Redox Dependent Interactions of the Metal Sites in Carbon Monoxide-Bound Cytochrome *c* Oxidase Monitored by Infrared and UV/Visible Spectroelectrochemical Methods†

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ABSTRACT: Spectroelectrochemical titration studies involving the binding of the infrared-active probe ligand carbon monoxide (CO) to the heme a_3/Cu_B site of bovine heart cytochrome *c* oxidase (C*c*O) have been reexamined. The spectroelectrochemical cell employed was constructed to monitor both the infrared (IR) and visible/Soret spectra of the C*c*O−CO complex as a function of the overall oxidation state of the enzyme. A number of commonly used electron transfer mediators were employed to shuttle electrons between the redox active sites within the enzyme and the electrode surface. The well-documented shift in the CO infrared stretch band maximum from 1963.3 cm^{-1} (C*c*O fully reduced) to 1965.5 cm^{-1} (C*c*O partially oxidized) was carefully titrated electrochemically. Deconvolution of the asymmetric CO stretches indicates the existence of two different states of CO vibrators within the enzyme, presumably due to two conformers which are present in a ratio of approximately 5:1. Upon incrementally stepping the potential from the fully reduced state to the partially oxidized state, we found it possible to follow the decrease in the intensity of the original pair of these conformers and the concomitant increase of a resultant pair while maintaining this 5:1 ratio between the conformers. By plotting the change in the deconvoluted CO peak intensities vs the redox potential, as well as the absorbance changes in the visible/Soret spectra vs the redox potential, we found not only that both fit an $n = 1$ electron process but also that the spectral changes tracked each other identically within experimental error. Furthermore, analysis of the second derivative of the Soret spectra allowed for the qualitative monitoring of the oxidation state of the Fe_a site which again tracked identically to that of the CO shift in the IR region. These results would seem to confirm earlier suggestions that perturbing the oxidation state of Fe_a causes a conformational change in the enzyme which affects the binding site for CO, namely heme a_3. As a consequence of the CO IR stretching frequencies changing by only 2 cm^{-1} during this redox titration, with no accompanying changes in half band width, we suggest that it is impossible that this small but significant change seen in the CO stretching frequencies could be due to an oxidation state change in Cu_B, given the known sensitivity of the CO stretching frequency to perturbations and the close proximity of Cu_B to the CO binding site at heme a_3 (4.5 Å). Therefore, it would appear that Cu_B must remain reduced as long as CO is bound to the heme a_3 site. This is consistent with earlier proposals that Fe_{a_3} and Cu_B are acting together as a two-electron donor to dioxygen.

Cytochrome *c* oxidase (C*c*O) is a multiple subunit heme/copper protein integral to the inner mitochondrial membrane of eukaryotes and the cell membrane of many prokaryotes (Wikstrom et al., 1981). The major function of this enzyme is the catalysis of O_2 to water using cytochrome *c* as the reductant. This reaction is accompanied by the development of trans membrane electrical and pH gradients, the resultant energy of which is used to promote the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) (Caughey et al., 1976). In addition to this biologically essential reaction of O_2 reduction to water, the bovine heart enzyme also appears capable of catalyzing at least three other reactions: the reduction of NO to N_2O and the oxidations of CO to CO_2 and NO to NO_2 (Breckenridge, 1953; Tzagoloff & Wharton, 1965; Young et al., 1979; Brudwig et al., 1980; Young & Caughey, 1980; Sampath et al., 1993; Zhao et al., 1993, 1995; Ignarro et al., 1993). The possible physiological significance and mechanisms of these three additional reactions are intriguing but remain poorly understood. And, although recent studies of this complex protein have become much more extensive and elaborate, the present understanding of the reaction mechanism by which O_2 is reduced to water by four cytochrome *c* molecules, as well as the "proton pumping" ability of this enzyme, and the control of these processes (i.e., respiratory control) in terms of the protein structure remains limited.

The bovine heart enzyme isolated and purified by the same procedure as the enzyme employed in the present study has recently been crystallized and a 2.8 Å resolution structure reported (Tsukihara et al., 1995). The enzyme was found to contain two heme A chromophores, heme *a* (Fe_a) and heme a_3 (Fe_{a_3}), and three coppers. Two of the coppers

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comprise the dinuclear Cu_A site and the third, Cu_B, is closely associated with the O_2 binding site at heme a_3 (Einarsdottir & Caughey, 1985; Steffens et al., 1987; Soulimaine & Buse, 1995; Tsukihara et al., 1995). The dinuclear Cu_A site and mononuclear Cu_B site each appear to function as one-electron redox centers in the native enzyme (Kronek et al., 1991; Steffens et al., 1993; Malmstrom & Aasa, 1993). Additionally, the enzyme contains one zinc and one magnesium, both of unknown function (Einarsdottir & Caughey, 1985; Tsukihara et al., 1995). Six histidines are totally conserved among all known sequences for subunit I of C*c*O which contains heme *a*, heme a_3, and Cu_B (Cu_A is located in subunit II of the 13 protein subunits in bovine heart C*c*O) (Saraste, 1990; Calhoun et al., 1993; Tsukihara et al., 1995).

Earlier studies involving visible/Soret spectra provided important insight into the reactions of the hemes of C*c*O (Wharton & Tzagoloff, 1964; Beinert et al., 1980). Additional investigations of the enzyme employing optical dispersion and circular dichroism (CD) spectra (King et al., 1971) as a function of the oxidation state of the enzyme, as well as the oxidation state dependence of the enzyme to proteolysis (Yamamoto & Okukuki, 1970), suggested that C*c*O was undergoing a relevant structural rearrangement which was triggered by the oxidation states of the redox active metals within the enzyme. Although these earlier methods along with many others have added valuable information to that which is already available, they tell us very little about the binding site for O_2 and the interactions therein. However, more recently, infrared spectra of IR-active ligands [i.e., carbon monoxide (CO), cyanide (CN^-), azide (N_3^-), and nitric oxide (NO)] at metal sites within the protein were found to provide direct evidence as to the nature of the binding of these ligands in analogy to O_2 binding (Yoshikawa & Caughey, 1982; Caughey et al., 1993; Zhao et al., 1994; Yoshikawa et al., 1995). The study of CO at O_2 binding sites in hemeproteins has been especially extensive given the comparative biochemistry between CO and O_2 in addition to the ease of locating the CO stretching frequency in the infrared region.

