Resonance Raman Studies of Hemoglobin with Selectively Deuterated Hemes. A New Perspective on the Controversial Assignment of the Fe−CO Bending Mode

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Abstract: In an effort to resolve an existing controversy involving the assignment of the bending fundamental [δ(FeCO)] of CO−ligated heme proteins, resonance Raman studies of the CO adducts of native hemoglobin (Hb), together with Hb containing selectively deuterated hemes, have been conducted. Hemes were utilized which were deuterated at the peripheral methyl positions (d12) or at the methine carbon positions (d4). Several CO isotope sensitive modes were observed in the low-frequency region (300−400 cm^{-1}) by generating difference spectra from the absolute spectra of the natural abundance (NA) and doubly labeled $^{13}C^{18}O$ (DI) CO-Hb. These features included one at ∼370 cm^{-1}. Deconvolution of the low-frequency region revealed that a 1−2 cm^{-1} shift of a heme mode at 367 cm^{-1} is responsible for this difference feature. In the mid-frequency spectral region (650−1300 cm^{-1}), features which were previously suggested to be ascribable to combination bands and overtones involving the proposed ∼370 cm^{-1} δ(FeCO) fundamental showed sensitivity to heme deuteration, a fact which suggests that these modes are combinations involving heme modes rather than two internal fundamentals of the FeCO fragment. These results imply, therefore, that the observation of weak isotope-sensitive features in the 700−900 cm^{-1} region does not support the assignment of the ∼370 cm^{-1} feature to the δ(FeCO) fundamental, but these features are more reasonably interpreted to arise from combinations involving the relatively strong ν(Fe−CO) and lower frequency heme modes, an interpretation that is consistent with the long-standing assignment of the weak band near 580 cm^{-1} to the δ(FeCO) fundamental.

Introduction

The ability of the oxygen carrying heme proteins, myoglobin (Mb) and hemoglobin (Hb), to bind carbon monoxide has profound physiological implications and a considerable amount of effort has been expended in attempting to identify the protein structure and electronic factors which discriminate against CO binding in favor of dioxygen.[1] The fact that CO binds to d^6 transition metal complexes in a linear geometry, while the corresponding O_2 complexes are inherently bent, led Collman and co-workers[2] to propose that steric hindrance to formation of an FeCO fragment, imposed by the imidazole side chain of the pocket histidine, lowers the affinity of Hb and Mb for binding of CO relative to situations in which steric hindrance is absent. Naturally, many attempts to accumulate direct experimental evidence for off-axis distortions of the FeCO fragment in the CO adducts of these proteins have been made over the past two decades.[1−6]

While studies of crystals of the CO adducts by neutron[3] or X-ray diffraction[4] show apparent deviations from linearity (by ∼120−140°), a potentially more sensitive and reliable technique, which is applicable to the proteins in the physiologically more relevant solution phase, is vibrational spectroscopy, i.e., infrared and resonance Raman.[7−13] However, as is summarized in more detail later, the validity of structural and bonding parameters derived from resonance Raman studies of these systems remains questionable because of a controversy surrounding the identification and definitive assignment of one of the three fundamental modes of vibration of the FeCO fragment, namely the δ(FeCO) bending mode. While the ν(CO) and ν(Fe−CO) modes have been unambiguously assigned to features observed near 1960 and 500 cm^{-1}, respectively, disagreement has arisen as to the location of the bending fundamental.

The earliest assignment for δ(FeCO) was made for HbCO by Tsubaki et al.,[9] who observed a typical "zigzag" isotopic

(1) Kerr, E.; Yu, N.-T. *Biological Applications of Raman Spectroscopy*, Vol. 3: Resonance Raman Spectra of Heme and Metalloproteins, Spiro, T. G., Ed.; Wiley: New York, 1988; p 39.
(2) Collman, J. P.; Brauman, J. L.; Halbert, T. R.; Suslick, K. S. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3333.
(3) (a) Cheng, X.; Schoenborn, B. P. *Acta Crystallogr* **1991**, *B46*, 195. (b) Norvell, J. C.; Nunes, A. C.; Schoenborn, B. P. *Science* **1975**, *190*, 568. (c) Hanson, J. C.; Schoenborn, B. P. *J. Mol. Biol.* **1981**, *153*, 117.
(4) Kuriyan, J.; Wilz, S.; Karplus, M.; Petsko, G. A. *J. Mol. Biol.* **1986**, *192*, 133.
(5) Jewsbury, P.; Yamamoto, S.; Minato, T.; Kitagawa, T. *J. Am. Chem. Soc.* **1994**, *116*, 11586−11587.
(6) (a) Ray, G. B.; Li, X.-Y.; Ibers, J. A.; Sessler, J. L.; Spiro, T.G. *J. Am. Chem. Soc.* **1994**, *116*, 162. (b) Ghosh, A.; Bocian, D. F. *J. Phys. Chem.* **1996**, *100*, 6363. (c) Kozlowski, P. M.; Spiro, T. G. Manuscript in preparation.
(7) Hu, S.; Vogel, K. M.; Spiro, T. G. *J. Am. Chem. Soc.* **1994**, *116*, 11187.
(8) Li, X.-Y.; Spiro, T. G. *J. Am. Chem. Soc.* **1988**, *110*, 6024.
(9) Tsubaki, M.; Srivastava, R. B.; Yu, N.-T. *Biochemistry* **1982**, *21*, 1132.
(10) Ivanov et al. *J. Am. Chem. Soc.* **1994**, *116*, 4139−4140.
(11) Hirota, S.; Ogura, T.; Kitagawa, T. *J. Am. Chem. Soc.* **1995**, *117*, 821.
(12) Hu, S.; Smith, K. M.; Spiro, T. G. *J. Am. Chem. Soc.* **1996**, *118*, 12638.
(13) Hu, S.; Morris, I. K.; Singh, J. P.; Smith, K. M.; Spiro, T. G. *J. Am. Chem. Soc.* **1993**, *115*, 12446.

