Vol. 123, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1984 Pages 247-253

INFRARED SPECTRA OF CARBON MONOXIDE BOUND TO MITOCHONDRIA FROM DIVERSE SPECIES AND TISSUES REVEAL STRUCTURALLY SIMILAR CYTOCHROME c OXIDASE DIOXYGEN REACTION SITES

Lawrence J. Young†, Ólöf Einarsdóttir, Charles R. Vossbrink*, and Winslow S. Caughey

Department of Biochemistry, Colorado State University
Fort Collins, Colorado 80523

Received July 12, 1984

Summary. Infrared bands for CO bound to mitochondria from bovine and porcine hearts, bovine brain, rat kidney, and blowfly flight muscle and to intact blowfly flight muscle have been measured in the carbon-oxygen stretch region. Each spectrum contains a narrow band near 1963 cm^{-1} similar to the major band found earlier for the carbonyl cytochrome c oxidase purified from bovine heart. A second band near 1959 cm^{-1} ascribed to a less stable conformer of the purified oxidase carbonyl is also detected in mitochondria. These spectra support very similar CO (and O_2) binding sites among all the oxidases examined whether the enzyme is purified or is still within mitochondria or intact tissue and therefore suggest that the reduced heme A ligand binding site has been highly conserved during evolution.

Introduction. Cytochrome c oxidase (CcO), a complex membrane protein that occurs widely among animals, plants, and microorganisms, is a terminal oxidase with a key role in bioenergetics. This enzyme provides for the efficient use of energy derived from the reduction of dioxygen to water with electrons donated by four reduced cytochrome c molecules per O_2 (1,2). The enzyme can also serve as a carbon monoxide dioxygenase by catalyzing the reaction: $2CO + O_2 \rightarrow 2CO_2$ (3). A common feature of reduced CcO from different sources is a visible spectrum with a major band near 600 nm due to the presence of heme A, an iron porphyrin uniquely found in CcO (4). The few enzymes from diverse species examined carefully thus far contain two A-type hemes and two coppers in the mini-

†Present address: Department of Biochemistry, Duke University School of Medicine, Durham, NC. 27710
*Department of Entomology, University of Illinois at Urbana-Champaign, Urbana, IL. 61801

Abbreviations: CcO, cytochrome c oxidase; CcO·CO, carbonyl of fully-reduced cytochrome c oxidase; ν_{CO}, C-O stretch frequency; $\Delta\nu_{\frac{1}{2}}$ = band width at one half maximum band absorbance.

0006-291X/84 $1.50

mum O_2 reduction unit. Although oxidases from only a limited number of species have been studied, the widespread occurrence of proteins with similar visible spectra and oxidase function raises the possibility that the detailed structures and reaction mechanisms at the O_2 reaction sites have been highly conserved during biological evolution.

Infrared spectra of metal-bound CO provide a sensitive probe of hemeprotein dioxygen binding sites. Frequency and width parameters of C-O stretch bands vary widely among hemeprotein carbonyls as a result of differences in bonding and environment of the CO ligand (5). The unusually narrow band at 1963 cm^{-1} found for the carbonyl of cytochrome c oxidase (CcO·CO) purified from bovine heart was used to demonstrate that CO binds only to one heme A Fe^{2+} in a site well-isolated from the external medium (6,7). In contrast, cyanide infrared spectra show that CN^- may bind to one of the coppers as well as to one of the irons (8). The one iron and one copper shown by infrared studies to be accessible to external ligands appear involved in reactions at the dioxygen binding site (3,7-10). The study of CO binding is relevant to O_2 binding in that O_2 and CO are expected to compete for the same binding site.

Yoshikawa and Caughey recently reported that the highly purified bovine heart CcO·CO exhibits at least two C-O bands at 1963.5 and 1959.5 cm^{-1} (9). The two bands were ascribed to two protein conformers in analogy to the rapidly interconverting multiple conformers found in infrared spectra of myoglobin and hemoglobin carbonyls (10-14). The frequencies, widths, and relative intensity of the two CcO·CO bands change little with changes in temperature, pH, and overall oxidation state (9,15). These findings plus the extreme narrowness of both bands ($\Delta\nu_{\frac{1}{2}} \sim 4$ cm^{-1}) indicate the immediate environment about the CO ligand in the bovine CcO·CO is unusually stable for a hemeprotein carbonyl. We report here an extension of these CO infrared studies to compare CcO active sites in mitochondria of different species and/or tissues. The spectra obtained reveal remarkably similar active site structures in mitochondria from a variety of sources and the enzyme isolated from bovine heart.