The discovery of a single CO vibrational band with a frequency at 1963 cm^{-1} for CO bound to fully reduced bovine heart C*c*O gave the first evidence that CO binds stably to Fe^{2+} (specifically $Fe_{a_3}{}^{2+}$) but not to Cu^+ at room temperature (Caughey et al., 1970). When photodissociated from ferrous heme a_3 at low temperatures, however, a new CO band at 2062 cm^{-1} appears, ostensibly due to transient formation of a Cu^+−CO complex, presumably with Cu_B (Alben et al., 1981; Einarsdottir et al., 1989; Lemon et al., 1993). On the basis of comparisons with various models, the CO stretching frequency (ν_{CO}) at 1963 cm^{-1} is fully consistent with CO being bound to a ferrous heme A moiety having a histidine ligand in the trans position (Alben & Caughey, 1968; Caughey et al., 1970; Potter et al., 1990). The very narrow half band width for the CO vibrational band ($\Delta\nu_{1/2}$, band width at half-maximum of the absorbance) of ∼4 cm^{-1} compared with that of 8−20 cm^{-1} found in other hemeproteins indicated that the CO ligand was located within a relatively immobile environment well isolated from the external medium in C*c*O. It only later became clear that the CO vibrational band could be deconvoluted into one major band (C_{II}) and one minor band (C_I) of Gaussian shape with a relative intensity ratio of 5:1 (Yoshikawa & Caughey, 1982; Einarsdottir et al., 1988). The C_I and C_{II} bands have been interpreted to represent discrete protein conformers that may interconvert rapidly.

The C*c*O CO vibrational band was found to be very sensitive to any structural changes at the CO (and thus, O_2) binding site caused by extremes of pH and elevated temperature (Einarsdottir & Caughey, 1988; Einarsdottir et al., 1988). However, compared to the carbonyls of other hemeproteins (e.g., hemoglobin, myoglobin, and cytochrome P_{450}), the C*c*O CO bond was unusually insensitive to moderate deviations from physiological pH and other changes in the medium and temperature. Furthermore, the frequencies of both the major and minor bands were observed to change only by 2 cm^{-1} with changes in the overall oxidation state of the enzyme. For example, reduction of the fully oxidized enzyme by increasing amounts of β-nicotinamide adenine dinucleotide/phenazine methosulfate (NADH/PMS) resulted in CO complexes that exhibited CO vibrational band maxima that progressively shifted from 1965.5 to 1963.3 cm^{-1} (Yoshikawa & Caughey, 1982). This study, employing chemical reductants, appeared to suggest that only one electron needed to be added to the fully oxidized enzyme in order for CO to bind to Fe_{a_3}. An earlier study by Anderson et al. (1976) had come to a similar conclusion on the basis of coulometric titration data. They reported that the fully reduced C*c*O−CO complex underwent a reversible three-electron oxidation with different midpoint potentials for each of three one-electron components. Other chemical (Babcock et al., 1978) and electrochemical (Lindsay et al., 1975; Wilson & Nelson, 1982) titrations of the enzyme in the presence of CO, however, have suggested that *two* electrons must be added to the enzyme in order for CO to bind to Fe_{a_3}. Finally, in contrast to the apparent effect of CO on the enzyme, there is strong experimental support for the view that CN^- (which is isoelectronic with and structurally similar to CO) binds with high affinity to $Fe_{a_3}{}^{3+}$ regardless of whether the other redox active centers (Fe_a, Cu_A, and Cu_B) are oxidized or reduced (Yoshikawa et al., 1995).

As indicated above, previous electrochemical studies performed on cytchrome *c* oxidase have often led to significantly differing interpretations (Lindsay & Wilson, 1974; Anderson et al., 1976; Wilson & Nelson, 1982; Hendler & Westerhoff, 1992; Harmon et al., 1994). In an attempt to clarify the effects of the overall redox state of the enzyme on the protein conformation of the binuclear reaction site, we have carried out a spectroelectrochemical study in which both IR and visible/Soret spectra of the enzyme are monitored as a function of applied potential. The conditions employed allowed the enzyme to be repeatedly cycled through its various oxidation levels while obtaining accurate spectral data as the potential was varied. When an initial potential of −605 mV vs NHE (normal hydrogen electrode) is applied to the solution, the enzyme turns over any excess dioxygen in the system by reducing it to water and allows CO to bind to the fully reduced enzyme, thus eliminating the problems that were inherent in earlier chemical titrations (Yoshikawa & Caughey, 1982). The possibility remains that undesirable interactions between the mediators and/or the electrode and the protein may occur in the electrochemical method. However, if they do exist, they appear to be independent of mediator concentrations between 0.05 and 1.5 mM and also appear to be independent of the composition of the mediators in the final solution, provided

a substantial number of mediators exist within the potential window one is interested in probing.

Clear evidence of a fully reversible effect of applied potential on the protein conformation as exhibited by the CO stretching band was found. Furthermore, the data suggest that the oxidation states of heme *a* and/or Cu_A affect the structure and properties of the O_2 reaction site involving Fe_{a_3} and Cu_B. Moreover, we will provide arguments that Cu_B, as well as Fe_{a_3}, not only must be reduced in order for CO to bind to Fe_{a_3} but also must remain reduced while CO is ligated to Fe_{a_3}.

