Assignment of Protoheme Resonance Raman Spectrum by Heme Labeling in Myoglobin

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Abstract: Resonance Raman (RR) spectra are reported for myoglobin reconstituted with seven heme isotopomers which are labeled with ^{15}N and *meso*-D_4 in the porphyrin skeleton or at the vinyl and propionate substituents. The RR bands are assigned to the porphyrin in-plane and out-of-plane modes as well as to the internal vibrations of substituents on the basis of the observed isotope shifts. The issue of vinyl substituent effects is revisited, and bands are assigned to the 2- or 4-vinyl group from selective deuteration shifts. Contributions of the aliphatic propionate groups are also revealed in the RR spectrum. The protein influence on the heme structure is reflected in the activation of several out-of-plane modes in the low-frequency region.

Introduction

Resonance Raman (RR) spectroscopy plays a key role in the study of heme proteins because of the strong signal enhancement and detailed vibrational spectrum afforded by the heme group[1] in its varied protein settings. However, a completely satisfactory interpretational framework is not yet available despite a wealth of published data and despite the discovery of useful marker bands for the porphyrin core size, for the electron density of the frontier orbitals, and for the vibrations of specific axial ligands.[2] The spectra are rich and variable, and the effects of peripheral substituents and of distortions imposed by the protein are not easy to decipher. These effects, however, are of considerable interest with respect to heme protein structure and functions.

The first requirement for sound interpretation is a set of reliable vibrational assignments. There has been substantial progress in this direction through detailed isotopic studies coupled with normal coordinate analyses of model compounds, particularly of the nickel complexes of porphine, tetraphenylporphine (TPP),[3] octaethylporphyrin (OEP),[4] and etioporphyrin-I (EPI).[5] Using the framework developed in these model studies, we have recently assigned the very rich RR spectra of cytochrome *c*[6] using an enzymatic technique to replace the covalently bound endogenous heme with isotopically labeled hemes. This study revealed strong RR signatures of protein-induced distortion of the heme.

In this work we assign the RR spectrum of myoglobin (Mb), again using several isotopic labels. Mb is a representation of the large class of proteins carrying noncovalently bound protoheme. The vinyl substituents in protoheme are conjugated with the porphyrin π system and have a strong influence on the RR spectra.[7] The nature of the influence has been a matter of debate, and an important objective of this work is to clarify this influence in the context of a protein-binding pocket. We are also able to gauge the role of the propionate substituents through propionate deuteration and have assigned a low-frequency band to a propionate bending mode. With assignments in hand, we have explored the effects of different oxidation and ligation states of Mb on the heme structure through changes in low-frequency RR spectra.

Experimental Section

Materials. Horse heart myoglobin and natural abundance heme were purchased from Sigma, while other heme isotopomers were synthesized according to published procedures.[8] The propionate-labeled heme, designated as 6,7-di(d-D_2)-heme, is also deuterated at the vinyl C_b positions and at two methyl substituents: 2,4-di(b-D_2)vinyl-1,3-bis-(deuteriomethyl)-6,7-di(d-D_2).[8b]

Preparation of Heme Isotopomer Reconstituted Myoglobins. Apomyoglobin was prepared by Teale's butanone method[9] and dialyzed against three successive changes of buffers: 10 mM $NaHCO_3$ (4 h), 10 mM potassium phosphate (pH 7.0, 4 h), and finally 100 mM potassium phosphate (pH 7.0, 12 h). The precipitate of denatured protein was removed by centrifugation and discarded. The apomyoglobin, containing less than 5% heme, was used within 24 h. To prepare heme-labeled Mb, a slight excess of the appropriately labeled heme was dissolved in 0.1 N NaOH and added to the apomyoglobin solution with gentle stirring. Excess heme was removed by passage through a Sephadex G-25 column previously equilibrated with 0.1 M potassium phosphate buffer (pH 7.0). Deoxymyoglobin was prepared by addition of freshly prepared aqueous sodium dithionite in a NMR tube which had been flushed with argon. The CO adduct was formed by passing

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(1) For recent reviews on RR spectroscopy of heme proteins, see: (a) Abe, M. In *Advances in Spectroscopy*; Clark, R. J. H., Hester, R. E., Eds.; Wiley: New York, 1986; Vol. 13, 347−393. (b) Kitagawa, T.; Ozaki, Y. *Struct. Bonding* **1987**, *64*, 71−114. (c) Spiro, T. G.; Czernuszewicz, R. S.; Li, X.-Y. *Coord. Chem. Rev.* **1990**, *100*, 541−571. (d) Spiro, T. G.; Smulevich, G.; Su, C. *Biochemistry* **1990**, *29*, 4497−4508. (e) Procyk, A. D.; Bocian, D. F. *Annu. Rev. Phys. Chem.* **1992**, *43*, 465−496.

(2) Spiro, T. G., Ed. *Biological Applications of Raman Spectroscopy*; Wiley: New York; 1988, Vol. III.

(3) Li, X.-Y.; Czernuszewicz, R. S.; Kincaid, J. R.; Su, Y. O.; Spiro, T. G. *J. Phys. Chem.* **1990**, *94*, 31−47.