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shift pattern, which reflects greater sensitivity to the mass of the central carbon atom, as expected for a linear FeCO linkage. In the case of HbCO, the specific frequencies observed were the following: 578 cm $^{-1}$ ($^{12}C^{16}O$), 563 cm^{-1} ($^{13}C^{16}O$), 576 cm^{-1} ($^{12}C^{18}O$), and 560 cm^{-1} ($^{13}C^{18}O$). Though an alternative interpretation, suggesting the 578 cm^{-1} band might be the first overtone of a much lower energy (289 cm^{-1}) δ(FeCO) fundamental, was proposed early on,[14] the original assignment to the feature near 580 cm^{-1} had become generally accepted and the corresponding mode has been identified for the CO adducts of a large number of heme proteins.[15a−d]

Recently, it has been suggested that the feature near 580 cm^{-1}, conventionally assigned to δ(FeCO), is not the fundamental, but is a combination band involving an unidentified low-frequency heme macrocycle mode and a proposed δ(FeCO) fundamental that is observed only as a very weak feature occurring near 370 cm^{-1} in the $^{12}C^{16}O/^{13}C^{18}O$ difference spectrum.[16] This proposal was quickly questioned by Hu et al.[7] in a study of the infrared spectra of simple, well-characterized model compounds, where it was shown that a distinct infrared-active, isotope sensitive feature is observed near 580 cm^{-1} with no evidence of such modes below 500 cm^{-1}. After the appearance of the IR study, Hirota et al.[11] observed a set of complicated isotope-sensitive features appearing between 700 and 900 cm^{-1}. According to these workers, the interpretation of these difference patterns required the presence of the δ(FeCO) fundamental near 370 cm^{-1} and their existence is presented as support for this assignment. While the observation of distinct, isolated isotopically sensitive difference features near 580 cm^{-1} in the IR spectra of model compounds would seem to provide unequivocable evidence for the validity of the assignment of the ∼580 cm^{-1} feature in the RR spectra of CO adducts of heme proteins, the undisputed observation of the weak difference patterns near 370 cm^{-1}, as well as those between 700 and 900 cm^{-1}, then remains unexplained.

The most obvious approach to resolve this controversy is to attempt to induce shifts in the heme modes which are proposed to participate in the supposed combination mode at 578 cm^{-1} and in those which are CO isotope sensitive in both the low-frequency (300−400 cm^{-1}) and mid-frequency spectral regions, by deuterium labeling of the macrocycle. Herein are reported detailed RR studies of such deuterium-labeled hemoglobins which clearly show that the mode near 575 cm^{-1} is insensitive to heme deuteration, supporting its assignment as δ(FeCO). Deconvolution and simulation studies were also done to extract absolute frequencies for the other CO isotope sensitive spectral features. The present studies provide a new perspective on this controversy, clearly showing that the weak difference feature near 370 cm^{-1} arises from very slight shifts in low-frequency heme modes, and that all of the difference features between 700 and 900 cm^{-1} are reasonably assignable to combinations involving the relatively strong, allowed ν(FeC) with various heme macrocycle deformation modes.

Experimental Section

Preparation of Selectively Deuterated Hemins. (A) Protoheme-d4. Protoporphyrin IX dimethyl ester (PPIXDME) was obtained from Porphyrin Products (Logan, Utah) and used only after checking for purity by absorption spectrophotometry, TLC, and ^{1}H NMR.[17a,b] Deuterium exchange at the four methine positions was accomplished, according to previously reported procedures,[17c] by refluxing 60 mg of the PPIXDME in 15 mL of a solution of MgI_2 (50 mg/mL) in pyridine containing 12% (v/v) of CH_3OD for 72 h. Following removal of complexed Mg^{2+} and reesterification (at −20 °C) in 5% (W/W) H_2SO_4 in CH_3OH, the resulting methine-deuterated PPIXDME (PPDME-d4) was extracted into CH_2Cl_2 and chromatographed on Al_2O_3, using CH_2Cl_2 as eluent. ^{1}H NMR spectroscopy[17c,e] revealed no impurities and confirmed nearly complete (>95%) deuteration at the methine positions. Iron was inserted according to established procedures[17d] as was hydrolysis of the remaining ester groups[17e] to yield the final product, which was checked by ^{1}H NMR.

(B) Synthesis of Protoheme-d12. The peripheral methyl groups of protoheme were deuterated by using the method of Godziela et al.[17e] Approximately 65 mg of Fe(III)-protoporphyrin IX was stirred in dimethyl sulfoxide-d6 (Me_2SO-d6), 6 mM) containing 3 to 7 equiv of tetrabutylammonium hydroxide ((TBA)OH) for 72 h. The reactions were quenched at the end of 72 h through precipitation of the iron porphyrin by the addition of 1 M aqueous HCl (added slowly until precipitation occurred). The iron porphyrin was then separated by centrifugation, washed 3 times with deionized water, and dried in a vacuum desiccator. Deuteration of the methyl groups was confirmed by ^{1}H NMR.[17c]

Isolation of Hb and Reconstitution with Deuterated Hemes. Hb was isolated from red blood cells, obtained from a local blood center, according to well-established procedures.[17f] ApoHb was generated by using the acid−acetone procedure[17g] and reconstituted with a selected deuterated hemin with standard procedures.[17h] The resulting Hb containing deuterated hemin was purified on a DEAE column followed by reduction with dithionite under a CO atmosphere to produce the CO derivative. Subsequent removal of excess dithionite and buffer change for Raman measurements was accomplished by passage of the Hb through a Bio-gel P6-DG column equilibrated with 50 mM phosphate buffer, pH 7.4. Final concentrations were 0.2−0.3 mM Hb. CO-Hb (natural abundance) was made by gently bubbling CO gas through the Hb solution, which was kept on ice for approximately 10−15 min. Doubly labeled $^{13}C^{18}O$ gas was transferred to an NMR tube containing deoxyHb (obtained from oxyHb by using a vacuum line to first pull off O_2). This was done 3 times to ensure complete ligation. The sample was kept on ice and allowed to equilibrate for at least half an hour before measurements were taken. Complete ligation was checked by RR measurements.

Resonance Raman Measurements. Resonance Raman measurements were done on a Spex Model 1269 spectrometer equipped with a UV-enhanced CCD detector (Princeton Instruments, NJ) and appropriate notch filters (Kaiser Optical). The 413.1 nm excitation from a Coherent Model Innova 100 Kr+ laser was used for native CO-Hb as well as for protoheme-d12 and protoheme-d4 reconstituted CO-Hb. A cylindrical lens was used and power was kept at 0.8 mW to avoid photodissociation of the samples. The absence of photodissociated species (i.e., deoxyHb) was confirmed by the absence of the ν_4 mode for deoxyHb.