MATERIALS AND METHODS

Enzyme Preparation. Recrystallized cytochrome *c* oxidase was isolated from fresh bovine heart muscle by the method developed by Yoshikawa and Caughey with Brij 35 as the detergent (Volpe & Caughey, 1974; Yoshikawa et al., 1977, 1991). The final product in 10 mM sodium phosphate buffer (pH 7.4) was dialyzed against the same buffer and concentrated to ~2 mM in terms of heme A concentration employing an Amicon Diaflow apparatus. The heme A concentration of fully reduced, recrystallized C*c*O was determined by using a $\Delta\epsilon$ equal to 23.6 mM^{-1} cm^{-1} for the 603 nm minus 630 nm transition (Yoshikawa et al., 1995). The recrystallized C*c*O was stored in the dark at −70 °C until used. Nitrogen (N_2) (99.9%) and carbon monoxide (CO) (99.5%) gases were obtained from General Air Service and Supply.

Spectroelectrochemical Studies. A group of biologically compatible mediators were tested for their electron transfer reversibility and stability between the potential range of −605 and +695 mV vs NHE using cyclic voltammetry. This potential range allowed access to the fully reduced and fully oxidized forms of the enzyme as well as all intermediate forms. The fully reduced spectrum at −605 mV was identical to the spectrum of the fully reduced enzyme obtained with excess sodium dithionite. Similarly, the spectrum obtained at +695 mV corresponded to the spectrum of the fully oxidized enzyme. Nine different mediators were chosen to cover this entire potential range.

Solutions of the following mediators (5 mM each) were freshly prepared before each experiment in 200 mM sodium phosphate buffer. The nine mediators employed were as follows: $[Fe(4,4'\text{-dimethyl-}2,2'\text{-bipyridine})_3](Cl)_2$ (E_m = 885 mV vs NHE), synthesized according to Serr and co-workers (Serr et al., 1988); (ferrocenylmethyl)trimethylammonium iodide (E_m = 645 mV), Pfaltz and Bauer; 1,1-ferrocenedimethanol (E_m = 460 mV), Aldrich; potassium ferricyanide (E_m = 430 mV), Fisher Scientific; 2,6-dichloroindophenol (E_m = 230 mV), Aldrich; 5-hydroxy-1,4-naphthoquinone (E_m = 40 mV), Aldrich; 2-hydroxy-1,4-naphthoquinone (E_m = −150 mV), Aldrich; benzyl viologen (E_m = −320 mV), Aldrich; and methyl viologen (E_m = −460 mV), Aldrich. All mediators were used as received. It should be noted that, once specific redox processes were identified, it was generally possible to reduce the number of mediators to only those in the potential regions where the C*c*O redox processes occurred [e.g., (ferrocenylmethyl)trimethylammonium iodide, potassium ferricyanide, 2,6-dichloroindophenol, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine, 5-hydroxy-1,4-naphthoquinone, and methyl viologen]. However, unless otherwise stated, all nine of the originally specified mediators listed above were present in solution.

The C*c*O (~1 mM in heme A) solution with the nine mediators (0.1 mM each) was divided into two equal portions and placed into Eppendorf tubes, and the tubes were sealed with rubber septa. Two syringe needles (one for inlet, the other for outlet) were attached to the rubber septa. The two solutions were then thoroughly degassed by exposure to N_2 gas which had been passed through a gas-washing bottle containing doubly distilled water. Without the CO gas being passed through the solution, one of the Eppendorf tubes was then flushed with CO gas (which had also passed through a gas-washing bottle containing doubly distilled water) for approximately 2 h to assure complete saturation of the highly concentrated protein solution. The remaining Eppendorf tube of C*c*O solution, used as the blank in the infrared and visible/Soret experiments, was further flushed with N_2. A solution containing only the nine mediators (of similar concentration in the same buffer solution degassed with N_2) was employed as a background/blank for the visible spectroscopic experiments. These blanks were handled identically to the C*c*O solution containing CO (excluding the saturation with carbon monoxide).

The CO-saturated C*c*O samples were placed in a glovebag which was first flushed with nitrogen gas and then filled with carbon monoxide. The spectroelectrochemical cell, described previously by Bauscher and co-workers (1990), was loaded with the C*c*O−CO sample and assembled in the glovebag. Briefly, the optically transparent thin layer spectroelectrochemical cell consists of CaF_2 windows, a gold grid working electrode (250 lines/in.), a platinum counter electrode, and a Ag^+/AgCl reference electrode. Unless otherwise stated, however, all potentials are reported against a normal hydrogen electrode (NHE). The path length of the cell was carefully calibrated by measuring the visible absorbance of a hemoglobin A solution of known concentration and extinction coefficient and found to be 24 ± 4 μm. Electrical contact between the working electrode and reference electrodes was made using a solution of the appropriate mediators (0.1 mM in each) in 200 mM sodium phosphate buffer.

Instrumentation and Data Handling. A Bioanalytical Systems-100 electrochemical analyzer was used to control the potentials applied to the working electrode. Infrared spectra of C*c*O−CO complexes in the spectroelectrochemical cell were measured at 20 °C using a Perkin-Elmer Model 1800 Fourier transform infrared spectrophotometer equipped with an MCT detector and a 7700 computer system. Each spectrum consisted of an average of 100 individual interferometer scans at a resolution of 2 cm^{-1} and an interval of 1 cm^{-1}. A reference base line for the C*c*O sample without CO but with the appropriate mediators was recorded at each individual potential and subtracted from all subsequent IR spectra taken at that particular potential.[1] A water vapor spectrum was obtained by taking the difference of repeated scans of the same buffer solution. This spectrum was subtracted from subsequent IR spectra as well. Interferences from water vapor absorbances occur at frequencies similar to the C*c*O CO stretching frequency and can be different

[1] The same results were obtained when the background correction was made by independent subtraction of the spectrum of the native enzyme at a fixed potential and the spectrum of the appropriate mediators in buffer solution at the relevant applied potential from that of the sample spectra.

from sample to sample even with dry air purging of the IR cell compartment (Caughey et al., 1993; Dong & Caughey, 1993). The final infrared difference spectra were subjected to nine-point smoothing with a Savitsky−Golay function. Deconvolutions of the infrared bands were carried out using the CURVEFIT function of Spectra Calc Software (Galactic Industries Corp.).