(4) (a) Abe, M.; Kitagawa, T.; Kyogoku, Y. *J. Chem. Phys.* **1978**, *69*, 4526−4534. (b) Li, X.-Y.; Czernuszewicz, R. S.; Kincaid, J. R.; Stein, P.; Spiro, T. G. *J. Phys. Chem.* **1990**, *94*, 47−61. (c) Li, X.-Y.; Czernuszewicz, R. S.; Kincaid, J. R.; Spiro, T. G. *J. Am. Chem. Soc.* **1989**, *111*, 7012−7023.

(5) Hu, S.; Mukherjee, A.; Piffat, C.; Mak, R. S. W.; Li, X.-Y.; Spiro, T. G. *Biospectroscopy* **1995**, *1*, 395−412.

(6) Hu, S.; Morris, I. K.; Singh, J. P.; Smith, K. M.; Spiro, T. G. *J. Am. Chem. Soc.* **1993**, *115*, 12446−12458.

(7) (a) Choi, S.; Spiro, T. G.; Langry, K. C.; Smith, K. M. *J. Am. Chem. Soc.* **1982**, *104*, 4337. (b) Choi, S.; Spiro, T. G.; Langry, K. C.; Smith, K. M.; Budd, L. D.; La Mar, G. N. *J. Am. Chem. Soc.* **1982**, *104*, 4345.

(8) (a) Kenner, G. W.; Smith, K. M.; Sutton, M. J. *Tetrahedron Lett*, **1973**, 1303−1306. (b) Budd, D. L.; La Mar, G. N.; Langry, K. C.; Smith, K. M.; Nayyir-Mazhir, R. *J. Am. Chem. Soc.* **1979**, *101*, 6091−6096. (c) Smith, K. M.; Morris, I. K. *J. Chem. Res., Synop.* **1988**, 16−17.

(9) Teale, F. W. J. *Biochim. Biophys. Acta* **1959**, *35*, 543.

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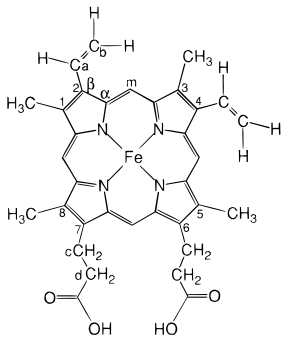

Figure 1. Molecular structure and labeling scheme of iron protoporphyrin-IX.

CO over the deoxymyoglobin solution, while cyanometmyoglobin was prepared by adding a few crystals of potassium cyanide to 0.5 mL of ~0.1 mM metmyoglobin solution.

Resonance Raman Spectroscopy. RR spectra were acquired with a scanning double monochromator (Spex 1404) equipped with a photomultiplier tube. The excitation sources were a Coherent Innova 100K krypton laser (406.7 and 413.1 nm) and a Liconix helium−cadmium laser (441.7 nm). Samples contained in a 5-mm NMR tube were positioned in a backscattering geometry and kept spinning during laser illumination. To minimize photolysis of carbonmonoxymyoglobin, a cylinder lens was used to focus the laser beam into a line 0.5 cm in height. The laser power (less than 20 mW at the sample point) was slightly adjusted for each sample to ascertain that no shoulder at 1355 cm^{-1} (ν_4 for deoxyMb) occurs. To accurately determine the isotope shifts, the monochromator position was calibrated against Rayleigh scattering before the start of each sample and checked at the end of scanning. No more than a 0.2 cm^{-1} shift of the Rayleigh line was found.

Results and Discussion

In making assignments, we take advantage of the extensive analysis available for metalloporphyrin vibrational modes.[4,5] NiOEP is the primary reference molecule because of its high symmetry and because its ethyl substituents have the basic C_β-substituent pattern of protoheme (Figure 1). The NiOEP-based analysis of porphyrin vibrational modes was recently extended to NiEPI,[5] in which alternating ethyl substituents of OEP are replaced by methyl groups. This substituent pattern resembles that of cytochrome *c*, which has four methyl and four $-CH_2C_2X$ substituents; the two protoheme vinyl groups become saturated by condensation with two cysteine side chains. The RR spectra of cytochrome *c*,[6] however, are considerably more complex than those of NiEPI because the protein distorts the porphyrin significantly. Despite the effect of distortion, and of the asymmetric substituent pattern, the NiOEP normal mode structure is retained by cytochrome *c*. Its spectra were successfully assigned,[6] with the aid of isotopic substitution, by recognizing the effects of symmetry reduction.

This framework is now extended to Mb, whose RR spectra are further complicated by the electronically conjugated vinyl substituents which perturb the porphyrin skeletal modes and which contribute distinctive internal vibrations to the spectra.[7] As in the cytochrome *c* study, we consider first the in-plane porphyrin skeletal modes, which dominate the spectra and resemble those of NiOEP rather closely, and then the substituent internal modes, and finally the out-of-plane porphyrin modes in the low-frequency region of the spectra.