Materials and Methods. Bovine and porcine heart mitochondria were isolated by the method of Smith (16); only the heavy fraction was used. High yields of mitochondria were obtained from the cortical gray matter of bovine brain by following the procedure of Bernard and Cockrell (17). Rat kidney mitochondria were isolated from tissue which had been at -70°C for 24 hours. The procedure of Rasmussen and Ogata (18) was employed except for using solution A of Bernard and Cockrell (17) as the homogenization medium. Flight muscle was removed intact from the thorax of the blowfly Sarcophaga nodosa by manual expression. The techniques of Slack and Bursell (19) were used to prepare mitochondria from blowfly muscle.

The pelleted mitochondria were frozen immediately following isolation and stored at -20°C. The presence of mitochondria in the pellet was confirmed by optical difference spectroscopy with an Aminco DW-2 spectrophotometer; reduced minus oxidized spectra contained band maxima near 602 nm for cytochrome c oxidase as well as bands near 560 and 550 nm for cytochromes b and c, respectively. The pellets were thawed the following day and placed directly on a CaF_2 infrared cell window, flushed with dry CO gas (99.7%, General Air Supply) for 10 min. and drops of neutralized substrate solution (10 mM Na succinate or 10 mM Na pyruvate) were applied to the mitochondrial paste to ensure reduction. CO flushing was continued for 45 minutes and then a cell spacer (0.1 mm thick) and a second CaF_2 were placed over the mitochondria. The mounted cell in a Beckman FH-01 variable temperature infrared cell was used for recording spectra at 30°C with a Perkin-Elmer Model 180 infrared spectrophotometer in a constant I_o, linear absorbance dual beam mode at a resolution less than one third the half-band width and a scan speed of 3-6 cm^{-1} per minute. The reference cell contained distilled water. Data points were accumulated at 0.1 cm^{-1} interval with the aid of an interfaced Tektronix 4051 computer which permitted spectral averaging, baseline correction, and deconvolution into bands of Gaussian shape by methods described earlier (11). The spectra reported represent the average of 4 to 9 single scans over the range from 2000 to 1900 cm^{-1} without utilizing smoothing procedures.

Results and Discussion. Representative C-O infrared spectra for CO bound to mitochondria from a variety of sources and to intact blowfly flight muscle are shown in Fig. 1. Panels F to J also show theoretical curves that represent attempts at deconvolution of the observed spectrum. In each case the major band near 1963 cm^{-1} can be reliably described by the band parameters (ν_{CO} and $\Delta\nu_{1/2}$) obtained upon deconvolution. The other theoretical bands are less reliably determined since they arise from a less unique deconvolution of the observed spectrum. Band parameters of the 1963 cm^{-1} band are compared in Table I.

CO bound to the fully-reduced CcO highly purified from bovine heart muscle exhibits a major band at 1963.3 ± 0.3 cm^{-1} with a half-band width of 4.2 ± 0.5 cm^{-1} (9). The assignment of the major band of bovine heart mitochondria (Fig. 1, B and G) with ν_{CO} = 1963.7 cm^{-1} and $\Delta\nu_{1/2}$ = 4.3 cm^{-1} to CcO·CO is therefore entirely reasonable and suggests little, if any, effect upon the active site as a result of removing CcO from the mitochondrial membrane. These band parameters are highly characteristic of CcO. The carbonyl of no hemeprotein other