Deconvolution Procedure. In the low-frequency region (200−400 cm^{-1}), deconvolution studies were done in an effort to identify the low-frequency modes and their ligated frequencies. Spectracalc@ software was used for this routine. Briefly, initial parameters (frequencies, bandwidths, and intensities) for 9 modes were selected based on consideration of results from previous studies of MbCO[12,18] and HbO_2[19]

(14) Tsuboi, M. *Indian J. Pure Appl. Phys.* **1988**, *26*, 188.

(15) (a) Han, S.; Madden, J. F.; Siegel, L. M.; Spiro, T. G. *Biochemistry* **1989**, *28*, 5477. (b) Uno, T. et al. *J. Biol. Chem.* **1987**, *262*, 4549. (c) Evangelista-Kirkup, R.; Smulevich, G.; Spiro, T. G. *Biochemistry* **1986**, *25*, 4420. (d) Smulevich et al. *Biochemistry* **1988**, *27*, 5486.

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

(17) (a) *Laboratory Methods in Porphyrin and Metalloporphyrin Research;* Fuhrhop, J.-H., Smith, K. M., Eds.; Elsevier Publishing Co.: New York, 1975. (b) Dinello, R. K.; Dolphin, D. H. *Anal. Biochem.* **1975**, *64*, 444. (c) Barbush, M.; Dixon, D. W. *Biochem. Biophys. Res. Commun.* **1985**, *129*, 70. (d) Kenner, G. W.; Smith, K. M.; Sutton, M. J. *Tetrahedron. Lett.* **1973**, *16*, 1303. (e) Parrish, D. W. Ph.D. *Thesis*, University of California, Davis, 1984. (f) Godziela, G. M.; Kramer, S. K.; Goff, G. M. *Inorg. Chem.* **1986**, *25*, 4286. (g) Riggs, A. *Methods in Enzymology*; Antonini, E., Rossi-Bernardi, L., Chiancone, E., Eds.; 1981; Vol. 76: Hemoglobin, p 5; Academic Press: New York. (h) Jeyarajah, S.; *Thesis* **1990** Marquette University, Milwaukee, WI.

(18) Wells, A. V.; Sage, J. T.; Morikis, D.; Champion, P. M.; Chiu, M. L.; Sligar, S. G. *J. Am. Chem. Soc.* **1991**, *113*, 9655.

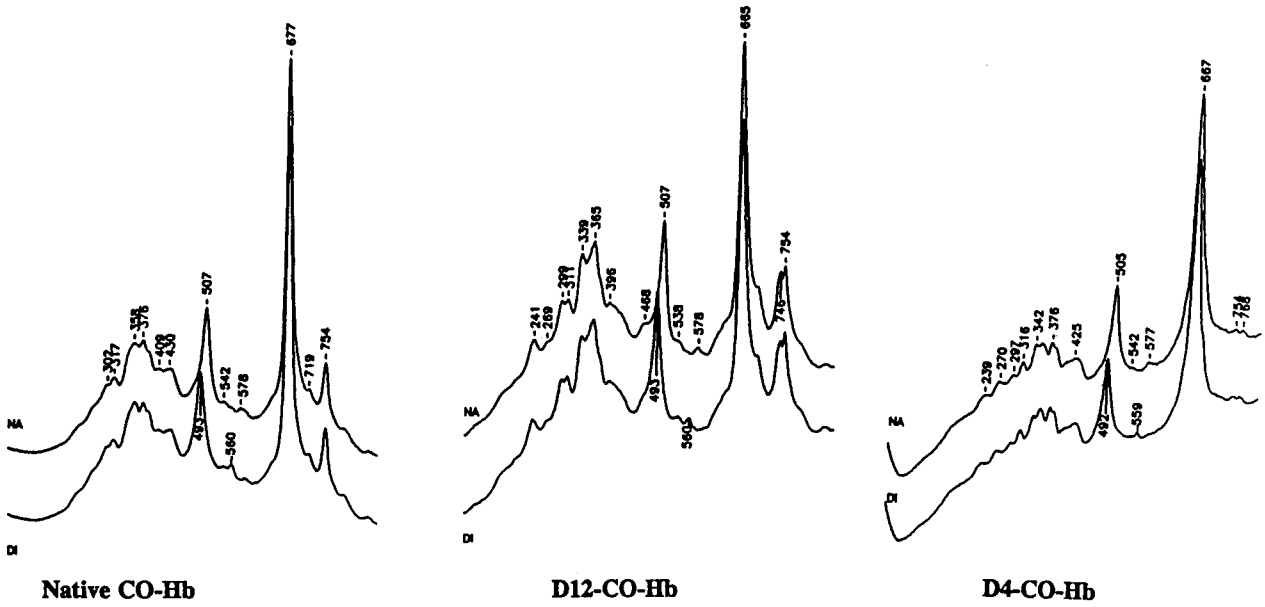

Figure 1. Low-frequency (100−700 cm^{-1}) absolute spectra for native, d4, and d12-CO−Hb species (both natural abundance = NA and doubly labeled = DI CO isotopomers).

for the spectral region of interest. A 50/50% Lorentzian/Gaussian band shape for all bands was assumed and fixed. Deconvolution was performed for both the natural abundance and double isotope spectra for each Hb species by using these initial estimates. The bandwidths obtained were then averaged between NA and DI for each species and the procedure was then repeated with the same fixed bandwidths for each spectrum (i.e., NA and DI). Next, an average intensity for each band between CO isotopomers was found and the deconvolution procedure repeated with nonfixed bandwidths. This systematic procedure of fixing and unfixing bandwidths and intensities was repeated until the best fit (CHISQ) was obtained for both isotopomers. The final frequencies (which were allowed to vary throughout) were those obtained when the CHISQ value was minimized.

Simulation Procedure. Simulation studies conducted as described previously[20d] were performed to extract absolute frequencies of the CO isotope sensitive modes from the isotopic shift patterns seen in the difference spectra. Initial estimates for the input frequencies were based on the deconvolution studies unless the mode, such as ν(Fe−CO), was clearly resolved in the absolute spectrum. Bandwidths were initially estimated as the sum of the bandwidths for the low-frequency mode (from deconvolution) and that of ν(FeCO). In the program, frequency, bandwidth, and intensity were varied systematically until the experimentally derived difference pattern was well-reproduced by the simulated data (within 1−2 cm^{-1}). The bandwidth and intensity of a given band were not allowed to vary between CO isotopomers of the same Hb species.

