Observation of Nonfundamental Fe−O_2 and Fe−CO Vibrations and Potential Anharmonicities for Oxyhemoglobin and Carbonmonoxyhemoglobin. Evidence Supporting a New Assignment of the Fe−C−O Bending Fundamental†

Shun Hirota, Takashi Ogura, and Teizo Kitagawa*

Graduate University for Advanced Studies and Institute for Molecular Science Okazaki National Research Institutes Myodaiji, Okazaki 444, Japan

Received June 30, 1994

The Fe−ligand interaction is a matter of fundamental concern in the chemistry of heme proteins, and resonance Raman (RR) spectroscopy has provided basic information on Fe−ligand vibrations for a variety of heme proteins and ligands.[1] The Fe-O_2 stretching mode (ν_{Fe-O_2}) was found at ~570 cm^{-1} for oxyhemoglobin (HbO_2),[2−4] oxymyoglobin (MbO_2),[4,5] and oxycytochrome *c* oxidase ($CcO{\cdot}O_2$),[6] at 530−560 cm^{-1} for oxyperoxidases,[7] and at ~540 cm^{-1} for oxycytochromes P-450.[8] Vibrational coupling between the O−O stretching and other modes causes splitting of the isotope-sensitive band and/or causes the observed frequency to be shifted from its intrinsic one.[9] The Fe−O−O bending (δ_{FeOO}) RR band has been recently identified at ~430 cm^{-1} for HbO_2 and $CcO{\cdot}O_2$[2,4] and at ~490 cm^{-1} for oxylactoperoxidase.[7b] The Fe−CO stretching (ν_{Fe-CO}) and Fe−C−O bending (δ_{FeCO}) modes were first assigned to the RR bands around 510 and 570 cm^{-1}, respectively,[10] but the latter assignment has not achieved full agreement.[11] Recently, a new CO-isotope-sensitive band assignable to δ_{FeCO} was found at ~360 cm^{-1} for various heme proteins.[12] Observation of nonfundamental bands would not only solve the assignment controversy but also provide new information on anharmonicities of potential functions, which are quite important for molecular dynamics calculations for the ligand. Accordingly, we have carefully examined the RR spectra of HbO_2 and HbCO and now report several nonfundamental Fe−ligand modes for the first time.

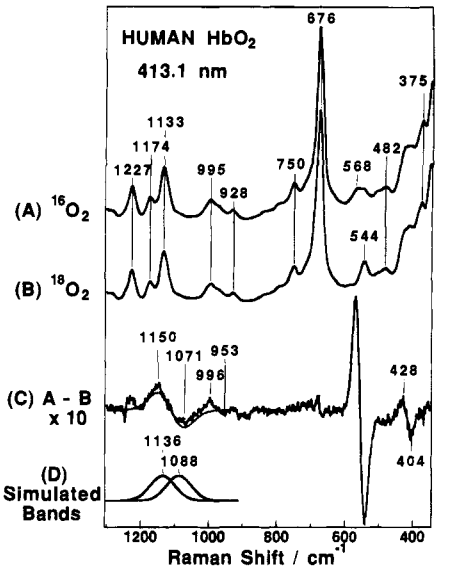

Figure 1. RR spectra in the 1300−350 cm^{-1} region for $^{16}O_2$ (A) and $^{18}O_2$ (B) adducts of HbA and their difference spectrum (C). The ordinate scales of spectra A and B are normalized by using intensities of porphyrin bands. The ordinate scale of spectrum C is expanded by a factor of 10. Trace D shows two Gaussian bands with bandwidths 86 cm^{-1}. Their difference spectrum is depicted by a broken line in trace C. Samples were contained in a spinning cell (3500 rpm), and the measurements were carried out at room temperature. Exposure time for one measurement was 320 s, and 10 measurements were carried out with each sample. The 10 spectra were almost similar, but the spectra shown here are those from one measurement. When partial oxidation occurred during the measurement, it was noticed easily from the noise levels and such spectra were discarded. Raman shifts were calibrated with ethanol and acetone as a secondary standard. Uncertainties of peak frequencies are ±1 cm^{-1} for raw spectra and ±2 cm^{-1} for difference spectra. Spectra A and B were observed with the following conditions; slit width, 200 μm; slit height, 10 mm; laser, 413.1 nm, 5 mW at the sample; laser beam, 50 μm at the sample; sample, 50 μM (heme) in 50 mM phosphate buffer, pH 7.2.