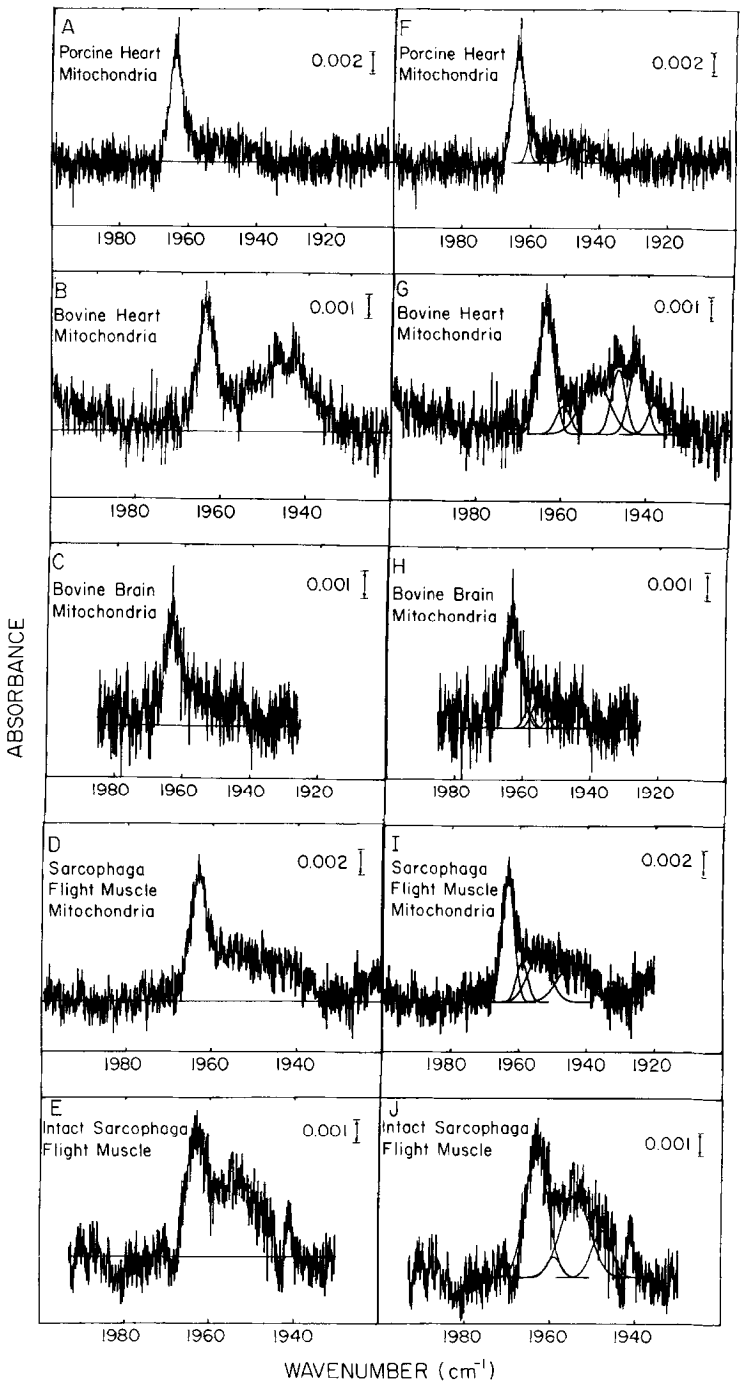

Figure 1. Infrared spectra of C-O bound to mitochondria from porcine heart, bovine heart, bovine brain and Sarcophaga flight muscle and to intact Sarcophaga flight muscle. Panels A to E show the infrared spectra slope corrected along a straight line that represents the baseline. Panels F to J represent spectra A to E with superimposed theoretical curves of Gaussian shape. In each case the difference between the observed spectrum and the sum of the Gaussian curves is essentially flat and corresponds closely to the baseline. (A) Porcine heart mitochondria. (B) Bovine heart mitochondria. (C) Bovine brain mitochondria. (D) Sarcophaga flight muscle mitochondria. (E) Sarcophaga

Table I. Parameters of the Major C-O Stretch Band for CO Bound to Reduced Cytochrome c Oxidase from Different Sources

Preparation	Source	ν_{CO} (cm^{-1})	$\Delta\nu_{\frac{1}{2}}$ (cm^{-1})
Mitochondria	Bovine heart	1963.7 ± 0.3	4.3 ± 0.5
"	Bovine brain	1963.2	5.9
"	Porcine heart	1963.7	4.0
"	Rat kidney	1965.1	3.0
"	Blowfly flight muscle	1963.2	4.2
Intact muscle	Blowfly flight muscle	1963.1	6.2
Pure protein[a]	Bovine heart muscle	1963.3	4.2

[a]From reference 9.

than cytochrome o from the myxobacterium Vitreoscilla exhibits a major band at this frequency and the cytochrome o is much wider with $\Delta\nu_{\frac{1}{2}} = 9$ cm^{-1} (20). No other hemeprotein carbonyl has yet been reported to have such a narrow band (5). For example, ν_{CO} ($\Delta\nu_{\frac{1}{2}}$) values in cm^{-1} for the major bands of human hemoglobin A CO of 1951 (8) and bovine myoglobin CO of 1944 (9) and 1938 (18) have been found (11-13). Furthermore the major CO binding protein in mitochondria is expected to be CcO. Therefore the band near 1963 cm^{-1} found in all the mitochondria examined as well as in the blowfly flight muscle, and earlier in mouse heart (21), is reasonably assigned to CcO·CO.

