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Effects of Systematic Peripheral Group Deuteration on the Low-Frequency Resonance Raman Spectra of Myoglobin Derivatives

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Abstract: *Resonance Raman spectra are reported for a series of systematically deuterated analogues of myoglobin in its deoxy state as well as for its CO and O_2 adducts. Specifically, the myoglobin samples studied are those that have been reconstituted with deuterated protoheme analogues. These include the methine deuterated, protoheme-d4; analogue bearing C^2H_3 groups at the 1, 3, 5, and 8 positions, protoheme-d12; the species bearing C^2H_3 groups at the 1 and 3 positions only, 1,3-protoheme-d6; and the species bearing C^2H_3 groups at the 5 and 8 positions only, 5,8-protoheme-d6. While the results are generally consistent with previously reported data for synthetic metalloporphyrin models and previous studies of labeled heme proteins, the high-quality low-frequency RR data reported here reveal several important aspects of these low-frequency modes. Of special interest is the fact that, using the two d6-protoheme analogues, it is shown that certain modes are apparently localized on particular pyrrole rings, while others are localized on different rings; i.e., several of these low-frequency modes are localized on one side of the heme.* © 2004 Wiley Periodicals, Inc. Biopolymers 75: 217–228, 2004

INTRODUCTION

Over the past 3 decades resonance Raman (RR) spectroscopy has been employed as an especially effective probe of the active site structures of many heme proteins.[1–4] Furthermore, the recognized utility of this technique was extended, with the use of high peak power pulsed lasers, to study fleeting reaction intermediates by so-called transient or time-resolved resonance Raman (TR3) methods.[5–7] In most of these

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impressive applications, key structural parameters of the heme are elucidated by analysis of so-called "marker modes" in the high-frequency region; i.e., modes which are sensitive to changes in spin, oxidation, or coordination state of the central iron. In addition, in favorable cases, these techniques permit observation of iron-ligand modes which appear in the low-frequency region (between 100 and 600 cm^{-1}) and serve as direct probes of the strength of bonding between the heme iron and various exogenous and endogenous axial ligands.[8–15]

Appearing in the low-frequency region, along with the iron-axial ligand vibrations, are numerous modes that, in general, are complex motions involving both in- and out-of-plane deformations of the heme macrocyclic core and its various peripheral substituents.[16–22] In fact, in recent years it was recognized that the low-frequency spectral patterns observed for various heme proteins are apparently sensitive to quite subtle structural changes; i.e., in some spectral comparisons, larger changes are seen in the low-frequency region than are observed for the high-frequency marker modes.[23–28] This variability of low-frequency spectral patterns was most reasonably attributed to changes in the orientation of particular heme peripheral substituents brought about by altered interactions with key active site amino acid residues; e.g., in the case of the common protoheme prosthetic group, the two vinyl groups and the two propionyl substituents may be found in various orientations in different proteins or in the same protein under different conditions.[23–28]

Given the documented sensitivity of these low-frequency spectral patterns to active site structural changes, it is important to attempt to derive a better understanding of the nature of these modes; i.e., to establish which particular molecular fragments contribute to the various observed modes. This task is most effectively approached by reconstituting a given heme protein with isotopically labeled prosthetic groups. While a number of elegant studies employing protohemes labeled with deuteriums at one or both vinyl groups or at the four methine positions have been reported,[16–22] further work is needed to help clarify the nature of the observed low-frequency modes. For example, while the deuterium shifts observed for several modes appearing near 400–440 cm^{-1} prompted their designation as "vinyl bending modes," sensitivity of these same modes to deuteration at the four methine positions reveals the fact that the motions are complex, involving multiple fragments of the heme prosthetic group.[16–22] Therefore, it seems important to provide an additional labeling scheme that leads to relatively large shifts in particular modes so as to permit a systematic comparison of the isotopic sensitivity of key features observed in a series of related proteins.

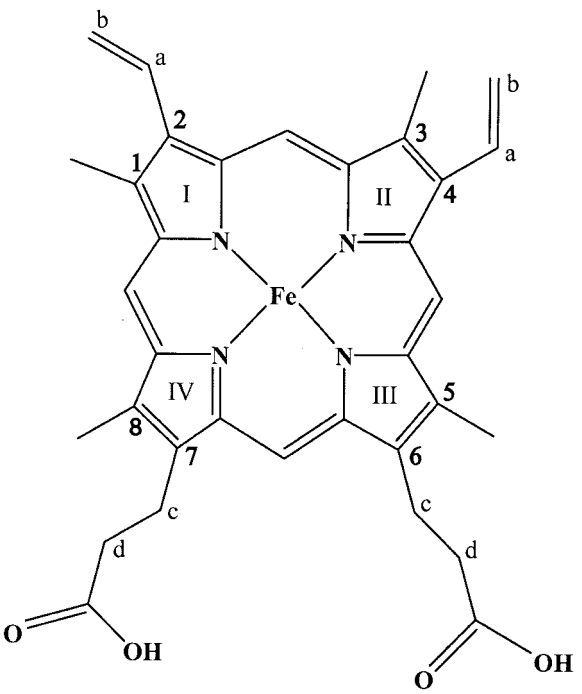

FIGURE 1 Structure and labeling scheme for protohemin IX.

Recent refinements, developed in our laboratory, of previously reported exchange procedures,[26] allowing for selective methyl group H/D exchange, have permitted the preparation of three useful deuterated protohemes, namely, 1,3-$(C^2H_3)_2$-protoheme (designated 1,3-d6-protoheme), 5,8-$(C^2H_3)_2$-protoheme (5,8-d6-protoheme), and 1,3,5,8-$(C^2H_3)_4$-protoheme (d12-protoheme); see Figure 1 for position labels. The deuteration of these methyl substituents leads to substantial shifts (up to 15 cm^{-1}) in certain key low-frequency modes. In the present work, this set of labeled protohemes is used to prepare corresponding labeled derivatives of the oxygen storage protein, myoglobin (Mb). Resonance Raman spectra are reported for the nonligated (deoxy) forms and for both the CO- and the O_2-ligated forms. Of special interest is the fact that, using the two d6-protoheme analogues, it is shown that certain modes are apparently localized on particular pyrrole rings, while others are localized on different rings; i.e., several of these low-frequency modes are localized on one side of the heme.