Visible/Soret spectra of the sample in the same spectroelectrochemical cell described above were taken at 20 °C using a Cary 2200 spectrophotometer. A reference base line for the solution of mediators was recorded under identical conditions (including the potential applied to the solution) and digitally subtracted from all subsequent spectra. The visible/Soret and the infrared CO vibrational spectra of the C*c*O−CO complex were each measured in sequence at each applied potential. The system was assumed to be at equilibrium following each potential step only after changes in the visible spectrum had ceased (5−20 min). The second derivative (SD) analysis ($\partial^2A/\partial\lambda^2$) of each Soret absorption spectrum with respect to the potential was achieved by the least-squares method of Savitsky−Golay with the CARY DS-15 Spectral Calculations Program supplied by Varian Instruments. The electrochemical data were treated using TABLE CURVE software by Jandel Scientific.

RESULTS

Infrared Spectroelectrochemical Titrations. During the titrations, visible/Soret spectra of the C*c*O−CO complex were employed to determine when the solution had reached redox equilibrium after each potential step. The changes in spectra were comparable to those reported by Yoshikawa and Caughey (1982). After a stable visible/Soret spectrum was obtained, the cell was transferred to the infrared spectrophotometer and an IR spectrum recorded. The CO IR absorbance spectra in Figure 1 show the changes in the CO stretching frequency and absorbance as the potential is incrementally stepped from −605 to 295 mV. Between −605 and 175 mV, the spectrum remains constant. As the potential is stepped between 175 and 320 mV, the band undergoes a fully reversible shift from 1963.3 to 1965.5 cm^{-1}. Furthermore, the intensity of the band first decreases (ongoing from 175 to 245 mV) and then increases (ongoing from 245 to 320 mV). In addition to this change in intensity, the half band width increases from ∼4.5 cm^{-1} (at 175 mV) to 5.5 cm^{-1} (at 245 mV) and then decreases back to ∼4.6 cm^{-1} (at 320 mV). During all of these spectral changes, however, the total area of the spectral band remains constant. Between 320 and 445 mV, no additional spectral changes occur. Stepping the redox potential to 495 mV results in the disappearance of the band for the bound CO ligand; however, if the potential is immediately stepped back to −605 mV, the CO stretching band partially (ca. 70%) reappears. On the other hand, when the potential is stepped to 695 mV, the CO vibrational band disappears and does not reappear upon stepping the potential back to −605 mV. Exposing the enzyme to additional CO gas at this point (at open circuit potential) and re-reducing the enzyme electrochemically (−605 mV) fully restores the CO vibrational band at 1963.3 cm^{-1}. Thus, stepping the potential to 695 mV does not appear to be destroying the CO binding ability of the oxidase; consequently, CO loss must be occurring via oxidation to CO_2 or some other pathway (Young & Caughey, 1987).

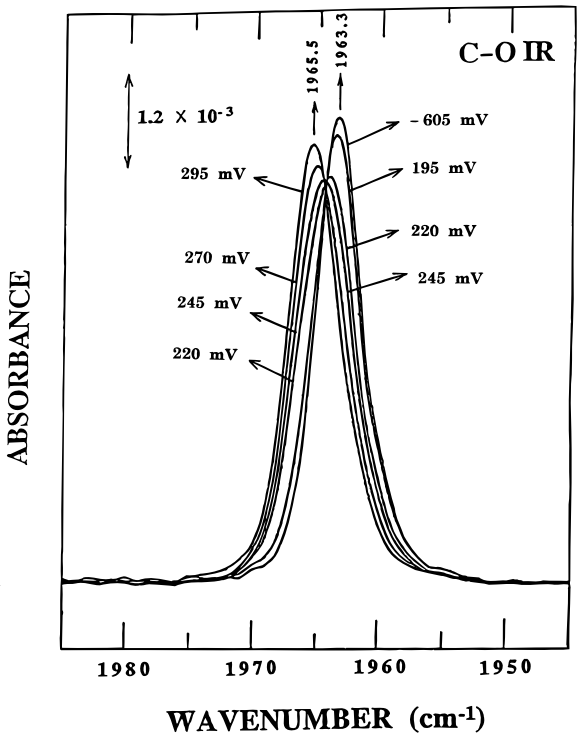

FIGURE 1: Difference spectra of the CO IR stretch (as described in Materials and Methods) for the C*c*O−CO complex obtained during an infrared spectroelectrochemical titration of recrystallized bovine heart cytochrome *c* oxidase. The infrared spectrum at each potential was acquired after the visible/Soret spectrum became constant with time. The redox potentials indicated in the figure are vs NHE at 20 °C. The conditions are as stated in Materials and Methods, employing all nine mediators.