The 1963 cm^{-1} band uniformly exhibits an asymmetry that Gaussian curve fitting indicates is the result of a second band near 1959 cm^{-1} with $\Delta\nu_{\frac{1}{2}} \sim 4$ cm^{-1}. As mentioned above such a band is clearly evident in spectra for the isolated bovine CcO·CO (9). The relative intensities of 1963 and 1959 cm^{-1} bands have not been determined with great accuracy but there are obvious variations among mitochondria; for pig heart the 1959 cm^{-1} band is about 30% as intense as the 1963 cm^{-1} band whereas in other mitochondria and flight muscle the value ranges from 15 to 20%.

intact flight muscle. (F) Spectrum A, porcine heart mitochondria, and four theoretical curves with wavenumber (ν_{CO}) and half-band width ($\Delta\nu_{\frac{1}{2}}$) values in cm^{-1} of 1963.7 (4.0), 1959.5 (3.0), 1951.6 (6.5), and 1945.3 (7.0). (G) Spectrum B, bovine heart mitochondria, and six theoretical curves with ν_{CO} ($\Delta\nu_{\frac{1}{2}}$) values in cm^{-1} of 1963.7 (4.3), 1959.5 (4.0), 1952 (8.0), 1946.5 (4.0), 1942 (4.5) and 1937.5 (4.3). (H) Spectrum C of bovine brain mitochondria and five theoretical bands with ν_{CO} ($\Delta\nu_{\frac{1}{2}}$) values in cm^{-1} of 1963.2 (5.9), 1958.3 (3.3), 1955 (4.6), 1950 (4.4), and 1944.2 (5.4). (I) Spectrum D of Sarcophaga flight muscle mitochondria and four theoretical curves with ν_{CO} ($\Delta\nu_{\frac{1}{2}}$) values in cm^{-1} of 1963.2 (4.2), 1959 (4.0), 1954 (8.0), and 1944 (10). (J) Spectrum E of intact Sarcophaga flight muscle and four theoretical curves with ν_{CO} ($\Delta\nu_{\frac{1}{2}}$) in cm^{-1} of 1963.1 (6.2), 1959.0 (3.9), 1954 (7.8), 1947.8 (5.4) and 1941.2 (2.0).

Other bands are also found in the infrared spectra. A relatively sharp band at $\sim$ 1940 cm^{-1} in some spectra results from water vapor. Bands at $\sim$ 1945 and $\sim$ 1950 cm^{-1} for bovine brain and heart and porcine heart mitochondria may result from contamination with myoglobin and hemoglobin, respectively, as was suggested for intact mouse heart (21). A band near 1955 cm^{-1} with $\Delta\nu_{\frac{1}{2}}$ of 4.6 cm^{-1} appears in the bovine brain spectrum. Deconvolution of the flight muscle spectrum also indicates the presence of a rather intense band at 1954 cm^{-1} with $\Delta\nu_{\frac{1}{2}}$ of 7.8 cm^{-1} (Fig. 1J). A similar but less intense band is found in the flight muscle mitochondria spectrum (Fig. 1I). The bands near 1954 cm^{-1} for both bovine brain and flight muscle may represent an as yet unidentified CO binding hemeprotein. However, an intriguing possibility is that the band results from an additional conformer of CcO·CO. This possibility becomes more attractive in view of recent findings of two low intensity bands (conformers) at $\sim$ 1969 and $\sim$ 1955 cm^{-1} for the purified bovine heart enzyme (14) in addition to the 1963 and 1959 cm^{-1} bands reported earlier (9). Thus, possibly the conformer giving rise to the 1954 cm^{-1} band is more stable in Sarcophaga flight muscle and bovine brain than in the isolated bovine heart oxidase. Species differences have been observed to markedly affect conformer relative stabilities in myoglobins (10, 12, 14) and hemoglobins (13, 14, 22).