Results and Discussion

Low-Frequency Region, 100−600 cm^{-1}. The absolute spectra (i.e., NA = natural abundance and DI = double isotope) obtained in the present study for the frequency range ∼100−700 cm^{-1} are shown in Figure 1, for all three Hb species studied (i.e., native Hb, d12-Hb, and d4-Hb). The spectrum for the native Hb sample is virtually identical with that reported by Hirota et al.[16] and others.[21,22] The isotopic shift most evident in all the spectra is for the mode that occurs at ∼507 cm^{-1} for $^{12}C^{16}O$ (NA) and at ∼495 cm^{-1} for $^{13}C^{18}O$ (DI). This feature has been unambiguously assigned to the ν(Fe−CO) mode. Clearly, there is also an isotopic shift observed for the feature at ∼580 cm^{-1}/560 cm^{-1}, which corresponds to the fundamental δ(FeCO) as assigned by most workers.[1,6−9,14]

Figure 2 shows the NA − DI difference spectra for the region near these obvious isotope sensitive modes. From the figure it is clear that these modes are essentially insensitive to heme deuteration, the derived maxima and minima for all three heme species being within 1 cm^{-1} of each other. The important point to be noted here is that such insensitivity of the ∼580 cm^{-1} feature to heme deuteration is consistent with the original assignment[1,6−9,14] of this feature to the δ(FeCO) fundamental. The alternative assignment,[11,14,16] which proposes that this feature is a combination band involving a lower frequency δ-(FeCO) with a low-frequency heme deformation mode, would suggest that some sensitivity to heme deuteration would likely be observed, given that all of the low-frequency heme modes show some sensitivity to deuteration at the methine and/or the peripheral methyl groups as shown below.

In the crowded spectral region between 100 and 500 cm^{-1}, wherein weak difference features were recently observed,[16] there are many potentially active, overlapping low-frequency heme macrocycle deformation modes.[12−14] Many previous workers have experimentally observed and assigned at least nine RR active heme-based modes in the spectral region between 200 and 400 cm^{-1}.[14,16,18,19] Based on this documentation, nine low-frequency heme modes were included in the deconvolution procedure described in the Experimental Section, which was used to attempt to extract the absolute frequencies, intensities, and bandwidths of all the features comprising the complicated spectral patterns observed for all six species studied (i.e., both $^{12}C^{16}O$ and $^{13}C^{18}O$ isotopomers of three different hemes; native,

(19) Jeyarajah, S.; Proniewicz, L. M.; Bronder, H.; Kincaid, J. R. *J. Biol. Chem.* **1994**, *269*, 31047.

(20) (a) Simianu, M. C.; Kincaid, J. R. *J. Am. Chem. Soc.* **1995**, *117*, 4628. (b) Rajani, C.; Kincaid, J. R. *J. Raman Spectroscopy* **1995**, *26*, 969. (c) Al-Mustafa, J.; Knicaid, J. R. *Biochemistry* **1994**, *33*, 2191. (d) Al-Mustafa, J.; Sykora, M.; Kincaid, J. R. *J. Biol. Chem.* **1995**, *270*, 10449. (e) Hu, S.; Treat, R. W.; Kincaid, J. R. *Biochemistry* **1993**, *32*, 10125. (f) Hirota, S.; Ogura, T.; Shinzawa-Itoh, K.; Yoshikawa, S.; Kitagawa, T. *J. Phys. Chem.* **1996**, *100*, 15274.

(21) Wang, J.; Takahashi, S.; Rousseau, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 9402.

(22) Jayaraman, V.; Spiro, T. G. *Biospectroscopy* **1996**, *2*, 311.

(23) Li, X.-Y.; Czernuszewicz, R. S.; Kincaid, J. R.; Su, Y. O.; Spiro, T. G. *J. Phys. Chem.* **1990**, *94*, 31.

(24) Jeyarajah, S.; Kincaid, J. R. *Biochemistry* **1990**, *29*, 5087.

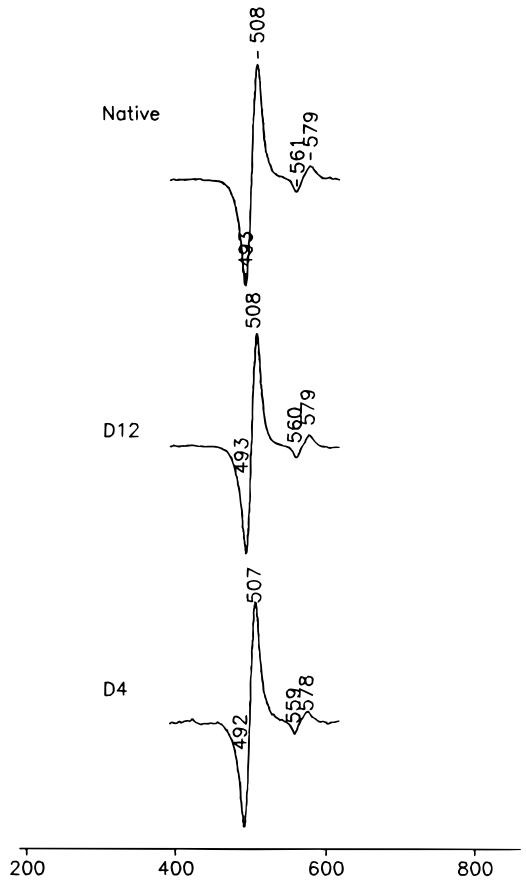

Figure 2. Difference spectra generated by subtraction (NA − DI) of two absolute spectra for the 400−600 cm^{-1} spectral region showing ν(FeC) and δ(FeCO) as assigned by Tsubaki et al.[9] for native, d4, and d12-CO-Hb.

d4, and d12). The results of this procedure are shown in Figure 3 and summarized in Table 1.

As is clearly shown in Figure 3, the composite spectra from the deconvolution studies satisfactorily reproduce the experimental spectra for each hemoglobin studied. For a given hemoglobin, the relative intensities and bandwidths are the same for both NA and DI isotopomers. In comparing the frequencies for the deuterated vs nondeuterated heme modes, all the derived shifts are in the right direction and are comparable to those observed by other workers for other heme proteins:[12,13,22] i.e., ν_8 downshifts 4 cm^{-1} for d4 and 8 cm^{-1} for d12 relative to native Hb; ν_{52} downshifts to 268 and 270 cm^{-1} for the deuterated species from its value of 274 cm^{-1} for the native Hb. From the summarized results of the deconvolution in Table 1, it can be seen that there is a consistent 1 cm^{-1} downshift in the doubly labeled isotopomer for the mode at ∼317 cm^{-1} for all the Hb species. Another mode at ∼365 cm^{-1} also experiences a consistent 1 cm^{-1} downshift for the $^{13}C^{18}O$ isotopomer for each of the three hemes studied.

