. Examination of [^{3}H]DM-labeled GBP by conventional 2D electrophoresis utilizing equilibrium isoelectric focusing in the first dimension failed to resolve the 92K GBP into discrete isoelectric components. This behavior was not representative of other, nonspecifically [^{3}H]DM-labeled proteins or proteins in general. Nonequilibrium pH gradient electrophoresis (NEPHGE) was therefore employed to achieve separation in the first dimension. Immunopurified, [^{3}H]DM-labeled GBP subjected to NEPHGE reached isoelectric equilibrium after 6 h of electrophoresis at 400 V. A single, broad peak of radioactivity was identified at pH ≃6.3. Second-dimension analysis of the NEPHGE-separated GBP by SDS-PAGE resolved this peak into two discrete, 92K, isoforms of apparent pI = 5.7 and 6.0-6.5. The GBP charge heterogeneity was confirmed by NEPHGE 2D analysis of [^{3}H]DM-labeled GBP prepared directly from crude cytosol. Two isoforms indistinguishable from those observed in immunopurified samples were identified. An additional, more acidic, isoform (apparent pI ≃ 5.2) was also identified. Thus, there are at least two, and perhaps three, isoforms of the GBP. These data therefore suggest that there is significant charge heterogeneity in the GBP of the glucocorticoid receptor.

Glucocorticoid steroid hormone receptors can exist in at least four biochemically relevant forms. These include the active and inactive steroid binding forms of the unoccupied receptor (Sando et al., 1977) and the unactivated and activated forms of the glucocorticoid receptor (GR)[1] complex (Munck et al., 1972; Baxter et al., 1972). Activation of the GR complex has been defined in intact cells as the temperature-dependent conversion of cytoplasmic GR complexes to a form capable of nuclear translocation (Baxter et al., 1972). In vitro, activation of GR complexes results in their increased affinity for nuclei and DNA (Baxter et al., 1972; Milgrom et al., 1973; Kalimi et al., 1975; LeFevre et al., 1979) and their altered chromatographic behavior on DEAE-Sephadex, DEAE-cellulose, and phosphocellulose (Parchman & Litwack, 1977; Sakaue & Thompson, 1977). This altered chromatographic behavior has been used to demonstrate that in intact cells activation precedes nuclear translocation of GR complexes (Munck & Foley, 1979; Markovič & Litwack, 1980). In addition, we have shown that mutants defective in activation of GR complexes are completely resistant to the biological activity of glucocorticoids (Schmidt et al., 1980; Harmon et

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[1] Abbreviations: β-ME, β-mercaptoethanol; DM, dexamethasone 21-mesylate; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NEPHGE, nonequilibrium pH gradient electrophoresis; NP-40, Nonidet P-40; GBP, glucocorticoid binding protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GR, glucocorticoid receptor. The trival names for the steroids used are triamcinolone acetonide for 9-fluoro-11β,21-dihydroxy-16α,17-[(1-methylethylidene)bis(oxy)]pregna-1,4-diene-3,20-dione and dexamethasone for 9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione.