QUANTITATIVE INFRARED SPECTROSCOPY OF CO COMPLEXES OF CYTOCHROME c OXIDASE, HEMOGLOBIN AND MYOGLOBIN: EVIDENCE FOR ONE CO PER HEME

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Received October 31, 1974

Summary. The carbonyl complex of fully reduced bovine heart cytochrome c oxidase in 0.01 M phosphate buffer at pH 7.4 exhibits an infrared absorption at 1963.5 cm^{-1} which is confirmed as due to the $^{12}C^{16}O$ stretch by shifts to 1919 and 1918 cm^{-1} with $^{12}C^{18}O$ and $^{13}C^{16}O$ respectively. A stoichiometry of one CO for each heme is demonstrated by the amount of HbCO formed upon exchange of CO from oxidase to Hb. This exchange can be followed by intensities of infrared or electronic spectra. The intensity of infrared absorption due to CO varies from 2.2×10^4 to 2.5×10^4 to 3.0×10^4 M^{-1} cm^{-2} for oxidase, Mb and Hb respectively, and represents an important property of bound CO characteristic of the particular binding site.

Introduction. The binding of CO to cytochrome c oxidase has been of interest since the classical photoactivation spectral studies of Warburg (1). Keilin and Hartree (2) concluded from their early work that CO bound only to cytochrome a_3 of an oxidase they described as containing a and a_3 cytochromes. Numerous estimates of CO/heme ratio have been made and range widely (i.e. 1:2.3 to 1:4) (3-6). However, in a preliminary report of infrared studies we noted a band presumably due to bound CO with an intensity suggestive of one CO bound to each heme of the oxidase (7). A CO/heme ratio of 1:1 indicates both hemes ($a + a_3$) of the reduced oxidase are available for ligand binding and is consistent with a mechanism of O_2 reduction in which there is formed a μ-peroxo linkage between two hemins (i.e. Fe^{III}-O-O-Fe^{III}), in analogy to autoxidation reactions of simple hemes (8). These preliminary findings have now been confirmed and extended to provide quantitative as well as qualitative information regarding CO binding to oxidase (Ox), hemoglobin (Hb) and myoglobin (Mb).

Methods and Materials: HbO_2 was prepared from whole blood (human) by the method of Geraci et al., (9). MbO_2 was isolated from bovine heart as previously described (10). Concentrations of Hb and Mb were obtained using extinction coefficients reported previously (11,12). Oxidase was prepared in detergent free media and assayed as previously described (13). The concentration of OxCO was determined from an A_{mM} of 63 at 431 nm based on total iron content.

Infrared spectra were measured in CaF_2 cell of either 0.0025 cm or 0.0055 cm in pathlength using a Perkin-Elmer 180 infrared spectrophotometer in the absorbance mode at a resolution of 3.0 cm^{-1}. Areas under absorption bands were measured over a 30 cm^{-1} region on both sides of the band center by planimetry. The apparent integrated absorption intensity (B) was calculated according to the equation

$$B = \frac{1}{c\ell}\int A \, d\nu$$

where c is the concentration in moles and ℓ is the pathlength in cm (14).

In the determination of CO by the infrared method, OxCO was prepared from 0.2 ml of enzyme solution ca. 1.5 mM in heme a with 0.01 M Na phosphate buffer pH 7.4 reduced with dithionite and maintained under a CO atmosphere for $\sim$ 1 hr to ensure complete saturation (15). In addition, as a blank the same buffer was maintained under a CO atmosphere for an identical time interval. Both oxidase and blank solutions were treated with an equal volume of 4.8 mM (in heme) HbO_2. The amount of HbCO formed in each case was determined by infrared spectroscopy from a standard curve of absorbance at 1951 cm^{-1} vs HbCO concentration. The amount of CO bound to oxidase was obtained as the difference between the total CO from the oxidase solution and the CO from the blank (i.e., dissolved CO). Inclusion of other proteins (i.e., albumin) did not alter the CO in the blank significantly. These reactions may be followed by visible spectra but the complex mixture of oxidized Ox, HbO_2 and HbCO is more readily examined by infrared methods.

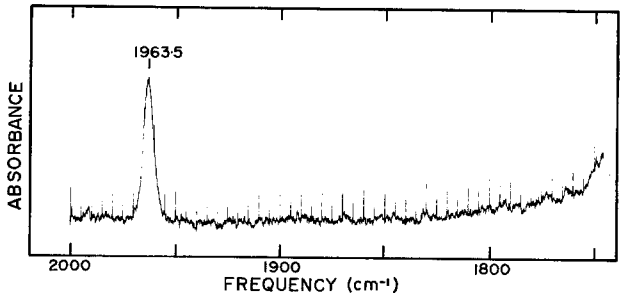

Figure 1: Infrared difference spectrum from 2000 to 1750 cm^{-1} for carbonyl oxidase vs oxidized oxidase at 2.4 mM in heme a in 0.01 M phosphate buffer pH 7.4.