There is a well-established precedence for weak sensitivity of certain heme localized deformation modes to XY isotopic substitution of FeXY fragments. For instance, studies of many CN^--ligated heme proteins, including both Hb and Mb, have revealed a number of low-frequency modes that are sensitive to isotopic substitution of the transaxial ligand.[20a−f] In cytochrome P-450, two CN^- isotope sensitive heme modes were seen at 328 and 350 cm^{-1}.[20a] In studies of CN^--ligated cytochrome *c* peroxidase, an isotope-sensitive feature was detected at 372 cm^{-1}.[20b] CN^--ligated horseradish peroxidase exhibited three isotope-sensitive heme modes in this spectral region at 378, 368, and 318 cm^{-1}.[20c] Both beef liver catalase and the catalase isolated from *Aspergillus niger* have CN^- isotope sensitive macrocycle deformation modes at 380 cm^{-1}.[20d] Lactoperoxidase has been found to have a CN^- isotope sensitive feature at 315 cm^{-1}.[20e] Hirota et al.,[20f] in an investigation of CN^--ligated Hb, Mb, and cytochrome *c* oxidase, noted the following isotope-sensitive features attributable to essentially porphyrin modes: for Hb, difference patterns were seen at 345, 357, and 313 cm^{-1}; for Mb, CN^- isotope sensitive features were detected at 425, 404, 385, 374, 302, and 257 cm^{-1}; for cytochrome *c* oxidase, an additional CN^- isotope sensitive mode was found at 369 cm^{-1}. All of the above modes exhibited small shifts, (i.e., 2−4 cm^{-1}) compared to the fundamental δ(FeCN$^-$) bending mode that typically showed ∼15 cm^{-1} shifts.

Table 1. Identity of Low-Frequency Modes in the 200−400 cm^{-1} Spectral Region Which Were Derived from Deconvolution Studies

	freq (cm^{-1})			
	NA	DI	fwhm	10^6 *I*
Native				
ν_9	242	242	23.2	1.029
ν_{52}	274	274	26.8	2.375
γ_7	299	299	22.0	3.951
γ_{16}	317	316	17.0	4.025
ν_8	343	343	18.9	4.851
γ_6	355	355	17.2	5.094
ν_{50}	365	363	14.8	4.340
$\delta(C_\beta C_c C_d)$	377	377	12.4	5.894
$\delta(C_\beta C_c C_d)$	388	388	12.4	3.809
d4				
ν_9	237	237	23.2	1.929
ν_{52}	268	268	26.0	2.951
γ_7	295	295	20.0	2.570
γ_{16}	316	315	15.6	3.838
ν_8	341	341	19.0	5.516
γ_6	355	355	16.0	3.683
ν_{50}	361	360	14.8	2.232
$\delta(C_\beta C_c C_d)$	375	375	12.4	4.806
$\delta(C_\beta C_c C_d)$	385	385	12.4	3.523
d12				
ν_9	240	240	23.2	4.096
ν_{52}	270	270	26.0	1.790
γ_7	297	297	20.0	5.746
γ_{16}	313	312	15.2	4.046
ν_8	337	337	16.0	9.256
γ^6	348	348	16.3	4.854
ν_{50}	358	358	15.6	5.011
$\delta(C_\beta C_c C_d)$	366	365	12.6	3.565
$\delta(C_\beta C_c C_d)$	377	377	11.2	3.996

When the composite spectra (derived from the deconvolution) for both CO isotopomers (i.e., NA and DI) were subtracted, the result (Figure 4) was a difference spectrum that was essentially superimposable on the difference spectrum generated from the experimental absolute spectra. This observation is obviously consistent with the proposal that the observed experimental difference patterns arise because of slight (1 cm^{-1}) isotopic shifts of two low-frequency heme deformation modes.

In summary of this section, it can be seen that the observation of weak difference patterns near 370 cm^{-1}, observed here and in previous work,[16] does not require the existence of a lower frequency δ(FeCO) fundamental, but those patterns arise naturally from a very weak sensitivity of certain heme deformation modes to CO isotopic substitution, a sensitivity for which there is a well-documented precedence.[20a−f] Carefully conducted deconvolution studies, employing only 9 heme-based modes for which there is literature precedence, reveal systematic heme deuteration shifts which are consistent with those expected[12−14] and produce composite absolute spectra whose