Raman scattering was excited at 413.1 nm with a Kr^+ ion laser (Spectra Physics 2016) and detected with a single polychromator (Ritsu Oyo Kogaku, DG-1000) equipped with a cooled diode array (PAR 1421HQ). Human adult Hb (HbA) was prepared according to the method of Geraci et al.,[13] and its concentration was adjusted to 50 μM (heme) with 50 mM sodium phosphate buffer pH 7.2. $Hb^{18}O_2$ was obtained by substituting the gas inside the cell with N_2, followed by substitution with $^{18}O_2$ (ICON, 99.5 atom %). $Hb^{16}O_2$ was obtained by exposing $Hb^{18}O_2$ to $^{16}O_2$ for 5 min. The $^{12}C^{16}O$ and $^{13}C^{18}O$ (ICON, 99 atom % for ^{13}C and 98 atom % for ^{18}O) adducts were obtained as described previously.[12]

Figure 1 shows the RR spectra in the 1300−350 cm^{-1} region for $Hb^{16}O_2$ (A), $Hb^{18}O_2$ (B), and their difference (C). In the raw spectra, the peak intensity of the 544 cm^{-1} band in B (largely $\nu_{Fe-^{18}O_2}$) appears stronger than that of the 568 cm^{-1} band in A ($\nu_{Fe-^{16}O_2}$) due to the presence of a porphyrin mode at ~540 cm^{-1}, but the difference spectrum yields the expected symmetric derivative pattern. The difference peak at 428/404 cm^{-1} was recently assigned to δ_{FeOO}.[4] We stress here that there are additional difference patterns at 1150/1071 and 996/953 cm^{-1}. The latter is easily interpreted as the stretching plus bending combination [(568 + 428)/(544 + 404)], while the former needs some discussion. The O−O stretching (ν_{OO}) IR bands of HbO_2 are reported at 1155 and 1106 cm^{-1} (1094 and 1065 cm^{-1} for $^{18}O_2$),[14] but their frequencies are clearly different from the frequencies in the present observation. Furthermore, the difference spectrum between $^{16}O_2$ and $^{16}O^{18}O$ adducts of

* Author to whom correspondence should be addressed.

† This study was supported by Grants-in-Aid from the Ministry of Education, Science, and Culture, Japan, to Priority Areas (bioinorganic chemistry) to T.K. (04225106) and (cell energetics) to T.O. (04780281).

(1) (a) Yu, N.-T. *Methods Enzymol.* **1986**, *130*, 350−409. (b) Yu, N.-T.; Kerr, E. A. In *Biological Applications of Raman Spectroscopy*; Spiro, T. G., Ed.; Wiley-Interscience: New York, 1988; Vol. 3, pp 39−95.

(2) Brunner, H. *Naturwissenschaften* **1974**, *61*, 129.

(3) Nagai, K.; Kitagawa, T.; Morimoto, H. *J. Mol. Biol.* **1980**, *136*, 271−289.

(4) Hirota, S.; Ogura, T.; Appelman, E. V.; Shinzawa-Itoh, K.; Yoshikawa, S.; Kitagawa, T. *J. Am. Chem. Soc.* **1994**, *116*, 10564−10570.

(5) Kerr, E. A.; Yu, N.-T.; Bartnicki, D. E.; Mizukami, H. *J. Biol. Chem.* **1985**, *260*, 8360−8365.

(6) (a) Ogura, T.; Takahashi, S.; Shinzawa-Itoh, K.; Yoshikawa, S.; Kitagawa, T. *J. Am. Chem. Soc.* **1990**, *112*, 5630−5631. (b) Han, S.; Ching, Y.-C.; Rousseau, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 2491−2495. (c) Varotsis, C.; Woodruff, W. H.; Babcock, G. T. *J. Am. Chem. Soc.* **1989**, *111*, 6439−6440.

(7) (a) Van Wart, H. E. V.; Zimmer, J. *J. Biol. Chem.* **1985**, *260*, 8372−8377. (b) Hu, S.; Kincaid, J. R. *J. Am. Chem. Soc.* **1991**, *113*, 7189−7194.