EXPERIMENTAL

Materials

Protoporphyrin IX dimethyl ester (PPIXDME) and ferriprotoporphyrin IX chloride (Fe(III)PPIXCl) were purchased from Porphyrin Products (Logan, UT). All porphyrins were used only after checking for purity by using thin-layer chromatography, proton NMR, and electronic absorption spectroscopy.[29–31] All solvents and chemical reagents used in this work were of HPLC or spectroscopic grade. Deuterated methyl sulfoxide (dimethylsulfoxide (DMSO)-d_6), deuterated methanol (CH_3OD), tetrabutylammonium hydroxide (TBAOH), (1.0 *M* solution in methanol), anhydrous pyridine, anhydrous acetonitrile, anhydrous tetrahydrofuran (THF), anhydrous hexane, and heptane were purchased from Aldrich Chemical Company (Milwaukee, WI) and used without further purification. Bio-gel P-6DG (Bio-Rad Laboratories, Hercules, CA) and CM cellulose (CM-52) (Whatman, Fairfield, NJ) were used for protein column chromatography.

Preparation of Selectively Deuterated Protohemes

Deuterium substitution at the four methine carbons (d4-protoheme) and synthesis of 1,3,5,8-$(C^2H_3)_4$-protoheme (d12-protoheme) were accomplished according to a previously published procedure.[32–35] Syntheses of 1,3-$(C^2H_3)_2$-protoheme (1,3-d6-protoheme) and 5,8-$(C^2H_3)_2$-protoheme (5,8-d6-protoheme) was conducted with small modifications of the procedure used for deuterium substitution of the four methyl groups of protoheme.[34] The reaction time for deuterium exchange of 1,3 methyl groups in 1,3-d6-protoheme was 3.5 h using a 3.5 molar excess of TBAOH (1.0 *M* solution in CH_3OH). For the synthesis of 5,8-d6-protoheme, the d12-protoheme was used as the starting material. Special care was taken to assure the purity of the d12-protoheme starting material. Reaction time was 3 h and a 5.0 molar excess of base was used. In this method, advantage was taken of differences in deuterium exchange rates, between the 1 and 3 methyl groups and the 5 and 8 methyl groups, caused by the local substituent effect (first-order inductive effect).[34–39] The optimum times of reactions and concentrations of base were established experimentally in order to obtain the required ratio of regio-specific deuterium exchange of the 1 and 3 methyl groups, while not exchanging the 5 and 8 methyl groups and vice versa. After deuteration, all samples were recrystallized from THF/heptane mixture.[33] Thin layer chromatograms,[40] pyridine hemochromogen electronic absorption spectra,[41] and proton NMR (300 mHz) spectra[34,42] were acquired to check the chemical and isotopic purity of the final samples. The ^1H NMR spectra of the methine-exchanged sample revealed that the meso-positions were deuterated to the extent of 95% with a little loss (less than 5%) of vinyl protons.[32,42] Corresponding spectra of the base-exchanged d12-protoheme samples showed that all methyl groups in the protoheme-d12 were deuterated to the extent of 95%. For the 1,3-d6-protoheme, the 1 and 3 methyl groups were deuterated to the extent of 95%, while the 5 and 8 methyl groups remained essentially protonated (90–95%). In case of the 5,8-d6-protoheme, the 5 and 8 methyl groups were exchanged (80% deuterated) while, as shown in Figure 2, the 1 and 3 methyl groups showed only ~10% deuterium substitution.[34]

Protein Preparation

Horse heart myoglobin (Sigma) (Mb) was purified as described previously.[43] Apomyoglobin was isolated from met-myoglobin by the acid–acetone method[44] and reconstituted[45] with the various deuterated protohemes to yield the corresponding labeled heme proteins; i.e., Mb-d12, Mb-d4, etc. The reconstituted myoglobins were then anaerobically reduced by sodium dithionite and purified according to established procedures.[43] The myoglobin samples were converted to CO form for storage by passing CO over a stirred solution of reduced protein (the solution was placed in a small Erlenmeyer flask immersed in ice bath and slowly stirred under a stream of CO gas for about 30 min) and then before measurements turned into oxy or deoxy form. All procedures were performed at 4°C.

Sample Preparation for Raman Measurements

All samples were prepared in 0.030 *M* phosphate buffer, pH 7.0, using the following procedures. The carbon monoxide adducts were prepared as stated above. The dioxygen adducts were obtained by photodissociation (visible source, 250 W) of CO from the CO form of the given heme protein at 0°C (sample immersed in an ice-water bath) while gently purging the protein sample with O_2 for about 30 min. The deoxy samples were generated by evacuation of the oxygenated samples on a vacuum line. The solution of oxygenated protein was placed in J. Young NMR tubes (528-JY-7, Wilmad, Buena, NJ) and a small magnetic stirring bar was placed inside the tube. Next, the tube was sealed and hooked up to a vacuum line. The oxygen evacuation was followed by intermittent flushing with dry, oxygen-free nitrogen. The sample was constantly cooled down by immersing the tube in an ice-bath.

Resonance Raman Measurements

Resonance Raman spectra were acquired with a Spex Model 1403 Czerny-Turner double monochromator equipped with a Hamamatsu R928 photomultiplier and Spex monochromator control hardware with SpectraMax/32 software for windows from Instruments SA, Inc. Excitation at 413.1 nm (krypton ion laser, Coherent Model Innova (I) 100-K3) was used to measure spectra of the oxy and carbonmonoxy adducts of Mb. For the carbonmonoxy samples, the power at the samples was maintained below 1.0 mW to prevent photodissociation of CO from the heme as judged by ab-