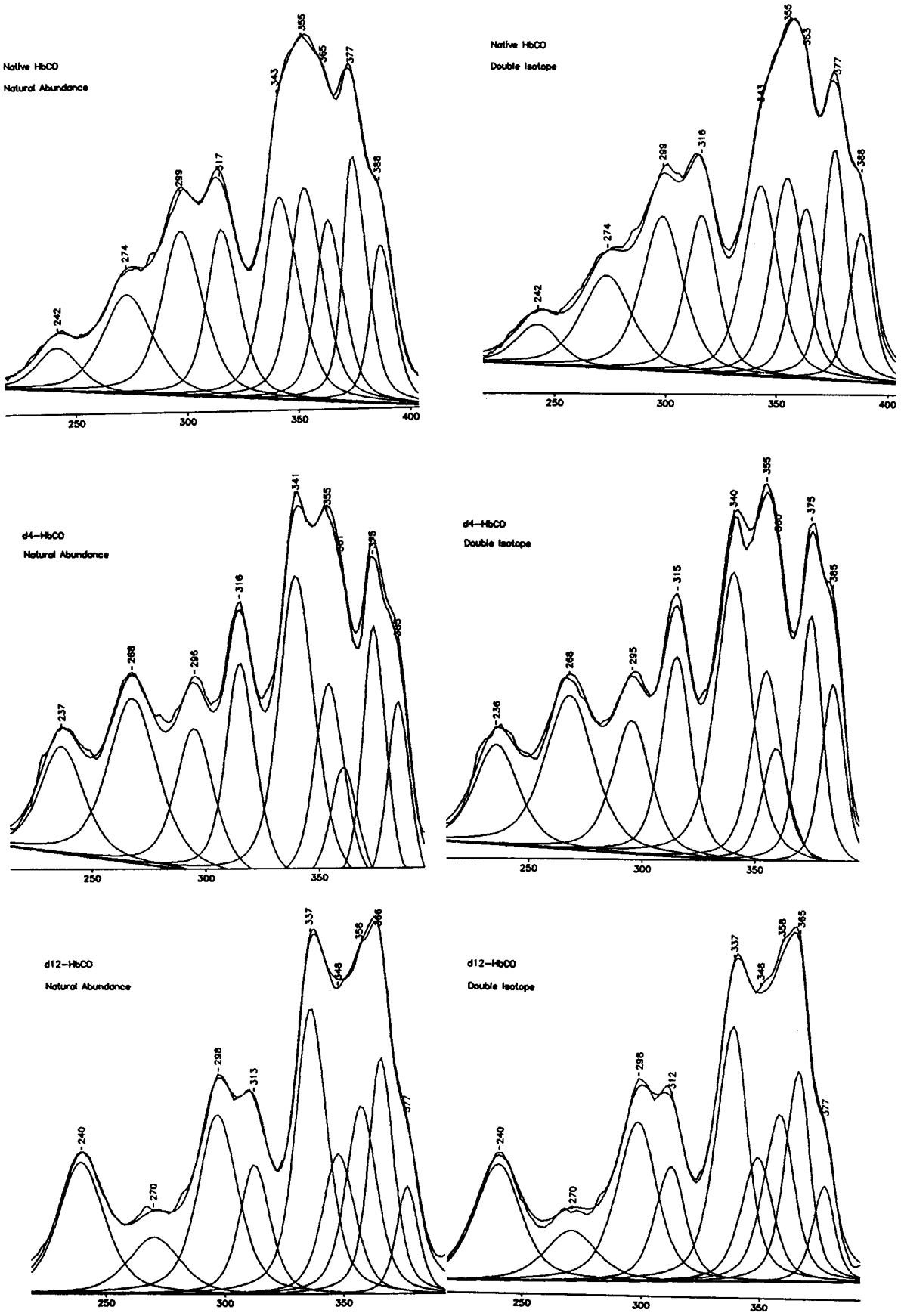

Figure 3. Deconvolution of the low-frequency modes for native, d12, and d4-CO-Hb. Values for frequencies, bandwidths, and intensities are tabulated in Table 1.

difference spectral patterns match very closely those observed (Figure 4).

Combinations and Overtones in the 700−1300 cm^{-1} Region. As shown in Figure 5, the two strongest CO isotope

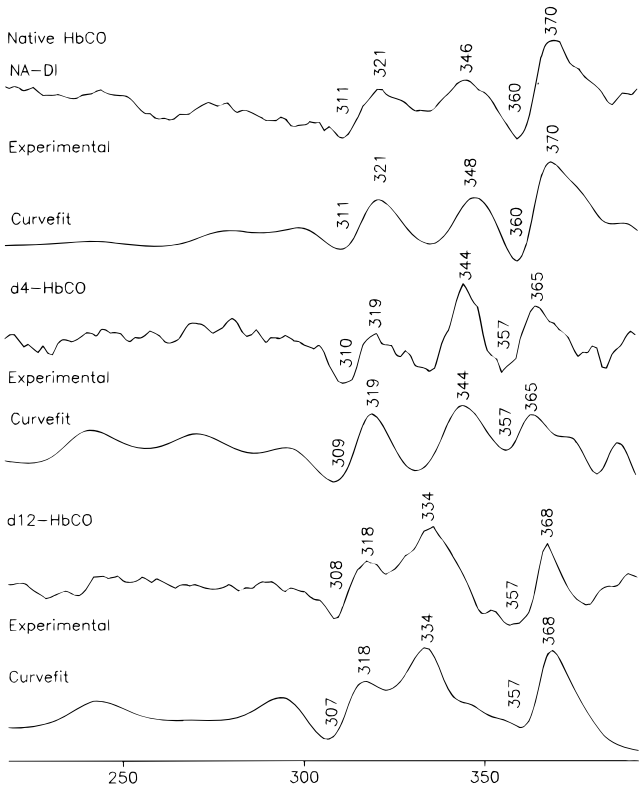

Figure 4. Difference spectra generated by subtraction (NA − DI) of absolute spectra for the 300−400 cm^{-1} spectral region and the corresponding difference spectra (NA − DI) from the composites generated from deconvolution studies done on all three Hb species studied.

sensitive difference features appearing between 700 and 1300 cm^{-1} are observed near 1000 and 1180 cm^{-1}. Both Hirota et al.[11] and Wang et al.[21] have reported and assigned two difference patterns seen in this mid-frequency region: a feature at 1004/974 cm^{-1}, which had been previously assigned to the overtone 2υ(Fe−CO),[11,21] and another at 1183/1165 cm^{-1}, which was assigned to a combination band, $\upsilon_7 + \upsilon$(Fe−CO).[11,21] The assignment of the latter to a combination band is confirmed in the present study by the d4 and d12 data, as the downshift in this mode corresponds well to the observed downshift in υ_7 (678 → 668 cm^{-1}) for the deuterated Hb species. The feature occurring near ∼1000 cm^{-1} is not downshifted for any of the deuterated Hb samples; behavior consistent with its assignment as the first overtone of υ(Fe−CO).

In the region between 700 and 900 cm^{-1} a complex pattern of weak CO isotope sensitive features is observed for all three hemoglobins studied here. The pattern observed for the native protein essentially matches that first reported by Hirota at al.,[11] who suggested that the appearance of such a difference pattern in this region supports assignment of the δ(FeCO) fundamental to the weak difference feature occurring near 370 cm^{-1}. Thus, the individual components of the $^{12}C^{16}O/^{13}C^{18}O$ difference pattern observed for native Hb were assigned by Hirota et al.[11] as follows: (a) a difference feature occurring at 738/718 cm^{-1} was assigned to an overtone of the proposed δ(FeCO) fundamental [2(369)/2(355)], the calculated resultant frequencies being 738/710 cm^{-1}, and (b) two positive features at 859 and 890 cm^{-1} associated with one negative feature at 836 cm^{-1} (equal in intensity to the sum of the intensities of the two positive features). The two positive features were suggested to arise from "vibrational coupling" with an unobserved heme mode and the unperturbed frequency of this feature was