In an alternate procedure involving the use of visible spectra and removal of dissolved CO by degassing, a solution of oxidized Ox (20-30 μM in heme a) in 0.01 M phosphate buffer pH 7.4 was degassed slowly with N_2 for 1 hr and then reduced with a slight excess of dithionite. CO was introduced for 2 min. The mixture was allowed to stand for 1 hr and then degassed for 10 min with nitrogen to remove dissolved CO. (No CO was detected in similarly degassed controls.) One ml of OxCO was mixed with 1 ml of an equivalent concentration of HbO_2 based on heme content in the sample cell. A solution of oxidized Ox of the same concentration was placed in the reference cell. The spectrum was recorded and the saturation determined from the A_{568nm}/A_{538nm} ratio. Various amounts of HbO_2 were added to determine in terms of the A_{568nm}/A_{538nm} ratio the concentration of HbO_2 required for complete exchange of CO from oxidase to Hb and thus establish the amount of CO bound to oxidase.

Results. Figure 1 illustrates the infrared difference spectrum of OxCO versus oxidized Ox. OxCO exhibits a single band at 1963.5 cm^{-1} with a $\nu_{1/2}$ of 5.5 cm^{-1}*. No deviation in band position or $\nu_{1/2}$ has been observed with more than 20 preparations of oxidase. Isotopic substitution with $^{12}C^{18}O$ or $^{13}C^{16}O$ results in shifts to 1919 cm^{-1} and 1918 cm^{-1} respectively.

The results of the Hb exchange reaction with the degassed method followed in the visible region gave a CO/heme a ratio of 1.0 ± 0.1. The infrared meth-

*This value is from a plot of absorbance vs ν whereas the value reported earlier was obtained from a transmission vs ν plot (16).

Table I: Infrared Absorption Maxima and Integrated Intensities(A) for Carbonyl Hemoglobin, Myoglobin and Cytochrome c Oxidase

Protein	$\nu_{CO}(cm^{-1})$	$A(M^{-1}cm^{-2})^a$
Hemoglobin	1951.0 ± 0.5	3.0×10^4
Myoglobin	1944.0 ± 0.5	2.5×10^4
Cytochrome c Oxidase	1963.5 ± 0.5	2.2×10^4

[a]True integrated absorption intensity (15) which represents an average of apparent integrated absorption intensities (B) from at least 8 different concentrations for each protein with maximum deviation from the average of ± 0.2×10^4. There was no apparent dependence of B upon concentration.

od without degassing to remove dissolved CO gave similar results; in a specific instance the oxidase concentration was 1.36 mM in heme and the amount of bound CO found was 1.26 mM. Thus use of the visible and infrared methods, either with or without degassing, yielded identical results.

The true integrated absorption intensities for carbonyl Hb, Mb and Ox are given in Table I.

Discussion. The effects of isotopic substitution with $^{12}C^{18}O$ and $^{13}C^{16}O$ provide conclusive proof that the 1963.5 cm^{-1} band for the OxCO is due to a C-O stretch. The presence of a single band suggests all CO molecules are similarly bound and the frequency indicates the CO is bound to Fe^{II}, not Cu^{I}, and is a terminal (Fe-C-O) rather than a bridging ligand (i.e., Fe-C-Fe) (16,17). The very narrow band width demands a highly ordered nearest neighbor environment about the bound CO with significant exclusion of randomly oriented external aqueous solvent from the site (17). A similar band was noted in intact heart muscle after exposure to CO (18).

The previous suggestion that one CO is bound to each heme a on the basis of a comparison of the intensity of the 1963.5 cm^{-1} band with the intensities of CO bands for HbCO and MbCO (7) required independent confirmation since no information on the variation of intensities with hemeprotein structure was

available. The high affinity of Hb for CO permits the quantitative determination of both bound and dissolved CO. Also, oxidation of the oxidase upon loss of CO prevents the transfer of CO from HbCO back to Ox. Therefore confirmation of the CO/heme stoichiometry was attained through the reactions in which CO was transferred from oxidase to Hb. For this the heme a concentration was obtained by iron analysis which proved more reliable than the pyridine hemochromogen method. All iron appeared to be in heme a since no non-heme iron was detected and the CO to Fe ratio was 1:1 with only a single C-O stretch band in the infrared.

The CO to heme a ratio of 1:1 found does not agree with earlier observations which varied from 1:2.3 to 1:4.1 (3-6). Possible reasons for this variability are differences in the oxidase preparations, in the degree of completeness of reduction and/or CO binding, and in the accuracy with which heme a and CO could be determined. The visible spectra of our preparations compared favorably with each of the other preparations. The methods used here are convenient, rapid, and require only 0.1 to 0.2 ml of protein solution, and gave consistent results over a large number of preparations.

Determination of the CO bound permitted the integrated absorption intensity for the C-O stretch in the oxidase to be established as a value somewhat lower than that found for MbCO and much lower than for HbCO (Table I). In analogy to simple metal carbonyls (19), π-electron density of the metal-C bond can be an important influence on intensities in carbonyl metal proteins but effects of non-linearity of the Fe-C-O bond and of interactions of the bound CO with protein and solvent must also be considered (20). Studies are presently underway to evaluate the relative contributions of such factors. Clearly the band intensity represents another important infrared parameter in addition to frequency and band width for use in the study of carbonyl and other hemeprotein derivatives.

The methods described here have also been applied successfully to determine the stoichiometry and infrared intensity for CO bound to hemocyanin and will soon be described elsewhere.

Acknowledgment. This work was generously supported by grants from the U.S. Public Health Service (HL-15980) and the National Science Foundation (GP-36856) and by donation of bovine heart by Monfort of Colorado, Inc.

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