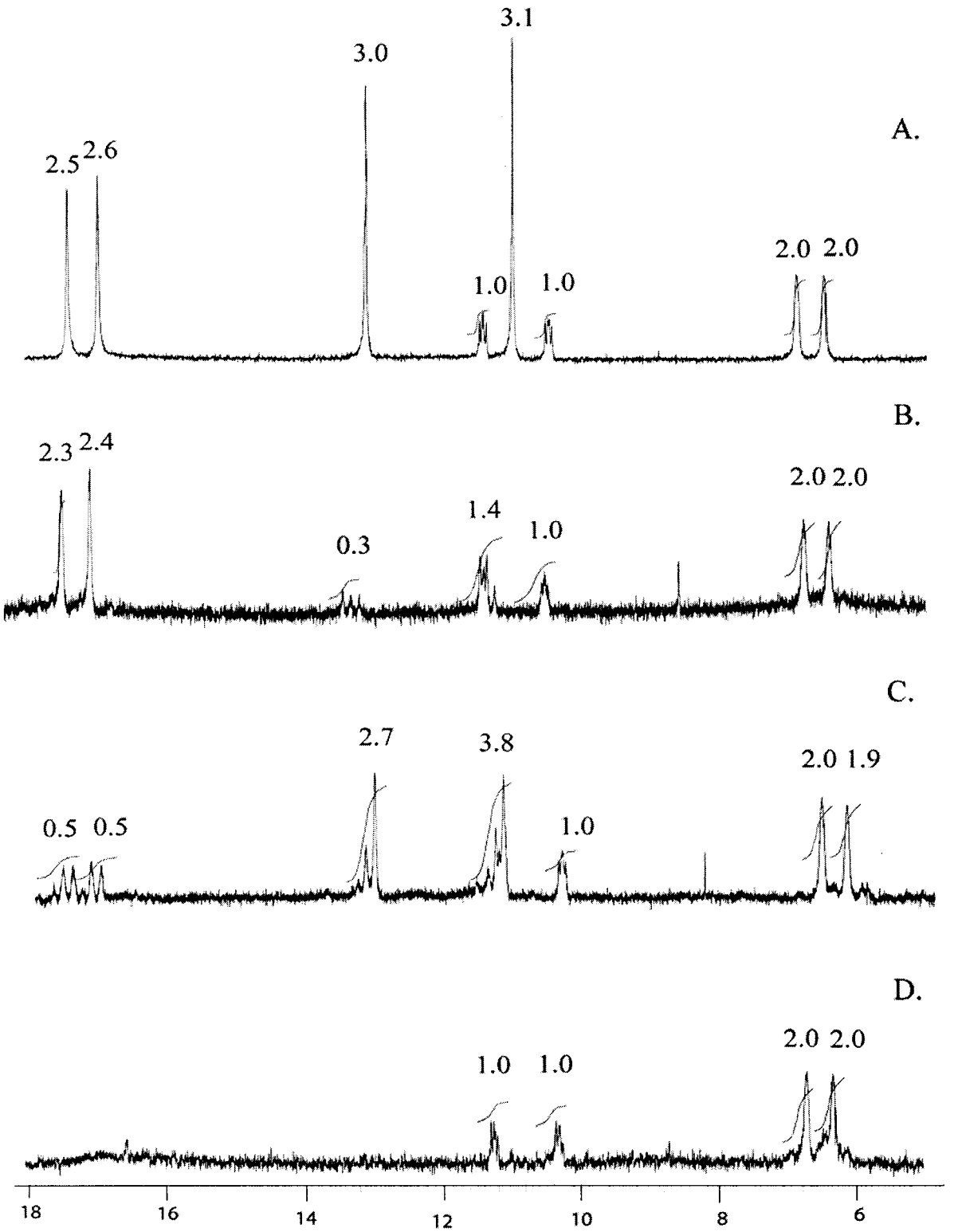

FIGURE 2 ^{1}H NMR spectra of dicyano complexes of protoheme IX (A), d12-protoheme 1,3-d6-protoheme (B), 5,8-d6-protoheme (C), and d12-protoheme(D) in DMSO-d_6.

sence of the 1358 cm^{-1} band, which is characteristic of the nonligated deoxyMb.[1–4] The power was held at approximately 15 mW for the less photosensitive oxy samples. The oxy samples were checked for traces of met form by electronic absorption spectra (Hewlett-Packard Model 8452A diode spectrometer). Resonance Raman spectra of the deoxy forms were obtained by excitation at 441.6 nm (helium : cadmium laser, Liconix model 4240NB). Power at the samples was 16 mW. Before and after measurements, the samples were checked for traces of oxy adducts or met forms (monitoring the ν_4 band using the 413.1 nm excitation line provided by the krypton ion laser). All RR measurements were done at room temperature in spinning NMR tubes (WG-5 *M*-ECONOMY, Wilmad). In order to lessen local heating, a small magnetic stirring bar was placed inside the NMR tube and held in a fixed position by an external magnet while the tube was being spun; i.e., to provide efficient mixing of the protein solution at the point of illumination. Typical concentrations of approximately 0.2 m*M* (total heme concentration) were used. The spectral band-pass was set to 4 cm^{-1} and the accuracy of the frequency readings was 0.5 cm^{-1}.

RESULTS AND DISCUSSION

The high-quality spectral data reported for the three forms of myoglobin studied here reveal the presence of a relatively large number of modes, many occurring as unresolved shoulders on more intense bands previously reported in other works.[1–4,9,16–24] Inasmuch as the origins of all of these low-frequency modes are not yet firmly established, acquisition of data for additional labeled analogues can help provide a better understanding of their nature; i.e., additional selective labeling of the heme periphery, as is being carried out here, can help identify contributions of various molecular fragments to the different features. Furthermore, the relatively large shifts induced by deuteration of the methyl groups, as well as by methine deuteration, are useful in correlating modes observed under different conditions, e.g., in attempting to correlate modes observed for deoxy Mb with those observed for a ligated form, it is necessary to establish that mode formulations are comparable in the sense that, if so, one expects that the sensitivities to various types of deuteration would not change substantially. Thus, analysis of the RR data acquired for the extensive set of deuterium-labeled analogues included here can provide a more reliable correlation of modes observed in the different forms being studied. The data presented in the following sections provide this documentation of isotopic sensitivities with respect to contributions made by the four peripheral methyl groups and the methine positions.