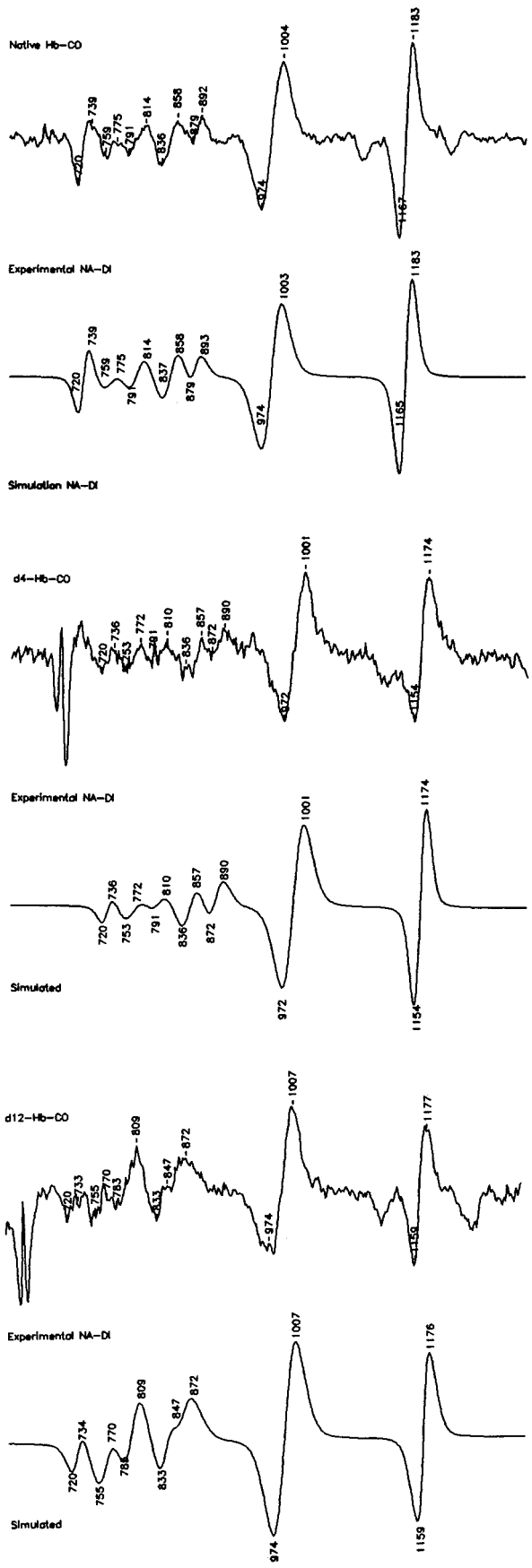

Figure 5. Difference spectra generated by subtracting the doubly labeled from the natural abundance (NA − DI) absolute, mid-frequency spectra. Simulated spectra are shown below each experimental difference spectrum.

Table 2. Experimental Difference Pattern Frequencies and Calculated (Simulation Studies) Absolute Frequencies for CO-Isotope Sensitive Bands of Native, d4, and d12-CO-Hb in the Low-Frequency (500−600 cm^{-1}) Region

	exptl (difference) (cm^{-1})		absolute (simulation) (cm^{-1})			
	max	min	NA	DI	fwhm	$10^6 I$
native						
ν(Fe−CO)	508	493	506	495	18	15.335
δ(Fe−C−O)	579	561	578	560	13	1.372
d4						
ν(Fe−CO)	507	492	505	494	18	14.275
δ(Fe−C−O)	578	559	576	558	11	0.965
d12						
ν(Fe−CO)	508	493	506	495	16	15.725
δ(Fe−C−O)	579	560	578	560	11	1.313

estimated to be 875 cm^{-1}. It was postulated that this feature was the result of a combination of ν(Fe−CO) + δ(FeCO) [(505 + 369)/(492 + 355)] = 874/847 cm^{-1}.

Several problematical issues arise from such an interpretation. First, while the positive components at 738 and 875 cm^{-1} (i.e., the approximate average of 859 and 890 cm^{-1}) are in agreement with the proposed assignments to the overtone and the combination of δ(FeCO) + ν(Fe−CO), the negative components at 718 and 836 cm^{-1} are approximately 10 cm^{-1} too high or too low (i.e., the predicted values being 710 and 847 cm^{-1}). Furthermore, while the difference patterns observed between 700 and 900 cm^{-1} are quite weak, and it is difficult to extract very reliable absolute frequencies and relative intensities from them, what is obvious from the present work is that the patterns are distinctly different for the individual (deuterium labeled) hemoglobins studied here. The previous assignment of these to overtones and combinations of ν(Fe−CO) and δ(FeCO) would suggest that these patterns should be relatively insensitive to heme deuteration. Similiarly, the previous assignment[11,16] of the more prominent difference feature at 578/559 cm^{-1} to a combination band involving the 370 cm^{-1} "δ(FeCO)" with an unidentified low-frequency heme mode would suggest that the difference feature would be sensitive to heme deuteration, given the fact that essentially all of the low-frequency heme modes are shifted by one or the other type of heme deuteration (vide supra). Significantly, the data obtained herein clearly show that the 578/559 cm^{-1} difference pattern is *not* sensitive to heme deuteration.

Given these observations, it seems more reasonable to suggest that the 578/559 cm^{-1} difference feature is attributable to the δ(FeCO) fundamental and the weak defference patterns observed between 700 and 900 cm^{-1} are ascribable to combination bands involving various low-frequency heme deformation modes and the strongest (i.e., allowed) CO isotope sensitive feature, the ν(Fe−CO) fundamental that occurs at 506/495 cm^{-1}. As is shown below, the observed difference patterns for all three Hb species studied here can be satisfactorily explained by this interpretation.

The difference pattern for native Hb, as shown in the top trace of Figure 5, is in essential agreement with that reported earlier.[11] However, rather than invoking a "vibrational coupling" interaction with an unidentified heme mode in this region, it can be shown that such patterns arise because of overlap of various combination bands. Thus, as is shown in the second trace, the experimentally observed difference pattern can be precisely reproduced by generating simulated difference patterns employing combination bands involving ν(Fe−CO) and several of the more prominent low-frequency heme modes (specific combinations being listed in Table 3). Similiarly, the patterns observed for the d4 and d12 hemoglobins also are satisfactorily reproduced by similar simulations.