Closer examination of Figure 1 reveals that each spectrum is asymmetric, consistent with the presence of two or more CO IR stretches. The extreme oxidation state spectra (i.e., at −605 and 320 mV) could each be cleanly fit to a pair of Gaussian bands [C_{IIA}, located at 1963.3 cm^{-1}, and C_{IA}, located at 1960.4 cm^{-1}, (−605 mV); and C_{IIB}, located at 1965.5 cm^{-1}, and C_{IB}, located at 1962.3 cm^{-1}, (320 mV)], each pair of roughly a 5:1 intensity ratio (top and bottom spectra in Figure 2, respectively). The data for the intermediate potentials were deconvoluted in one of two ways. First, the data were fit to linear combinations of the two pairs of bands obtained from the extreme oxidation state spectra (i.e., with fixed wavelength and band width) while the 5:1 ratio for each pair of bands was maintained. Second, the intermediate spectra were fit allowing the band widths and wavelengths to vary. Both approaches yielded equivalent quality fits and produced basically the same results, namely, four bands with the same frequencies, half band widths, and intensities. From these spectral deconvolutions, it becomes clear that, as the potential is stepped to progressively more positive values (beginning at 175 mV), the relative contribution of C_{IIA} and C_{IA} decreases with a concomitant increase in the contribution from C_{IIB} and C_{IB} (cf. Figure 2). Furthermore, the spectra are independent of the direction in which the potential is stepped (i.e., positive → negative or negative → positive), indicating that the C*c*O−CO complex is in redox equilibrium with the electrode.

Analysis of the Visible/Soret Spectroelectrochemical Titrations. Second derivative Soret absorption spectra are shown in Figure 3 at representative potentials. The absorbance at 428 nm has been assigned to both heme a_3-CO and

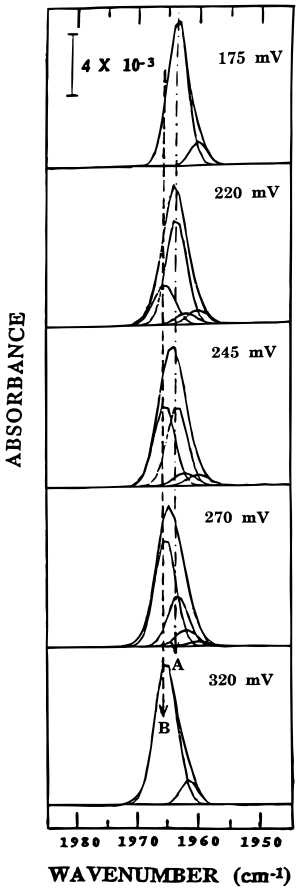

FIGURE 2: Deconvolutions of CO IR stretching bands shown in Figure 1 performed as described in Materials and Methods. A 100% Gaussian profile was employed in the fit. Arrows A and B correspond to 1963.3 and 1965.5 cm^{-1}, respectively.

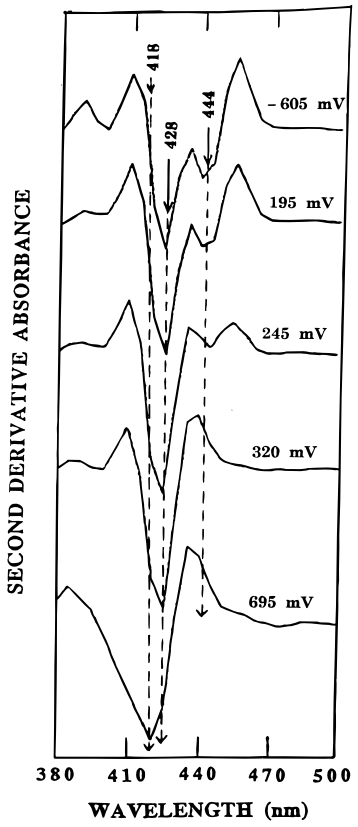

FIGURE 3: Second derivative absorption spectra (Soret) of the C*c*O−CO complex obtained during the spectroelectrochemical titration. The potentials reported are vs NHE. The conditions are as stated in Materials and Methods, employing all nine mediators.

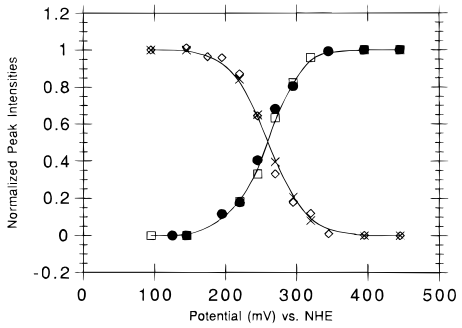

FIGURE 4: Deconvoluted and normalized CO IR peak intensity values at 1963.3 cm^{-1} (×) and at 1965.5 cm^{-1} (□) and the normalized Δ*A* of the absolute Soret spectra at 428 nm (●) and at 444 nm (◊) vs the applied potential (NHE). The solid lines are theoretical fits of the IR data to an $n = 1$ electron process. Visible and IR data were obtained in the same experiment.

heme a^{3+}, while the absorbance at 444 nm is almost exclusively due to heme a^{2+} (Sherman et al., 1991; Copeland, 1991).[2] The absorbance at 418 nm has been assigned to heme $a_3{}^{3+}$. Upon stepping the potential from −605 to 175 mV, essentially no changes in the second derivative spectra at 428, 444, or 418 nm are observed. Incrementally stepping the potential between 175 and 320 mV results in the disappearance of the absorbance at 444 nm, indicating that heme *a* has been oxidized. These changes in the redox state of heme *a*, determined from the visible/Soret spectra (heme $a^{2+} \rightarrow$ heme a^{3+}), parallel the changes in the CO stretching frequency. Finally, stepping the potential from 320 to 695 mV results in the appearance of a transition at 418 nm, signaling the loss of CO from $Fe_{a_3}{}^{2+}$ and its subsequent oxidation to $Fe_{a_3}{}^{3+}$.