Assignments are based on Soret-excited RR spectra which, because of the low effective symmetry, contain bands for most of the chromophore modes; no extra information was found to be available in Q-band-excited spectra (not shown). Isotope labeling in the porphyrin ring (^{15}N and *meso*-D_4) helped identify

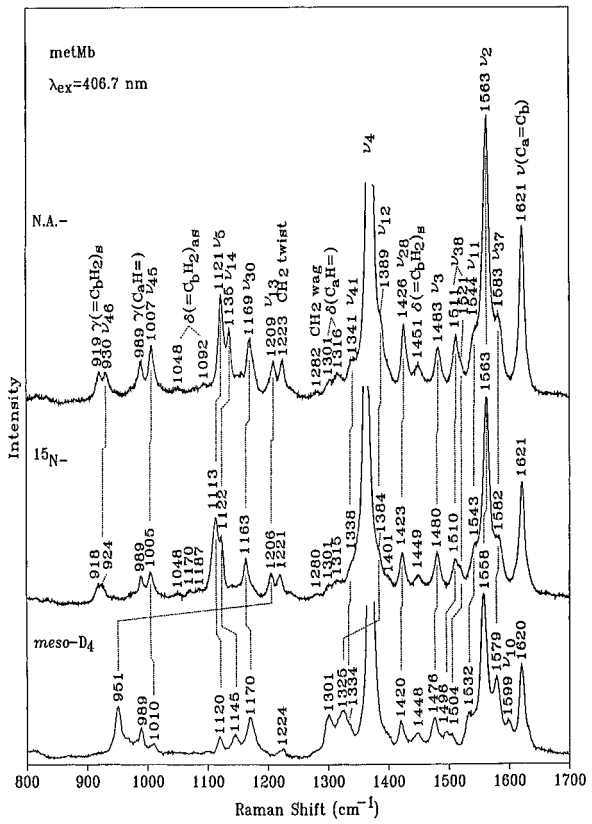

Figure 2. High-frequency resonance Raman spectra of metmyoglobin and its skeletally labeled *meso*-D_4 and pyrrole ^{15}N-heme isotopomers.

the skeletal modes, while the vinyl modes were disentangled via deuteration at the C_a or C_b atoms (Figure 1; 2,4-di(a-D_1), 2,4-di(b-D_2) isotopomers), including separate labeling of the 2- and 4-vinyl (2-a-D_1 and 4-a-D_1 isotopomer) groups. Contributions from the methyl and propionate groups were investigated with protoheme which was deuterated at the C_d atoms of the propionate groups (Figure 1) and at the 1- and 3-methyl groups as well as at the vinyl C_b atoms (abbreviated as the 6,7-di-(d-D_2) isotopomer). The high-frequency region of the isotopic spectra is shown in Figures 2−5 for metMb. Other Mb forms (not shown) exhibit the same pattern, but with skeletal modes shifted on the basis of oxidation and ligation state, as has been reviewed elsewhere.[10] Low-frequency spectra (Figures 6−10) are shown for several forms: deoxy, met, and the $Fe^{II}-CO$ and $Fe^{III}-CN^-$ adducts, because they display significant variations in frequency and enhancement patterns. The spectra are labeled according to the mode assignments and are correlated among the isotopomers via dotted lines. The assigned frequencies are listed in Table 1, while Tables 2 and 3 allocate the skeletal modes to the in- and out-of-plane local coordinates. This allocation is mainly for accounting purposes; the local coordinates are significantly mixed in the normal modes.[4,5]

The skeletal mode assignments are straightforward except for the modes allocated to the $C_\beta-C_1$ (Figure 1) stretching and bending coordinates. The eight $C_\beta-C_1$ bonds account for eight stretching and eight bending vibrations that can be divided into the D_{4h} symmetry classes as indicated in Table 2. However the $C_\beta-C_1$ bonds are not equivalent in protoheme, and the allocation to symmetry classes is therefore arbitrary. Moreover, the isotope shifts indicate that some of $C_\beta-C_1$ modes are significantly localized to particular bond types, i.e., $C_\beta-$vinyl and $C_\beta-$methyl. For consistency with previous work, we have

(10) Spiro, T. G.; Li, X.-Y. In ref 2, Chapter 1.

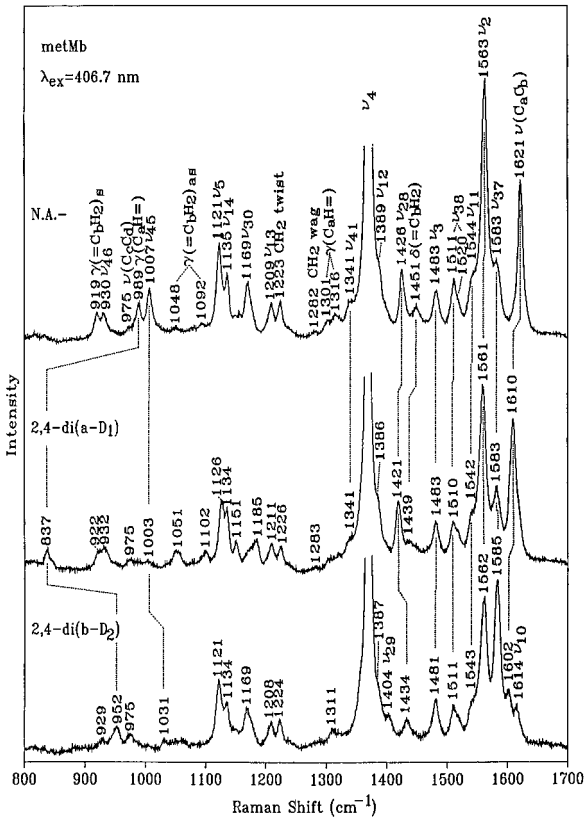

Figure 3. High-frequency resonance Raman spectra of metmyoglobin and its vinyl-deuterated isotopomers.