(8) (a) Bangcharoenpaurpong, O.; Rizos, A. K.; Champion, P. M.; Jollie, D.; Sligar, S. G. *J. Biol. Chem.* **1986**, *261*, 8089−8092. (b) Hu, S.; Schneider, A. J.; Kincaid, J. R. *J. Am. Chem. Soc.* **1991**, *113*, 4815−4822. (c) Egawa, T.; Ogura, T.; Makino, R.; Ishimura, Y.; Kitagawa, T. *J. Biol. Chem.* **1991**, *266*, 10246−10248.

(9) Proniewicz, L. M.; Bruha, A.; Nakamoto, K.; Kyuno, E.; Kincaid, J. R. *J. Am. Chem. Soc.* **1989**, *111*, 7050−7056.

(10) Tsubaki, M.; Srivastava, R. B.; Yu, N.-T. *Biochemistry* **1982**, *21*, 1132−1140.

(11) (a) Li, X. Y.; Spiro, T. G. *J. Am. Chem. Soc.* **1988**, *110*, 6024−6033. (b) Tsuboi, M. *Indian J. Pure Appl. Phys.* **1988**, *26*, 188−191. (c) Nagai, M.; Yoneyama, Y.; Kitagawa, T. *Biochemistry* **1991**, *30*, 6495−6503. (d) Ray, G. B.; Li, X. Y. Ibers, J. A.; Sessler, J. L.; Spiro, T. G. *J. Am. Chem. Soc.* **1994**, *116*, 162−176.

(12) Hirota, S.; Ogura, T.; Shinzawa-Itoh, K.; Yoshikawa, S.; Nagai, M.; Kitagawa, T. *J. Phys. Chem.* **1994**, *98*, 6652−6660.

(13) Geraci, G.; Parkhurst, L. J.; Gibson, Q. H. *J. Biol. Chem.* **1969**, *244*, 4664−4667.

0002-7863/95/1517-0821$09.00/0

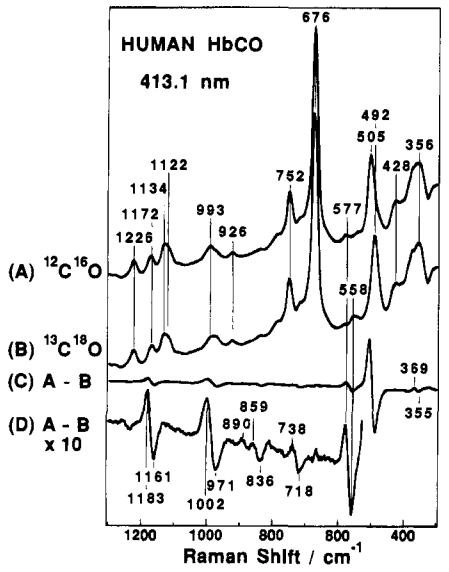

Figure 2. RR spectra in the 1300−300 cm^{-1} region for $^{12}C^{16}O$ (A) and $^{13}C^{18}O$ (B) adducts of HbA and their difference spectrum (C). Spectra A and B are the sums of five measurements with exposure time 320 s. These measurements were carried out twice for different samples, and the results were completely reproduced. Trace D depicts the ordinate scale expansion of trace C by a factor of 10. The ordinate scales of spectra A and B are normalized by using intensities of porphyrin bands. Experimental conditions were the same as those in Figure 1 except for the laser power, which was 3 mW at the sample.

HbA gave the corresponding difference peaks at 1150 and 1070 cm^{-1} (data not shown), indicating that the band is not associated with ν_{OO}. On the other hand, when Gaussian bands with bandwidth 86 cm^{-1} were assumed at 1136 and 1088 cm^{-1} as shown by trace D, their difference spectrum well reproduced the observed spectrum as drawn by a broken line in trace C. Consequently, it is reasonable to assign the 1150/1071 cm^{-1} peaks to an overtone of ν_{Fe-O_2}. Although its peak intensity appears to be only 18% of that of the fundamental, its area intensity is one-half that of the fundamental due to its large bandwidth. The large bandwidth might be due to the vibrational interaction with ν_{OO}. It is noted that the large overlapping between the $2\nu_{Fe-^{16}O_2}$ and $2\nu_{Fe-^{18}O_2}$ bands locates the difference peaks significantly outside of the true positions (by 14−17 cm^{-1}).