These studies demonstrate the usefulness of infrared spectroscopy for the comparison of ligand binding in isolated proteins, separated organelles, and intact tissue which can be supported by spectra of well-defined heme models. The remarkable similarities in 1963 and 1959 cm^{-1} band parameters found among the spectra for the isolated oxidase, mitochondria, and intact tissues thus far examined (Fig. 1 and Table I) provide strong support for very similar CO (and O_2) binding site structures. The apparent widespread conservation of the O_2 reaction site suggests that the chemistry of the active sites is very similar and that the reaction mechanisms for reductions of O_2 to water and for oxygenation of CO to CO_2 can be anticipated to be the same for all aa_3-type terminal oxidases.

Acknowledgement. This work was supported by U.S. Public Health Service Grant No. HL-15980.

References

1. Caughey, W.S., Wallace, W.J., Volpe, J.A. and Yoshikawa, S. (1976) in The Enzymes (Boyer, P.D., ed) Vol. 13, pp. 299-344, Academic Press, New York.
2. Wikstrom, M., Krab, K. and Saraste, M. (1981) Cytochrome Oxidase A Synthesis, Academic Press, New York.
3. Young, L.J. and Caughey, W.S. (1981) in Oxygen and Oxy-Radicals in Chemistry and Biology (Rodgers, M.A.J. and Powers, E.L., eds.) pp. 787-790, Academic Press, New York.
4. Caughey, W.S., Smythe, G.A., O'Keeffe, D.H., Maskasky, J.E. and Smith, M. L. (1975) J. Biol. Chem. 250, 7602-7622.
5. Caughey, W.S. (1980) in Methods for Determining Metal Ion Environments in Proteins: Structure and Function of Metalloproteins (Darnall, D.W. and Williams, R.G., eds) pp. 95-115, Elsevier/North-Holland, New York.
6. Caughey, W.S., Bayne, R.A. and McCoy, S. (1970) J. Chem. Soc. D, 950-951.
7. Yoshikawa, S., Choc, M.G., O'Toole, M.C. and Caughey, W.S. (1977) J. Biol. Chem. 252, 5498-5508.
8. Yoshikawa, S., Einarsdottir, O. and Caughey, W.S. (1984) Biophys. J. 45, 372a.
9. Yoshikawa, S. and Caughey, W.S. (1982) J. Biol. Chem. 257, 412-420.
10. Caughey, W.S., Shimada, H., Tucker, M.P., Kawanishi, S., Yoshikawa, S. and Young, L.J. (1982) in Oxygenases and Oxygen Metabolism (Nozaki, M. , Yamamoto, S., Ishimura, Y., Coon, M.J., Ernster, L. and Estabrook, R.W., eds) pp. 429-443, Academic Press, New York.
11. Caughey, W.S., Shimada, H., Choc, M.G. and Tucker, M.P. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 2903-2907.
12. Shimada, H. and Caughey, W.S. (1982) J. Biol. Chem. 257, 11893-11900.
13. Choc, M.G. and Caughey, W.S. (1981) J. Biol. Chem. 256, 1831-1838.
14. Caughey, W.S., Shimada, H., Hazzard, J.H., Houtchens, R.A., Potter, W.T. and Einarsdottir, O. (1983) Federation Proc. 42, 2000.
15. Choc, M.G., Einarsdottir, O. and Caughey, W.S. (unpublished results).
16. Smith, A.L. (1967) Methods Enzymol. 10, 78-86.
17. Bernard, P.A. and Cockrell, R.S. (1979) Biochim. Biophys. Acta 548, 173-186.
18. Rasmussen, H. and Ogata, E. (1966) Biochemistry 5, 733-745.
19. Slack, E.N. and Bursell, E. (1976) Biochim. Biophys. Acta 449, 491-499.
20. Choc, M.G., Webster, D.A. and Caughey, W.S. (1982) J. Biol. Chem. 257, 865-869.
21. Maxwell, J.C., Barlow, C.H., Spallholz, J.E. and Caughey, W.S. (1974) Biochem. Biophys. Res. Commun. 61, 230-236.
22. Caughey, W.S., Houtchens, R.A., Lanir, A., Maxwell, J.C. and Charache, S. (1978) (Caughey, W.S., ed.) pp. 29-56, Academic Press, New York.