Theoretical Fitting of the Resultant Spectroelectrochemical Data. Figure 4 is a composite showing the changes in the normalized absorbance (Δ*A*) for several spectral features of the C*c*O−CO complex plotted vs the applied potential (*E*). The deconvoluted infrared peak intensities of the 1963.3 cm^{-1} (×) and 1965.5 cm^{-1} (□) CO stretches as well as the absolute Soret absorbances at 444 nm (◊) and 428 nm (●) are included. The solid lines represent theoretical fits of the IR data to a one-electron redox process. The experimental spectral data (both visible/Soret and IR) exhibit very good agreement with the theoretical fits. A plot of the potential vs log $[(A_{max(1963.3)} - A_{(1963.3)})/A_{(1963.3)}]$ yields a straight line, the slope of which corresponds to an $n = 1$ electron process (not shown). The midpoint potential (E_m), as determined from the *y*-intercept for this particular data set, is 258 mV vs NHE. The spectral data (both the IR deconvolutions and changes in the visible/Soret) from four separate spectroelectrochemical experiments, employing both the intensity and the CO band area as well as the changes in the absorbances at 428, 444, and 604 nm (not shown) in the visible/Soret region, were treated statistically. The resulting average midpoint potential (E_m) was 255 ± 7 mV, and the Nernst slope was 59.6 ± 6.9 mV.

[2] Actually, there are two electronic transitions, one at 444 nm and one at 450 nm, in the second-derivative absorption spectrum that can be assigned to two different conformations of heme a^{2+} when CO is bound to heme $a_3{}^{2+}$ (Sherman et al., 1991; Copeland, 1991). Due to the limited sensitivity and low absorbance, only the transition at 444 nm for $Fe_a{}^{2+}$ can be resolved.

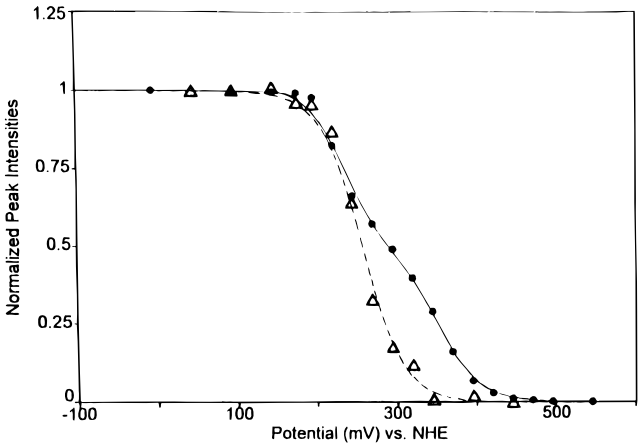

FIGURE 5: Normalized Δ*A* (at 444 nm) vs applied potential without CO (●) and with CO (△). The solid line is a fit of the data acquired without CO to two $n = 1$ electron processes ($E_{m1} = 235$ mV, $E_{m2} = 350$ mV). The dotted line is a fit of the data acquired in the presence of CO to a single $n = 1$ electron process ($E_m = 258$ mV).

Spectroelectrochemical data were also acquired for CO-free C*c*O. The solid line in Figure 5 is a theoretical fit of the Δ444 nm spectral data to two $n = 1$ electron processes. All of the points fall within a confidence level of 95% to the fit, and all but one fall within a confidence level of 99%. These two redox processes correspond to the oxidation of Fe_a and Fe_{a_3}. The open triangles correspond to the Δ444 nm transition for the C*c*O−CO complex. The dashed line is a fit of that data to a single one-electron transfer corresponding to the oxidation of Fe_a ($E_m = 258$ mV).[3]

DISCUSSION

The spectroelectrochemical cell employed in this study has allowed us to effectively obtain simultaneous visible/Soret and IR spectra of the C*c*O−CO complex as a function of the equilibrium solution potential. Therefore, we are able to directly cross-correlate both the visible/Soret and IR spectral data with the equilibrium solution potential. The results with CO present can be summarized most simply by the following. Between the potentials of −605 and 445 mV, there appears to be a single *spectrally detectable* one-electron redox event which occurs with a midpoint potential of 255 mV.[4] The changes which occur in the CO stretching frequency and the changes which occur in the visible/Soret due to heme *a* quantitatively correlate. No other spectral changes are observed until the potential is reached where the heme a_3 is oxidized (∼495 mV) and the bound CO is lost. The fact that these two spectral features (i.e., the CO stretch and the heme *a* visible/Soret) correlate implies that they are related. Before this result is discussed, however, it is worth reviewing the general nature of the IR spectral behavior of heme−CO complexes.

There is abundant evidence from studies on both biological and abiological heme−CO complexes that the CO stretching frequency and half band width are each extremely sensitive to the environment about the CO, particularly the distal environment. For example, a variety of IR studies conducted on hemoglobin (Hb) and myoglobin (Mb) carbonyls (Alben & Caughey, 1968; Yoshikawa et al., 1977; Caughey, 1980; Einarsdottir et al., 1988; Potter et al., 1990) have demonstrated that $\Delta\nu_{1/2}$ values for the CO stretching bands are anywhere from ∼8 to >20 cm^{-1} (as stated earlier). For isolated six-coordinate heme carbonyl complexes with pyridine or 1-methylimidazole as the sixth ligand, simply changing the solvent from CH_3Cl to CH_2ClCH_2Cl caused a 10 cm^{-1} shift in the frequency and a 4 cm^{-1} change in the half band width (Maxwell & Caughey, 1976). Changing the axial ligand, on the other hand, is a somewhat more subtle perturbation. The frequency shift in the CO stretch upon pyridine being exchanged for 1-methylimidazole is only on the order of about 6 cm^{-1}, with little or no change in the half band width (in weakly coordinating solvents) (Maxwell & Caughey, 1976).