Figure 2 shows the RR spectra of $Hb^{12}C^{16}O$ (A) and $Hb^{13}C^{18}O$ (B) excited at 413.1 nm and their difference (C). The most intense difference peak at 505/492 cm^{-1} in spectrum C has been assigned to ν_{Fe-CO}.[10-12] The difference peaks at 577/558 cm^{-1} have been assigned to δ_{FeCO},[10,11a,d] but those at 369/355 cm^{-1} are now reassinged to δ_{FeCO} by a new proposal.[12] Here we want to point out the presence of additional difference peaks at 738/718, 859−890/836, 1002/971, and 1183/1161 cm^{-1} as illustrated by spectrum D, a 10-fold expansion of trace C. The two positive peaks at 859 and 890 cm^{-1} presumably resulted from vibrational coupling, and the unperturbed frequency would be ~875 cm^{-1}, since the sum of their intensities appears to be equal to the intensity of the negative peak at 836 cm^{-1}. The 1002/971 and 738/718 cm^{-1} peaks are assignable to overtones of ν_{Fe-CO} [(2 × 505)/(2 × 492)] and δ_{FeCO}[(2 × 369)/(2 × 355)]. The 859−890/836 cm^{-1} pair can be interpreted in terms of combination of ν_{Fe-CO} and δ_{FeCO} [(505 + 369)/(492 + 355)]. We note that the expected frequencies of $2\delta_{FeCO}$ and $\delta_{FeCO} + \nu_{Fe-CO}$ do not agree completely with the observed ones partly due to appreciable uncertainties involved in the expected values, since the peak positions in the difference spectra are not always the same as the peak positions of raw spectra and therefore the δ_{FeCO} frequencies for each isotope cannot be determined precisely from Figure 2C. However, these observations indicate that the difference bands at 369/355 cm^{-1} arise from fundamentals and thus strongly support their assignment to δ_{FeCO}.[12] The difference peaks at 1183/1161 cm^{-1} are assigned to $\nu_7 + \nu_{Fe-CO}$ combination [(676 + 505)/(676 + 492)], in agreement with Wang et al.[15] who recently reported the observation of the $2\nu_{Fe-CO}$ and $\nu_7 + \nu_{Fe-CO}$ bands for Mb and CcO and discussed the anharmonicities of the Fe−CO stretching modes of a large number of proteins.

The 1002/971 cm^{-1} difference pattern was well reproduced when the difference was calculated for two Gaussian bands at 1000 and 972 cm^{-1} with widths of 30 cm^{-1}. Since the expected frequencies for the fundamental and overtone in the presence of anharmonicity are $\nu_e(1-2x)$ and $2\nu_e(1-3x)$,[16] respectively, the anharmonic constant (x) for ν_{Fe-CO} is calculated to be 0.010, which suggests that the corresponding frequency at the lowest temperature is $\nu_0 = 507$ cm^{-1} and $\Delta\nu = 10$ cm^{-1}.[16] The same calculations for ν_{Fe-O_2} yielded $x < 0.001$. Therefore, anharmonicity for the Fe-CO potential is much larger than that of the $Fe-O_2$ potential, although the anharmonicity for the former seems to depend on the protein.[15]

Acknowledgment. The authors are indebted to Prof. M. Nagai of Kanazawa University and Dr. E. H. Appelman of Argonne National Laboratory for their courtesy in giving them purified HbA and $^{16}O^{18}O$, respectively.

JA942107Q

(14) Potter, W. T.; Tucker, M. P.; Houtchens, R. A.; Caughey, W. S. *Biochemistry* **1987**, *26*, 4699−4707.

(15) Wang, J.; Takahashi, S.; Rousseau, D. L. In *Proceedings of the 14th International Conference on Raman Spectroscopy*; Yu, N.-T., Li, X. Y., Eds.;) Wiley & Sons: Chichester, 1994; pp 118−119.

(16) When an anharmonic term proportional to $-\Delta r^3$ is incorporated as a perturbation to a harmonic potential, the energy level corresponding to a quantum number, v, is represented with the anharmonic constant, x, as $E_v = h\nu_e(v + 1/2) - h\nu_e x(v + 1/2)^2$. Under this approximation, the frequency for the $v \rightarrow v + 1$ transition is $\nu = \nu_0 - v\Delta\nu$, $\nu_0 = \nu_e - 2\nu_e x$, $\Delta\nu = 2\nu_e x$, and the thermal average of frequency observable at T K is $\langle\nu\rangle \approx \nu_0 - 2\Delta\nu/[\exp(h\nu_0/kT) - 1]$.