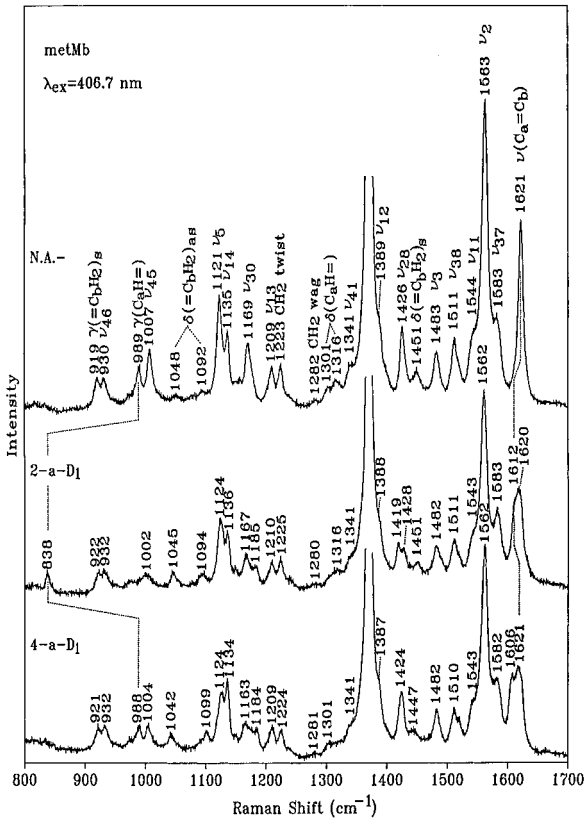

Figure 4. High-frequency resonance Raman spectra of metmyoglobin with selective labeling.

chosen to retain the NiOEP mode labels but have indicated modes which are localized to these bonds in Table 1 and 2.

A. In-Plane Heme Modes. 1. Skeletal Mode Assignments. Most assignments are readily made by extension of the

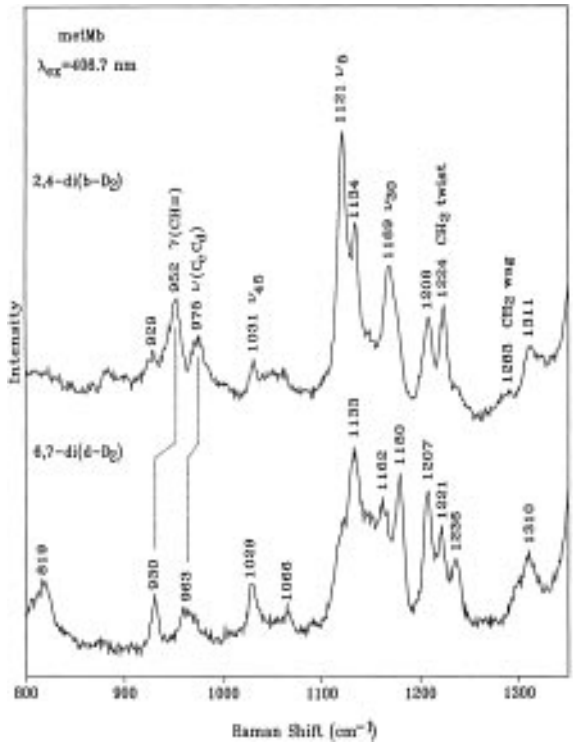

Figure 5. Effect of methyl and propionate labeling on the resonance Raman spectrum of metmyoglobin in the middle-frequency region.

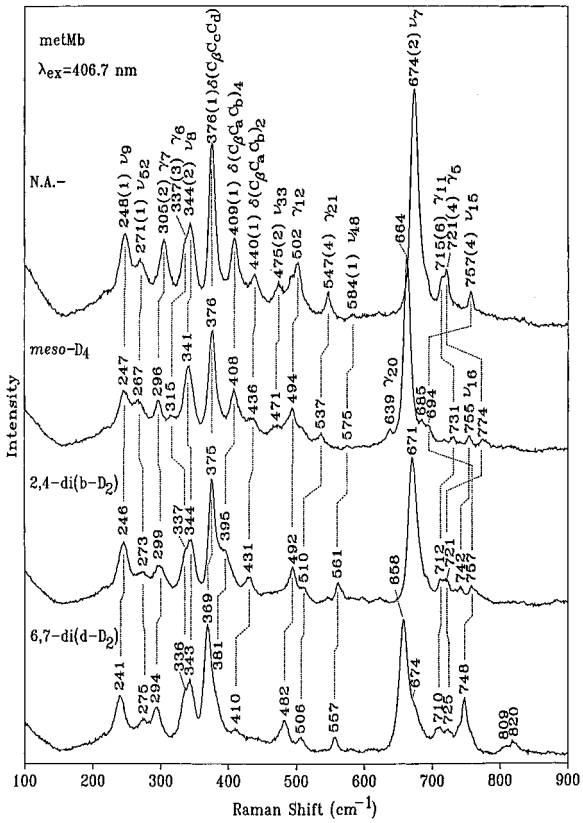

Figure 6. Low-frequency resonance Raman spectra of metmyoglobin and its isotopomers.