In contrast to the examples given above, the CO stretching bands in the C*c*O−CO complex are abnormally narrow (4 cm^{-1} for C*c*O−CO compared with ∼10 cm^{-1} for Hb−CO and 30 cm^{-1} for isolated heme−CO complexes) and appear to be quite insensitive to the environment outside of the protein (Caughey et al., 1993). For example, the addition of 25−50% (v/v) glycerol, ethylene glycol, halothane, chloroform, or dimethyl sulfoxide has been shown to have virtually no effect on either the CO stretching bands or the visible/Soret spectrum (Zhao, 1994). It has been suggested that the unusually narrow half band widths are indicative of a very well-ordered, immobile binding pocket which is well-isolated from the external environment (Yoshikawa & Caughey, 1982; Einarsdottir et al., 1988; Caughey et al., 1993). Given the extreme sensitivity of the CO stretching frequency to the distal environment, we argue that the subtle changes in the CO stretching band, which coincide with the redox event at $E_m = 255$ mV, must not involve significant changes in the structure, polarity, or charge of the CO binding pocket.

The existence of two CO IR stretching bands (C_I and C_{II}) having a 3 cm^{-1} difference has been rationalized as resulting from the existence of two discrete protein conformers in which the CO environment and/or the Fe−CO bonding are slightly different (Yoshikawa & Caughey, 1982; Einarsdottir et al., 1988; Caughey et al., 1993). Multiple CO IR bands are commonly encountered for heme proteins; for example, carbonyl complexes of hemoglobin A and myoglobin each exhibit at least four CO stretches which are attributed to four different conformers (Caughey et al., 1981; Potter et al., 1990). The relative concentrations of each of these conformers are, as expected, dependent upon such factors as temperature and pH (Einarsdottir et al., 1988). For the C*c*O−CO complex, the very narrow half band width (4 cm^{-1}) as well as the small change in the CO stretching frequency are consistent with both conformational forms, C_I and C_{II}, having similar well-ordered environments within the binding pocket. Until now, the interconversion of these two populations of CO vibrators could not be reproducibly monitored at intermediate potentials.

As indicated in the introduction, some disagreement has existed as to the level of C*c*O reduction necessary for CO

[3] It should be noted that a slight but reproducible deviation of the 444 nm data to the fit of an $n = 1$ electron model is observed in the low-potential region of Figures 4 and 5. This type of deviation has been reported by others (Ellis et al., 1986) and attributed to an interaction between the Fe_a and Cu_A sites. However, a deviation at low potentials is not consistently observed in the data at either 428 or 604 nm.

[4] The fact that there is a single *spectrally detectable* redox process does not necessarily mean that other spectrally nondetectable processes are not occurring (e.g., $Cu_A^{1+/2+}$); they are simply invisible to these experiments.

to bind to the enzyme. Lindsay and co-workers conducted potentiometric titrations on a C*c*O−CO complex prepared from oxidase isolated from pigeon breast muscle (Lindsay et al., 1975). From this work, they concluded that the most positive redox couple, and the one which involved the bound CO, was a two-electron process. They further speculated that the second redox center involved was the "invisible copper" (Cu_B), although this fact was not directly established. This speculation was primarily based on the presumed (at the time) proximity of Cu_B to the binding pocket. However, a coulometric study performed by Anderson et al. (1976) seemed to support the conclusion that only a single electron was needed in order for the enzyme to bind CO by showing that the fully reduced enzyme with CO underwent three reversible one-electron oxidations. Subsequently, Wilson and Nelson (1982) conducted coulometric titrations on C*c*O−CO samples prepared from the intact membranes of pigeon breast muscle mitochondria which again supported their original proposal that the enzyme needed to be reduced by two electrons in order to bind CO and also suggested that the second electron equivalent was accepted by the high-potential Cu (Cu_B) . At approximately the same time, Yoshikawa and Caughey (1982) presented evidence based on IR data from chemical redox titrations of beef heart C*c*O that suggested that only a single reducing equivalent was necessary for CO binding, contradicting the earlier reports of Lindsay and Wilson. The conclusion, however, in this later study employing chemical reductants was made suspect by the possiblity of complications from CO-induced autoreduction of the enzyme (Young & Caughey, 1987) and from inadequate exclusion of O_2 from the system, thus rendering the degree of reduction necessary for CO binding ambiguous. Also, as pointed out by Lindsay et al. (1975), experimental conditions such as differences in the pH, ATP concentration, and CO concentration can significantly change the redox properties of the metals within cytochrome *c* oxidase. Thus, differences in the enzyme preparation, its concentration, the mediators employed, and their relative and absolute concentrations as well as any differences in the pH might lead to apparently contradicting results. Nonetheless, it is generally well-accepted now that two electrons need to be added to C*c*O in order for it to bind an exogenous ligand such as CO (and presumably O_2) as suggested by these earlier studies (Wilson et al., 1976; Babcock et al., 1978). However, it has not previously been clear whether the Cu_B site can undergo oxidation while the CO is bound to Fe_{a_3}, assuming that Cu_B is the metal that initially accepts the second electron.

The results presented in the present study would seem to indicate that, indeed, the binding of CO at heme a_3 renders the Cu_B nonoxidizable as long as the CO remains bound. Over the entire potential range from −605 to 445 mV, only one redox event is spectrally observable.[4] During that event, the perturbation of the CO stretch, as observed in the IR, is extraordinarily modest. On the basis of the known sensitivity of the CO stretching frequency in heme carbonyls to its environment, it is not reasonable to propose that Cu_B changes in oxidation state over this potential region. If the interatomic distance between Cu_B and Fe_{a_3} in the reduced form of the enzyme remains ∼4.5 Å as it is in the resting form of the enzyme (Powers et al., 1981; Scott et al., 1986; Tsukihara et al., 1995), such an oxidation state change would constitute a major change in the polarity (and probably structure) of the binding pocket and in turn should translate into a significant change in both the frequency and half band width of the CO vibrational band (not observed). This prediction is also consistent with the fact that there has yet to be observed a *substantial* change in the electron paramagnetic resonance (EPR) signal attributable to a change in the redox state of Cu_B in the presence of CO. Thus, the original proposal that heme a_3 and Cu_B act as a concerted two-electron pair when carbon monoxide is present is consistent with our results. Furthermore, these present observations are also fully consistent with the argument that the second redox center is, indeed, Cu_B.