Deoxy Form

Figure 3 shows the observed RR spectra for the various deoxy Mb isotopomers of interest in this work. In Figure 3, the intensities of all the illustrated spectra have been normalized using the intensity of the previously assigned iron-histidine stretching mode, $\nu(Fe\text{-}N_{his})$, which occurs at 220 cm^{-1}; i.e., it is not expected that the intensity of this mode is sensitive to deuteration of the heme periphery. The spectra acquired here are of sufficiently high quality that a number of additional features are revealed as shoulders on the more intense bands previously reported in earlier works[9,16–28,46–49] and attempts are made here to suggest tentative assignments based on isotopic shift patterns with reference to corresponding studies on model compounds.[50–53]

As previously noted by other workers,[23,47,48] the low-frequency RR spectrum of deoxy Mb differs from that of deoxy Hb and its isolated chains mainly by the appearance of a relatively strong band occurring near 240 cm^{-1}, a feature which has been previously assigned[20,21,23] as ν_9, one of the principal totally symmetric modes of planar, high symmetry metalloporphyrin model compounds, such as nickel octaethylporphyrin (NiOEP)[50,51] and nickel etioporphyrin-I (NiEPI).[52] In detailed studies of planar and ruffled forms of the NiOEP model, the ν_9 mode was observed near 283 cm^{-1} and shown to shift down by 11 cm^{-1} for the methylene deuterated (NiOEP-d16) isotopomer in the case of the planar form.[51] In similarly comprehensive studies of NiEPI, actually a better model for the system being studied here, the ν_9 mode occurred at 255 cm^{-1} and shifted to 247 and 236 cm^{-1} (8 and 19 cm^{-1}, respectively) for the methylene deuterated (NiEPI-d8) and methyl deuterated (NiEPI-d12) analogues, respectively.[52] In the spectra reported here, the expected sensitivity to deuteration at the peripheral methyl groups is indeed observed, this mode shifting by about 7 cm^{-1} for the d12-protoheme isotopomer. Also consistent with studies of the NiOEP and NiEPI models is the relatively slight sensitivity of the ν_9 mode to deuteration at the methine carbons; shifts observed here for the protoheme-d4 isotopomer are no more than 3 cm^{-1} and those observed for the models are 0–1 cm^{-1}.[50,52]

While comparisons to such high symmetry models are often made in attempting to analyze the vibrational data obtained for naturally occurring heme proteins, it is important to recognize that symmetry lowering, by variation of substituents, apparently has dramatic consequences in terms of mode compositions; these are revealed only by careful study of selectively labeled protohemes, as has been initiated

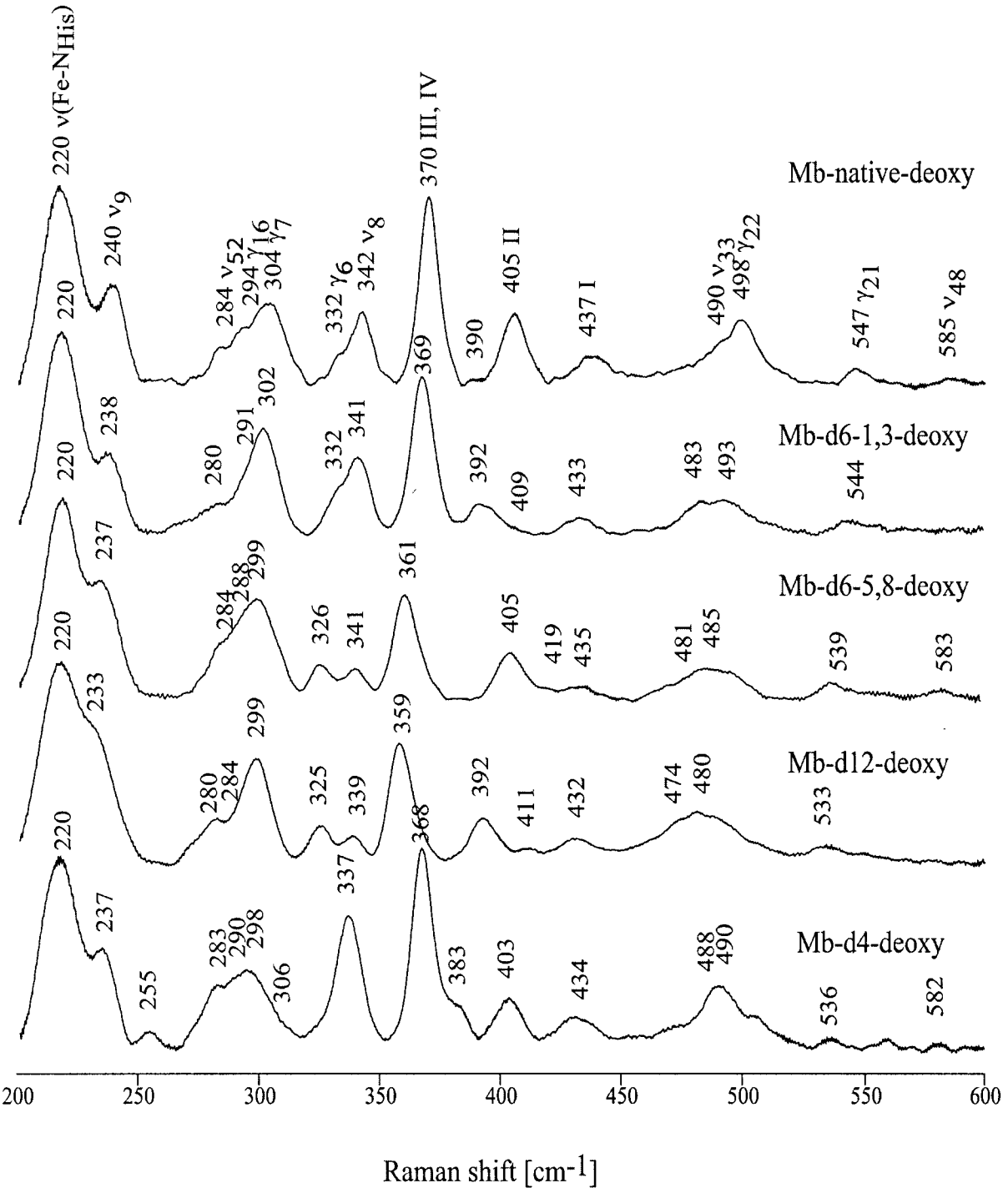

FIGURE 3 Resonance Raman spectra of deoxy myoglobin and its deuterated derivatives.