NiEPI[5] and cytochrome *c* assignments,[6] but we comment on features that are specific to protoheme. The highest-frequency $C_\alpha C_m$ asymmetrical stretching mode, ν_{10}, was not observed for metMb, owing to its overlap with the strong vinyl $C_a{=}C_b$ stretching band at 1621 cm^{-1}, but it can be seen at 1614 cm^{-1} in the RR spectrum of 2,4-di(b-d_2) vinyl deuterated heme (Figure

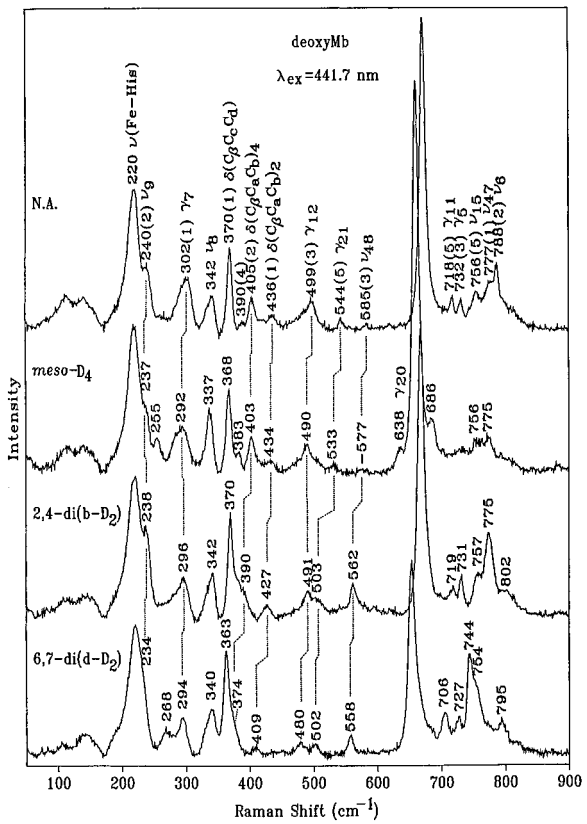

Figure 7. Low-frequency resonance Raman spectra of deoxymyoglobin and its isotopomers.

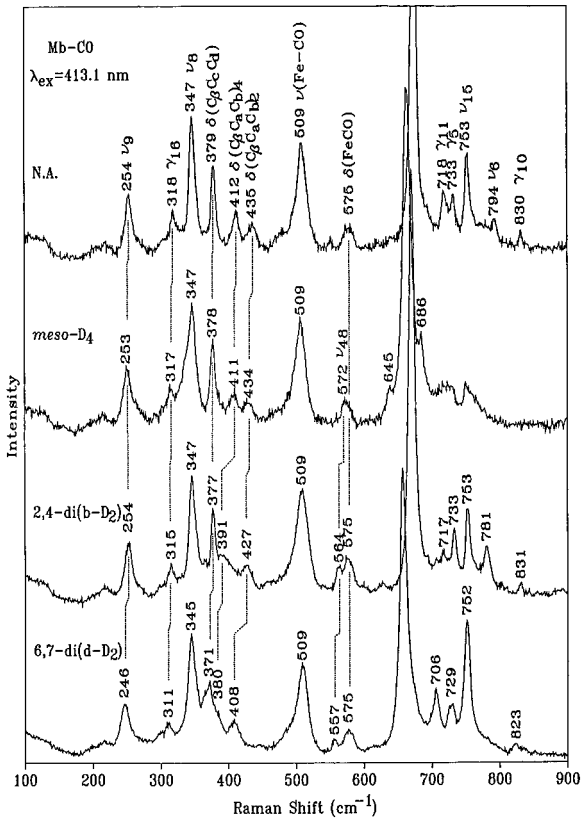

Figure 9. Low-frequency resonance Raman spectra of the carbon monoxide adduct of deoxymyoglobin and various isotopomers.