Table 3. Frequencies, Bandwidths, and Intensities (Extracted from Simulation Studies) for the Overtones and Combination Bands Observed in Difference Spectra (NA − DI) in the Mid-Frequency Region (700−1300 cm^{-1})[a]

	extracted freq				calcd freq		assignment
	NA	DI	fwhm	$10^5 I$	NA	DI	ν(FeC) + ?
native							
	732	725	20.2	8.48	736	725	ν(Fe−His) [230 cm^{-1}]
	772	766	44.8	8.19	780	769	ν_{52} [274 cm^{-1}]
	809	797	34.4	5.47	805	794	γ_7 [299 cm^{-1}]
	855	843	35.3	7.46	849	838	ν_8 [343 cm^{-1}]
	888	878	24.6	4.32	882	871	$\delta(C_\beta C_c C_d)$ [377 cm^{-1}]
	997	980	33.7	14.42	1012	990	2ν(FeC) [506 cm^{-1}]
	1178	1171	22.8	32.24	1184	1173	ν_7 [678 cm^{-1}]
d4							
	734	723	20.0	0.68	736	725	ν(Fe−His) [230 cm^{-1}]
	772	761	44.0	1.31	774	763	ν_{52} [268 cm^{-1}]
	809	793	36.0	0.78	800	789	γ_7 [295 cm^{-1}]
	850	837	34.0	1.59	847	835	ν_8 [341 cm^{-1}]
	885	872	30.0	1.38	880	869	$\delta(C_\beta C_c C_d)$ [375 cm^{-1}]
	996	976	35.0	3.94	1012	990	2ν(FeC) [506 cm^{-1}]
	1168	1158	20.6	5.36	1173	1162	ν_7 [668 cm^{-1}]
d12							
	733	723	22.0	1.89	736	725	ν(Fe−His) [230 cm^{-1}]
	770	761	37.8	5.20	776	765	ν_{52} [270 cm^{-1}]
	804	792	38.0	5.36	804	792	γ_7 [297 cm^{-1}]
	845	835	33.5	4.64	844	832	ν_8 [337 cm^{-1}]
	871	856	33.0	2.58	872	860	$\delta(C_\beta C_c C_d)$ [366 cm^{-1}]
	1005	976	30.0	4.85	1012	990	2ν(FeC) [506 cm^{-1}]
	1171	1163	24.0	8.18	1174	1163	ν_7 [668 cm^{-1}]

[a] Calculated values were obtained from the frequency values determined in the deconvolution studies + ν(FeC) [506/495 cm^{-1}].

The point to be emphasized here is that the most obvious difference features in all three data sets are well reproduced by the simulation, i.e., the shift of the higher frequency positive component in native Hb and d4-Hb (∼890 cm^{-1}) to ∼870 cm^{-1} for d12-Hb and the shift of the negative component at ∼838 cm^{-1} (Hb and d4-Hb) to ∼832 cm^{-1} (d12 Hb). While the additional apparent features between 820 and 750 cm^{-1} are extremely weak and not precisely reproducible from data set to data set, the approximate patterns are fairly well reproduced by simulation involving combinations of ν(Fe−CO) with γ_7 and ν_{52} (Tables 1 and 3).

The difference feature observed at ∼739/720 cm^{-1}, occurring near a relatively strong heme mode that sometimes contributes residual intensity in the digitally subtracted difference spectra, appears to be relatively insensitive to heme deuteration, i.e., this combination mode does not appear to involve a low-frequency heme macrocycle deformation mode. As was pointed out earlier, the validity of the previously suggested assignment of this difference feature to an overtone of a proposed δ(FeCO) fundamental occurring near 370 cm^{-1} is questionable because of the 10 cm^{-1} mismatch of the negative component (i.e., ∼720 cm^{-1} observed vs 710 cm^{-1} calculated). As is shown in Figure 5, the observed difference pattern is well reproduced by invoking a combination of ν(Fe−CO) with an apparently unobserved low-frequency (deuterium insensitive) mode having a frequency of ∼230 cm^{-1}. An interesting and attractive candidate for this mode is the iron−histidine stretching mode, ν(Fe−N_{His}). This (apparently RR inactive) mode is expected to occur near this frequency. Thus, phototransient studies of CO-Hb and CO-Mb have detected the ν(Fe−N_{His}) at ∼228 cm^{-1}, a value that is expected to be similar to that of the ligated value.[22]

Conclusions

The recent challenge[11,16] to the long-standing assignment of the weak feature occurring near 580 cm^{-1} to δ(FeCO) had been based primarily on the observation of a weak CO isotope difference pattern seen near 370 cm^{-1} and the detection of even weaker difference patterns in the region between 700 and 900 cm^{-1}. It had been argued[11,16] that the existence of these difference features required the frequency of the δ(FeCO) fundamental to occur near 370 cm^{-1}, a suggestion in direct conflict with the apparently unequivocable demonstration by IR spectroscopy that the inherent frequency of this mode should occur near 580 cm^{-1}.[7] The present work, employing two heme-deuterated hemoglobins as well as native hemoglobin, confirms the existence of these weak difference patterns near 370 cm^{-1} and between 700 and 900 cm^{-1}, but provides an alternative interpretation of their origin. The present interpretation suggests that these features arise from combinations of the strongest CO isotope sensitive fundamental, ν(Fe−CO), with the most prominent, well-documented, heme macrocycle deformation modes. The present results and interpretation thus resolve the apparent controversy, and confirm the long-standing assignment of δ(FeCO) to the feature appearing near 580 cm^{-1}.

The present study also provides strong support for previous assignments[11,21] of two other, more prominent, difference features occurring near 1000 and 1180 cm^{-1} in the RR spectra of CO-ligated heme proteins. Thus, in the mid-frequency region, the data observed herein reproduced the features reported by Hirota et al.[11] and Rousseau and workers.[21] An assignment for the ν(Fe−CO) overtone for the difference pattern at ∼1000/975 cm^{-1} is reinforced by the observed lack of sensitivity to heme deuteration. Similiarly, the previous assignment of the difference feature appearing at ∼1178/1171 cm^{-1} to a combination band involving ν(Fe−CO) and ν_7 is herein confirmed by the observation of the expected 10 cm^{-1} downshift of this pattern (1178 → 1168 cm^{-1}), given the observed 10 cm^{-1} downshift of ν_7 for both d4 and d12-Hb-CO.

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