earlier and is being extended here.[26,33,35] However, comparison of the spectra shown in the top three traces of Figure 3 reveals that the mode labeled ν_9 exhibits comparable shifts upon deuteration of the 5 and 8 methyl groups (3 cm^{-1}) and 1 and 3 methyl groups (2 cm^{-1}). This behavior is in contrast to previous results obtained in an elegant early study of nickel complexes of selectively deuterated analogues of protoporphyrin dimethyl ester. There, Lee et al. showed that a mode assigned as ν_9, observed at 269 cm^{-1}, showed a 6 cm^{-1} shift upon deuteration of the 5-methyl group, but insignificant shifts for those com-

plexes bearing CD_3 groups at the other three positions.[53]

In the region near 300 cm^{-1} three unresolved bands are distinguishable for deoxyMb, whereas in most earlier works a single (rather broad) band is reported in this region and assigned to the γ_7 out-of-plane mode.[16,17,20,21] In the present case, the strongest feature, occurring at 304 cm^{-1}, is assigned to this mode, in agreement with earlier studies. The rather significant (6 cm^{-1}) shift upon methine deuteration (bottom trace) is also consistent with data acquired for the ruffled NiOEP model compound, which exhibited this mode at 284 cm^{-1}, shifting to 277 cm^{-1} for NiOEP-d4.[50,51] Only small shifts of this mode are evident for the d6- and d12-protoheme isotopomers. The two weaker modes, located at 284 and 294 cm^{-1} in the top trace, are tentatively assigned to ν_{52} and γ_{16}, modes, which have been previously observed in this region for the ruffled form of NiOEP[50,51] and for met-Mb.[20,21]

In agreement with recently reported high-quality RR data obtained for a series of mutant myoglobins,[23] the spectrum shown in the top trace of Figure 3 exhibits two distinct bands near 340 cm^{-1}, one assigned to the out-of-plane γ_6 mode and the other to the in-plane ν_8 mode.[21] As noted in the previous comprehensive study of isotopically labeled myoglobin derivatives,[21] the γ_6 mode is quite sensitive to methine deuteration, shifting by 26 cm^{-1} to lower frequency; this shift is nearly identical to that observed (23 cm^{-1}) for the ruffled form of the NiOEP model.[51] While this mode was not specifically identified for deoxy Mb in the earlier study,[21] it was clearly seen for the met-Mb derivatives. In more recent reports,[23] including the present work, this mode is clearly identifiable as a low-frequency shoulder on the slightly stronger ν_8 mode. As expected, this feature disappears in the spectrum of the d4-protoheme analogue, with its counterpart possibly appearing as a weak high-frequency shoulder at 306 cm^{-1}, although other very weak features appear in this region (e.g., near 317 cm^{-1} in the spectrum of the native form shown in the top trace). In addition, the interpretation of the spectra in this region is made uncertain by the complicated shift patterns observed for the methyl deuterated analogues. Inasmuch as ν_8 is expected to shift substantially for the protoheme-d12 isotopomer (based on the shift of 12 cm^{-1} observed for the d12-NiEPI[52] and the 15–20 cm^{-1}shifts observed upon methylene deuteration (d16-NiOEP) for the two forms of NiOEP[50,51]), the proper assignments for the two weaker modes observed between 325 and 341 cm^{-1} in the spectra of the methyl deuterated isotopomers are not clear. It seems most reasonable to suggest that the feature centered at 342 cm^{-1} in the spectrum of native deoxy Mb is actually a superposition of two modes (e.g., ν_{8a} and ν_{8b}[20,21]) and that one of these modes, ν_{8a}, shifts only slightly upon methyl group deuteration (shifts of 1–3 cm^{-1}), while the other, ν_{8b}, shifts significantly (16–17 cm^{-1}), with some overlap with the γ_6 mode in the 5,8-d6 and d12 analogues (see Table I). Such shifts would be consistent with those observed for the ligated forms (vide infra), but in the absence of data acquired for additional isotopically labeled species (e.g., the methine/methyl-d16 labeled species), this interpretation remains speculative.

In the region between 360 and 450 cm^{-1} appear several modes that have commonly been associated with movements of the protoheme peripheral substituents; a mode appearing near 370 cm^{-1} has usually been designated as "propionate bending," while a pair of weaker features appearing near 400 and 450 cm^{-1} are normally referred to as "vinyl bending" modes.[16–28] In fact, in the case of myoglobin, on the basis of studies of heme labeled selectively at either the 2- or 4-vinyl positions, the higher frequency mode was attributed to the deformation of the 2-vinyl substituent, while the one at lower energy is ascribed to a mode involving the 4-vinyl substituent.[16–18,21] While such designations are in common usage, it is actually expected that the observed features are probably complex motions involving a number of internal coordinates. In fact, the data obtained for the samples bearing deuterated methyl groups, presented here for the first time, reveal that these modes involve movements of the methyl substituents, yielding shifts upon methyl deuteration that are at least comparable to those observed upon labeling of the adjacent peripheral groups (i.e., the vinyls or propionates). Thus, in earlier works, deuteration of the β-position of the vinyl groups led to shifts of between 9 and 15 cm^{-1} [16–18,21] while the "propionate bending" mode of met-Mb was observed to shift from 376 to 369 cm^{-1} for a species bearing a heme which contained propionate residues deuterated at the methylene carbons labeled d in Figure 1 (although it is also noted that this heme actually possessed additional deuterium atoms at the vinyl groups and at the 1 and 3 methyl positions).[21] The present results obtained for the 5,8-d6-protoheme and d12-protoheme analogues yield shifts of this "propionate bending" mode of 9 and 11 cm^{-1}. The point to be emphasized is that the present results indicate that these modes are more reasonably associated with the pyrrole rings and involve motions of both peripheral substituents on a given pyrrole ring, rather than being definitively ascribed to motions of a given peripheral substituent.