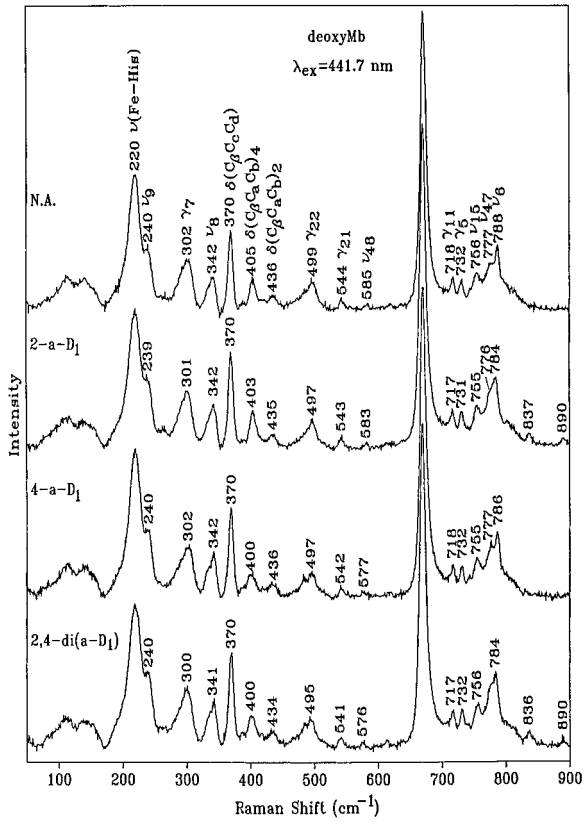

Figure 8. Low-frequency resonance Raman spectra of deoxymyoglobin and selective labeling.

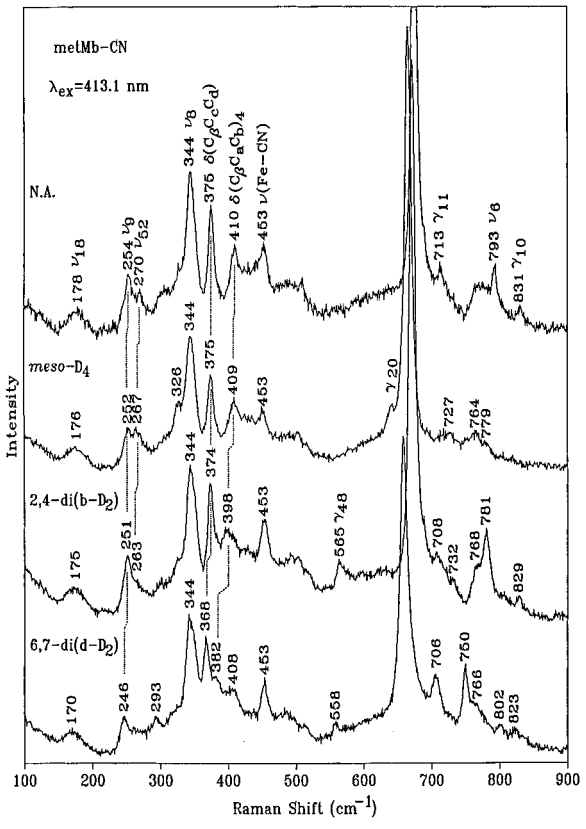

Figure 10. Low-frequency resonance Raman spectra of the cyanide adduct of metmyoglobin and various isotopomers.

3). This mode is shifted to 1599 cm^{-1} upon deuteration of the heme *meso*-protons (Figure 2).

The very strong ν_4 band at 1371 cm^{-1} has shoulders at 1341 and 1389 cm^{-1}. We assign them to ν_{41} and ν_{12}, respectively. These modes and ν_4 belong to the same local coordinate, the pyrrole half-ring symmetrical stretch, but with different phasing.[4] They are expected to show the same ^{15}N shifts but drastically different *meso*-D_4 shifts, because contributions from C_m−H

Table 1. Resonance Raman Frequencies and Their Normal Mode Assignments of Metmyoglobin and Its Isotopomers

N.A.	$\Delta(^{15}N)$	*meso*-D_4	2,4-di(a-D_1)	2,4-di(b-D_2)	2-a-D_1	4-a-D_1	6,7-di(d-D_2)	assignment[a]
1621	0	1620	1610	1602	1612/1620	1606/1621	1600	$\nu(C_a{=}C_b)$
[1608][b]		1599		1614	1583	1582	1614	ν_{10}
1583	1	1579	1583	1583	1583	1582	1583	ν_{37}
1563	0	1558	1561	1562	1562	1562	1559	ν_2
1544	1	1532	1542	1543	1543	1543	1543	ν_{11}
1521	1	1504	1518	1518	1520	1520		ν_{38}
1511	1	1496	1510	1511	1511	1510	1509	ν_{38}
1483	3	1476	1483	1481	1482	1482	1480	ν_3
1451	2	1448	1439	952	1428/1451	1447		$\delta(=C_bH_2)_s$
1426	3	1420	1421	1434	1419	1424	1439	ν_{28}
1402				1404			1403	ν_{29}
1389	5	1325	1386	1387	1388	1387		ν_{12}
1373	6	1372	1373	1373	1373	1373	1373	ν_4
1341	3		1341		1341	1341		ν_{41}
1316	1		975	1311	1316	974	1310	$\delta(C_aH{=})_4$
1301	0	1301	975	1289	974	1302		$\delta(C_aH{=})_2$
1282	2	1282	1283		1280	1281		
1223	2	1224	1226	1224	1225	1224		
1209	3	951	1211	1208	1210	1209		ν_{13}
1169	6	1170		1169	1167	1163		ν_{30}
1135	13	1145	1134	1134	1136	1134		ν_{14}
1121	8	1120	1126	1121	1124	1124		ν_5 (C_β-methyl stretch)
1092	5		1102		1094	1099		$\delta(=C_bH_2)_{as}$
1048	0		1051		1045	1042		$\delta(=C_bH_2)_{as}$
1007	2	1010	1003	1031	1002	1004		ν_{45} (C_β−vinyl stretch)
989	0	989	837	975	838	988		$\gamma(C_aH{=})$
930	6		932	929		932		ν_{46}
919	1		922		922	921		$\gamma(=C_bH_2)_s$
757	4	694	757	757	757	757	748	ν_{15}
721	4	774	720	721	721	721	725	γ_5
715	6	731	714	712	714	715	710	γ_{11}
674	1	664	673	671	674	674	658	ν_7
584	1	575	576	561	584		557	ν_{48}
547	4	537	545	547	546	546		γ_{21}
502	0	494	495	492	500	495	482	γ_{12}
475	2	471	467		468	471		ν_{33}
440	1	436	437	431	439	438	410	$\delta(C_\beta C_a C_b)_2 + \delta(C_\beta\ \mathrm{Me})$
409	1	408	407	395	409	407		$\delta(C_\beta C_a C_b)_4 + \delta(C_\beta\ \mathrm{Me})$
376	1	376	376	375	376	376	369	$\delta(C_\beta C_c C_d)$
344	2	341	344	344	344	344	343	ν_8
337	3	315	337	337	337	337	336	γ_6
305	2	296	302	299	303	304	294	γ_7
271	1	267	269	273	271	270	275	ν_{52}
248	1	247	247	246	248	248	241	ν_9