It is interesting to speculate as to the origin of the shift in the midpoint potential of Cu_B which accompanies CO binding. As pointed out earlier, there is evidence that CO will transiently bind to reduced Cu_B when photolyzed from reduced heme a_3 (Alben et al., 1981; Einarsdottir et al., 1989; Lemon et al., 1993). Moreover, it has been proposed that Cu_B may in fact be the entry point for exogenous ligands such as CO and O_2 (Woodruff, 1993). However, in the ground state enzyme, carbon monoxide is not bound to Cu_B in either copper oxidation state (in a bridged mode or otherwise); thus, it must follow that the potential shift of the copper has other origins. The $Cu^{2+/1+}$ couple is well-known from simple inorganic systems to be quite sensitive to the nature of coordinating ligands and to the coordination geometry of those ligands. As discussed previously, the extreme narrowness of the CO stretching band implies a very immobile (and thus structurally tight) binding environment. It is entirely reasonable, therefore, to propose that bound CO could impose a steric constraint on Cu_B either by forcing it into a geometry that favors the reduced form (i.e., more tetrahedral) and/or by sterically preventing ligand binding to the copper. The net effect would be to couple both the heme a_3 and Cu_B redox potentials to the process of CO binding without requiring any direct bondng between Cu_B and CO. Once a potential sufficiently positive to oxidize the heme a_3 site is reached (releasing the bound CO), the midpoint potential of Cu_B in the CO-free enzyme has been exceeded; consequently, the two events will titrate as a concerted two-electron process.

All else being equal, dioxygen and CO should have very similar steric requirements in their binding at the enzyme active site, especially if the CO ligand is forced to deviate from its preferred linear geometry by the steric constraints of the protein pocket causing a bent Fe−CO bond. Both are neutral ligands that initially, at least, bind to reduced heme a_3. It is reasonable to speculate that the initial act of O_2 binding should have a similar effect on the Cu_B redox potential as does the binding of CO. The obvious difference in these two ligands is that O_2 is reducible while CO is not. If, however, the analogy is valid, the effect would be to couple the oxidations of both metals such that they would be forced to function as a two-electron center, as originally proposed by Lindsay and co-workers (1975). This would be entirely consistent with the multielectron function of the enzyme.

Thus far, we have focused on what the small shift in the CO stretching frequency is not due to. It remains that the observed shifts correlate quantitatively with the visible/Soret changes in the redox state of heme *a*. The simplest explanation would then be that the CO stretching frequency is responding to the oxidation state of the heme *a* through some form of "allosteric" effect (possibly at the proximal

heme a_3 histidine) mediated by the intervening protein. Such coupling has been suggested to occur through helix X in the enzyme (Hosler et al., 1993; Tsukihara et al., 1995). The very modest 2 cm^{-1} shift would be consistent with such a process. However, in the C*c*O−CO complex, the midpoint potentials of heme *a* and Cu_A may be fortuitously degenerate as proposed by Yoshikawa and researchers (Yoshikawa et al., 1995; Tsukihara et al., 1995). If that were the case, the origin of the shift in the CO stretch could be due to an allosteric effect originating at either Cu_A or heme *a*.

CONCLUSIONS

The simultaneous acquisition of visible/Soret and IR data for the carbonyl complex of bovine heart C*c*O as a function of the solution redox potential has allowed us to arrive at a number of conclusions that have previously not been possible *via* chemical titrations or "one-mode" spectroelectrochemical experiments. First, the two conformers which produce CO stretches at 1963.3 and 1960.4 cm^{-1} in the fully reduced C*c*O−CO complex titrate as a Nerstian one-electron process with an $E_m = 255$ mV (vs NHE) to produce two conformers with the same relative concentration ratio with stretches at 1965.5 and 1962.3 cm^{-1}. By deconvoluting the CO IR bands as a function of the potential, we have further shown that the relative ratios of these conformers and the half band widths remain constant at intermediate oxidation levels throughout the redox transition.

We have also argued that it is unreasonable that the small shift in the CO stretch could be attributable to a change in the Cu_B oxidation state, and thus, it must follow that Cu_B must remain reduced as long as the CO is bound to the heme a_3 site. The changes in the absolute visible/Soret spectra due to the oxidation/reduction of heme *a* correlate quantitatively with the changes in the CO stretch. These shifts in the IR bands can be attributed to a long range interaction (allosteric effect) originating, most likely, at heme *a*. The redox chemistry of Cu_A is believed to be spectrally invisible in these experiments; however, the involvement of Cu_A cannot be ruled out.

The picture which emerges is that the binding of CO can only occur if both heme a_3 and the Cu_B are both reduced. Furthermore, the binding of CO at reduced heme a_3 stabilizes the reduced form of Cu_B relative to that in CO-free oxidase. The mechanism of this stabilization is speculative at this point but could arise from the steric requirements of the bound CO. In addition, heme *a* (or possibly Cu_A) can communicate information about its oxidation state to the bound CO through the intervening protein. This evidence reaffirms earlier studies which proposed such heme−heme interactions (Wilson & Leigh, 1972; Tiesjema & Van Gelder, 1974). Finally, the similarities in charge and spatial requirements for the binding of CO and O_2 suggest that each may produce similar perturbations on the Cu_B redox potential. If so, heme a_3 and Cu_B would be forced to perform as a concerted two-electron reductant for O_2, a predicted feature of the mechanism of O_2 reduction by this enzyme (Caughey et al., 1976).

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