Table I Summary of Observed Frequencies

	Mb-native-deoxy [Δ of d6-1,3/ d6-5,8/ d12/ d4]	Mb-native-CO [Δ of d6-1,3/ d6-5,8/ d12/ d4]	Mb-native-oxy [Δ of d6-1,3/ d6-5,8/ d12/ d4]
$\nu_{(Fe\text{-}His)}$	220 [0/ 0/ 0/ 0]		
$\nu_{.9}$	240 [2/ 3/ 7/ 3]	254 [3/ 6/ 9/ 3]	254 [1/ 11/ 12/ 3]
ν_{52}	284 [4/ 0/ 4/ 1]	270 [2/ 0/ 2/ 1[a]]	270 [2/ 0/ 2/ 1]
γ_{16}	294 [3/6/10/4]	318 [5/ 3/ 7/ 2]	312 [5/ 7/ 12/ 4]
γ_7	304 [2/ 5/ 5/ 6]	302 [3/ 4/ 6/ 5]	303 [1/ 2/ 5/ 4]
γ_6	332 [0/ 0/ 0/ 26]	347 [0/ 0/ 0/ 12]	
ν_{8a}	342 [1/ 1/ 3/ 5]	347 [3/ 5/ 10/ 0]	345 [5/ 5/ 7/1]
ν_{8b}	342 [1/ 16/ 17/ 5]	347 [3/ 15/ 17/ 0]	345 [5/ 15/ 16/1]
III, IV	370 [1/ 9/ 11/ 2]	379 [3/ 11/ 17/ 1]	377 [4/ 12/ 17/ 1]
	390 [?/ -/ -/ 7]		
II	405 [13/ 0/ 13/ 2]	412 [14/ 0/ 15/ 0]	409 [12/ 0/ 13/ 0]
I	437 [4/ 2/ 5/ 3]	435 [4/ 1/ 7/ 2]	439 [4/ 1/ 8/ 3]
ν_{33}	490 [7/ 9/ 16/ 2]	484 [10/ 11/ 22 /4]	479 [11/ 10/ 21/ 4]
γ_{22}	498 [5/ 13/ 18/ 8]		
$\nu_{(Fe\text{-}CO)}$		509 [0/ 0/ 0/ 0]	
γ_{21}	547 [3/ 8/ 14/ 11]	554 [5/ 9/ 18/ 9]	556 [11/ 11/ 21/ 11]
$\nu_{(Fe\text{-}O2)}$			572 [0/ 0/ 0/ 0]
$\delta_{(FeCO)}$		575 [0/ 0/ 0/ 0]	
ν_{48}	585 [?/ 2/ ?/ 3]	584 [0/ 0/ 0/ 0]	

Note. Raman shift (Δ in [cm^{-1}]) of deoxy myoglobin, their CO and O_2 adduct, and their deuterated isotopomers d6-1,3/ d6-5,8/ d12/ d4.
[a] Band position obscured by "new" 262 cm^{-1} component corresponding to 255 cm^{-1} feature in deoxy Mb spectrum.

While the results summarized above make it clear that the features attributed to the vinyl and propionate bending modes correspond to modes containing substantial contributions from both peripheral substituents on a given ring, it is also satisfying to note that the results obtained for the 1,3- and 5,8-d6-protoheme analogues are indeed consistent with previous designations. Thus, the vinyl bending modes observed between 400–450 cm^{-1} are sensitive mainly to 1,3-deuteration as expected, while the propionate bending mode observed near 370 cm^{-1} is sensitive mainly to deuteration at the adjacent 5 and 8 methyl positions, both sets of modes of course being sensitive to deuteration of all four methyl groups; i.e., the modes previously associated with a given peripheral substituent are shown here to be more properly described as motions of the particular pyrrole ring containing that substituent. Therefore, as indicated in Figures 3–5 and in Table I, these modes are now designated according to the pyrrole rings involved; i.e., the features near 370 cm^{-1} are labeled III, IV, while those associated with rings I and II are labeled separately, based on the earlier reported data from studies of selectively deuterated vinyl groups.[16–18]

Only a few modes are observed as weak features occurring between 450 and 600 cm^{-1} in the spectra of heme proteins. In agreement with previous works,[20,21] the two components of the unresolved doublet near 500 cm^{-1} are assigned to ν_{33} and γ_{22}, while the band at 547 cm^{-1} is assigned to γ_{21}. While some evidence is obtained for weak enhancement of the ν_{48} mode expected near 585 cm^{-1}, it is not observed in all spectra shown in Figure 3 and is not further discussed here.

Ligated Forms

The spectra of the two ligated forms, MbCO and MbO_2, and their deuterated analogues are shown in Figures 4 and 5. As expected, given the similarity of heme structures in these two derivatives, the frequencies and intensities of observed features are almost identical, with the exception of modes associated with the different coordinated ligands; i.e., the ν(Fe-C) and δ(FeCO) modes[11,12] of MbCO are observed at 509 and 575 cm^{-1}, while the ν(Fe-O) stretching mode of the oxygenated form is observed as a relatively weak feature at 572 cm^{-1}.[14,15,43] The frequencies and assignments of all the heme-based modes for both of the ligated forms are reported, along with those for deoxy Mb in Table I. Inspection of the data collected in Table I reveals that there is a good correspondence between the modes observed and already discussed for deoxyMb and those observed for the ligated forms; i.e., inasmuch as the basis for the validity of the assignments of the modes has already been summarized for the deoxy form, they are valid for the ligated forms.