[a] Mode compositions are expected to be similar to those of NiOEP, which are given in ref 4b. [b] This band is observed by the strong $\nu(C_a{=}C_b)$ band, but is readily located with excitation at wavelengths in the Q-region (see e.g., ref 22).

bending are forbidden in A_{1g} symmetry (ν_4) but allowed in B_{1g} (ν_{12}) and E_u (ν_{41}) symmetry. All these bands shift 3−5 cm^{-1} in the ^{15}N spectra, and ν_4 is insensitive to *meso*-D_4 substitution as expected. On the other hand, ν_{12} moves from 1389 to 1325 cm^{-1} in the *meso*-D_4 spectrum just as it does in NiEPI.[5] Because of its overlap with ν_4, ν_{12} had only been detected for NiOEP upon *meso*-D_4 substitution, but its position in the natural abundance spectrum was revealed by FT-Raman spectroscopy of NiEPI, with 1064-nm preresonant excitation.[5]

The 1341-cm^{-1} band was previously assigned by Choi et al. to one of the two vinyl $=C_bH_2$ scissor modes on the basis of an apparent 5-cm^{-1} 2,4-di(a-D_1) shift.[7] This shift, however, is not reproduced in the present spectrum (Figure 3), which is better resolved than the previous spectra. Upon *meso*-D_4 substitution, the 1341-cm^{-1} band shifts down about 7 cm^{-1}, appearing as a shoulder on the 1325-cm^{-1} ν_{12} band. The shift is that expected for ν_{41}, which is calculated at 1346 cm^{-1} in NiOEP, with 5- and 4-cm^{-1} ^{15}N and *meso*-D_4 shifts.

The 1000−1150-cm^{-1} region contains bands that are allocated to stretching of the porphyrin−substituent bands (C_β−C_1 Table 2). As noted above, assignment of these bands to NiOEP-derived modes is arbitrary, because of the inequivalence of the C_β−C_1 bonds. For example, the 1007-cm^{-1} band is assigned to ν_{45} because of its match to the frequency calculated for NiOEP (996 cm^{-1}) and observed for NiEPI (990 cm^{-1}). However, it is actually a superposition of porphyrin−vinyl stretches, as evidenced by its loss in intensity upon C_a-deuteration of either the 2- or 4-vinyl groups (Figure 4) and by its disappearance when both vinyl groups are deuterated (Figure 3). Choi et al.[7] had assigned the 1121-cm^{-1} band, now allocated to ν_5, to porphyrin−vinyl stretching based on its 5-cm^{-1} upshift upon C_a deuteration (Figure 3). However, this band undergoes a 12-cm^{-1} upshift in the 6,7-di(d-D_2) isotopomer (Figure 5) and probably involves porphyrin−methyl stretching; this coordinate would be depressed in frequency by interaction with the $-CH_3$ umbrella coordinate (∼1365 cm^{-1}), but raised in frequency when the interaction is relieved by deuteration of the 1- and 3-methyl groups. A similar effect was observed in NiEPI[5] in which ν_5 shifted from 1137 to 1152 cm^{-1} upon perdeuteration of the methyl groups. We note also that ν_{30}, allocated to a pyrrole half-ring coordinate (Table 2), likewise shifted up 14 cm^{-1} upon methyl perdeuteration in NiEPI,[5] implying substantial C_β−C_1 involvement, and similarly the 1169-cm^{-1} band assigned to ν_{30} in Mb shifts up to 1180 cm^{-1} in the 6,7-di(d-D_2) isotopomer (Figure 5).