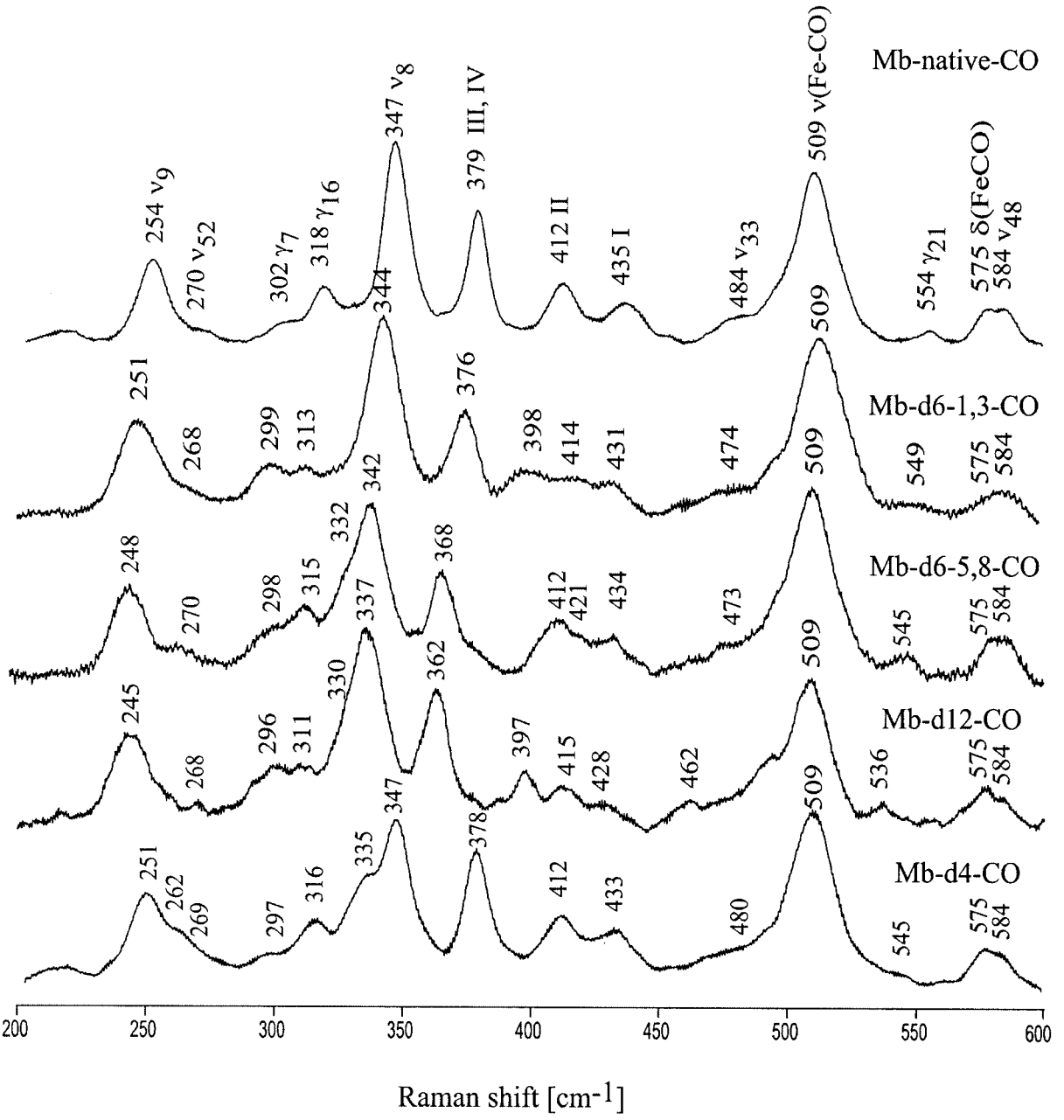

FIGURE 4 Resonance Raman spectra of carbon monoxide adducts of myoglobin and its deuterated derivatives.

As can be seen by comparison of Figures 4 and 5, most of the modes observed in the two sets of spectra exhibit comparable frequencies and nearly identical shift patterns, with only a few slight differences being notable. In the region near 300 cm^{-1}, an additional band appears in the spectrum of MbCO at 318 cm^{-1}, which has been previously observed and assigned to an out-of-plane mode designated γ_{16}.[21] We note that a weak feature near 312 cm^{-1} in the spectrum of oxyMb (Figure 5) is also assigned to this γ_{16} mode. The data obtained here show that this feature is somewhat sensitive ($\sim$7 cm^{-1}) to peripheral methyl deuteration, but only very slightly (2 cm^{-1}) to methine deuteration, behavior consistent with that observed for the γ_{16} mode of NiOEP, where a 10-cm^{-1} shift was observed for methylene deuterated NiOEP-d16 but only a 4-cm^{-1} shift for NiOEP-d4.[51]

In the region near 350 cm^{-1}, rather complex patterns are again observed, as in the case of the deoxy form (vide supra). In the spectrum of native MbCO

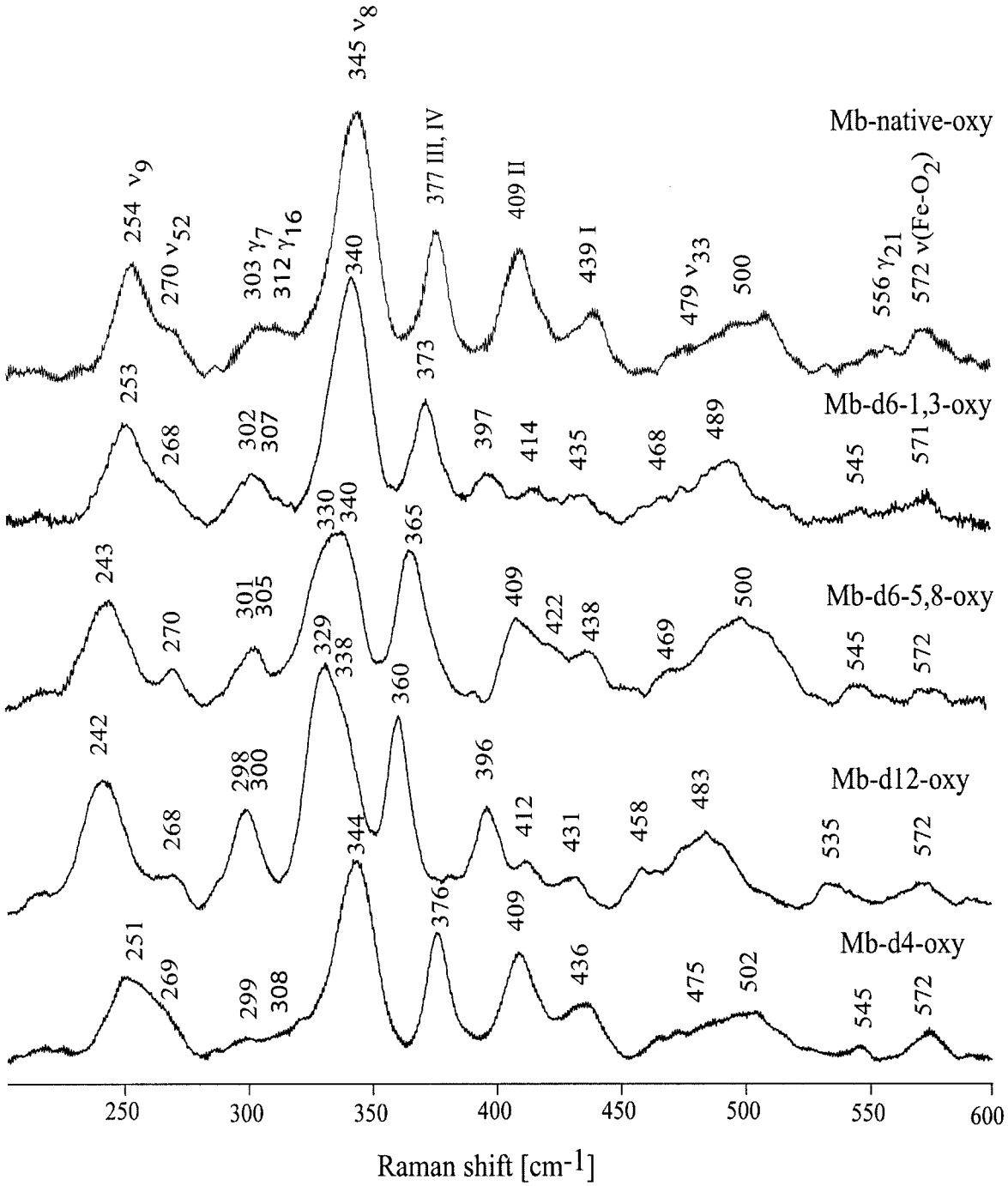

FIGURE 5 Resonance Raman spectra of dioxygen adducts of myoglobin and its deuterated derivatives.

(Figure 4) the relatively symmetrical strong feature observed at 347 cm^{-1} is seen to actually consist of at least three overlapping bands by considering the spectra obtained for the deuterated forms. Thus, comparing the spectra of MbCO with that containing d4-heme (bottom trace in Figure 4), the presence of a band sensitive to methine deuteration (most likely, γ_6) is revealed by its shift to 335 cm^{-1}, with a simultaneous loss of intensity of the stronger ν_8 mode. Also, in the methyl deuterated spectra, the entire envelope near 350 cm^{-1} broadens and shifts to lower frequency, especially for the 5,8-d6 and d12-protoheme isoto-

pomers; i.e., splitting of the ν_8 mode upon methyl deuteration, similar to that observed for the deoxy form of myoglobin, also occurs (see Table I entries for ν_8). While the spectra acquired for the oxygenated form of Mb (Figure 5) are generally similar to those obtained for the CO analogue (Figure 4), the intensities of the two out-of-plane modes, γ_6 and γ_{16}, do appear to be stronger for the MbCO samples.

In the region between 350 and 450 cm^{-1}, which contains the modes involving movements of the propionate and vinyl substituents, the behavior observed for the ligated derivatives (Figures 4 and 5) is similar to that seen for deoxy Mb (Figure 3). Thus, the strong mode observed near 380 cm^{-1} in the spectra of the ligated forms shifts only slightly (3–4 cm^{-1}) for 1,3-methyl deuteration, but by 11–12 cm^{-1} in the case of 5,8-d6-protoheme, again indicating that this mode is relatively localized to the propionate side of the macrocycle. In contrast, the modes having some contribution from vinyl bending coordinates, located between 400 and 440 cm^{-1}, are seen to be sensitive only to deuteration at the 1 and 3 methyl substituents, with much smaller changes being seen for the 5,8-d6-protoheme substituted forms (Figures 4 and 5). In the spectra of the ligated forms, clear evidence is obtained for the existence of three modes in the 400–440 cm^{-1} region; i.e., when the feature appearing near 412 cm^{-1} in Figure 4 shifts down to 398 or 397 cm^{-1} in the spectra of the 1,3-d6 or d12- analogues, a weak feature is observed near 415 cm^{-1}. Similar behavior is noted for the oxygenated Mb spectra (Figure 5). The existence of three modes in this region is also evident in the spectra of deoxy Mb (Figure 3) by the appearance of the very weak feature at 411 cm^{-1} in the Mb-d12 analogue.

SUMMARY

In summary, careful analysis of these newly acquired spectral data for all three forms of the isotopomeric Mb analogues yields a set of modes that is internally consistent in the sense that shifts observed for the d4-, d12-, and both d6-protoheme analogues are similar for all three forms being studied here (i.e., the deoxy Mb and two ligated forms). While the mode correlations given in Table I are consistent with previously reported data for synthetic metalloporphyrin models[50–53] and previous studies of labeled heme proteins,[16–22] the high-quality, low-frequency RR data reported here reveal several important aspects of these low-frequency modes, including the fact that those modes traditionally associated with movements of particular peripheral substituents (i.e.; the vinyl or propionate groups) are seen here to exhibit quite substantial shifts upon deuteration of the adjacent methyl substituents, indicating that the modes are more properly associated with given pyrrole rings (I-IV). On the other hand, while methyl deuteration effects eliminate this very localized picture of these modes, different shift patterns observed for the two selectively deuterated isotopomers (i.e., 1,3- and 5,8-d6-protohemes) document the fact that given modes are indeed reasonably attributed to different "sides" of the heme macrocycle. Furthermore, in the earlier work by Kitagawa and co-workers on nickel protoporphyrins bearing selectively deuterated methyl groups, it was shown that certain modes are sensitive to deuteration of only one methyl group; e.g., the ν_9 modes shifts only upon deuteration of the 5-methyl group, with no shifts being observed upon deuteration of the other methyl substituents.[53] Thus, the picture that emerges from consideration of the results obtained in the present work and earlier studies is that certain low-frequency modes are ascribable to given pyrrole rings and that the frequencies of these modes are apparently dependent upon the precise orientations of the attached vinyl or propionate substituents.[16–28]

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