Below 600 cm^{-1} (Figure 6), two A_{1g} modes ν_8 and ν_9 are

Table 2. Allocation of the Observed In-plane Skeletal Frequencies of metmyoglobin to the Local Coordinates[a]

local coordinate	A_{1g}	B_{1g}	A_{2g}	B_{2g}	E_u
$\nu(C_mH)$	ν_1 [3041]			ν_{27} [3041]	ν_{36} [3040]
$\nu(C_\alpha C_m)_{asym}$		ν_{10} 1614 *1655*	ν_{19} *1603*		ν_{37} 1583 *[1602]*
$\nu(C_\beta C_\beta)$	ν_2 1563 *1602*	ν_{11} 1544 *1577*			ν_{38} 1511/1521 *1604*
$\nu(C_\alpha C_m)_{sym}$	ν_3 1483 *1520*			ν_{28} 1426 *1483*	ν_{39} *1501*
ν(pyr quarter-ring)			ν_{20} *1393*	ν_{29} 1404 *1407*	ν_{40} *1396*
ν(pyr half-ring)$_{sym}$	ν_4 1373 *1384*	ν_{12} 1389 *1387*[b]			ν_{41} 1341 *[1346]*
$\delta(C_mH)$		ν_{13} 1209 *1220*	ν_{21} *1307*		ν_{42} *1231*
$\nu(C_\beta C_1)_{sym}$	ν_5 1121[c] *1138*	ν_{14} 1135 *1131*			ν_{44} *1153*
ν(pyr half-ring)$_{asym}$			ν_{22} *1121*	ν_{30} 1169 *1159*	ν_{43} *1133*
$\nu(C_\beta C_1)_{asym}$			ν_{23} *1058*	ν_{31} *1015*	ν_{45} 1007[d] *996*
δ(pyr deform)$_{asym}$			ν_{24} *597*	ν_{32} *938*	ν_{46} 930 *927*
ν(pyr breathing)	ν_6 *804*	ν_{15} 757 *751*			ν_{47} *766*
δ(pyr deform)$_{sym}$	ν_7 674 *674*	ν_{16} *746*			ν_{48} 584 *605*
δ(pyr rot.)			ν_{25} *551*	ν_{33} 475 *493*	ν_{49} *544*
ν(M−N)	ν_8 344 *361/343*	ν_{18} *168*			ν_{50} *[358]*
$\delta(C_\beta - C_1)_{asym}$			ν_{26} *[243]*	ν_{34} *197*	ν_{51} *328*
$\delta(C_\beta C_1)_{sym}$	ν_9 248[e] *263/274*	ν_{17} *305*			ν_{52} 271[e] *263*
δ(pyr transl)				ν_{35} *144*	ν_{53} *212*

[a] The italicized values are those of NiOEP.[4b] The bracketed values are the calculated frequencies for which the experimental values are not available. [b] Detected for NiEPI.[5] [c] Primarily $\nu(C_\beta$−methyl). [d] Primarily $\nu(C_\beta$−vinyl). [e] Coupled $\delta(C_\beta$−propionate) and $\delta(C_\beta$−methyl).

Table 3. Allocation of the Observed Out-of-Plane Modes of Metmyoglobin to the Local Coordinates[a]

local coordinates	A_{1u}	A_{2u}	B_{1u}	B_{2u}	E_g
$\gamma(C_mH)$		γ_4 *844*	γ_{10} 830[b] *853*		γ_{19} *841*
Pyr fold$_{asym}$	γ_1 *750*		γ_{11} 715 *729*		γ_{20}[c] *[713]*
Pyr fold$_{sym}$		γ_5 721 *739*		γ_{15} *704*	γ_{21} 547 *656*
Pyr swivel	γ_2 *[346]*		γ_{12} 502 *612*		γ_{22} *492*
Pyr tilt		γ_6 337 *360*		γ_{16} 318[d] *270*	γ_{23} *254*
$\gamma(C_\alpha C_m)$		γ_7 305 *[284]*	γ_{13} *[130]*		γ_{24} *230*
$\gamma(C_\beta C_1)_{sym}$		γ_8 *[108]*		γ_{17} *127*	γ_{25} *[91]*
$\gamma(C_\beta C_1)_{asym}$	γ_3 *[74]*		γ_{14} *[44]*		γ_{26} *[63]*
Pyr transl				γ_{18} *[30]*	
γ(NiN)		γ_9 *[32]*			

[a] The italicized values are those of NiOEP. The bracketed values are the calculated frequencies for which the experimental values are not available. [b] Seen in MbCO and metMbCN. [c] Seen at 639 cm^{-1} upon *meso*-D_4 substitution. [d] Seen in MbCO.

expected and were previously assigned at 344 and 271 cm^{-1}.[7] The ν_8 assignment is confirmed by the 2-cm^{-1} ^{15}N and 3-cm^{-1} *meso*-D_4 shifts of the 344-cm^{-1} band, but ν_9 is reassigned to the 248-cm^{-1} band because of the expected insensitivity of ν_9 to *meso*-deuteration.[4] The 4-cm^{-1} *meso*-D_4 shift of the 271-cm^{-1} band suggests assignment to ν_{52}, which is observed at 263 cm^{-1} in NiOEP, with a 3-cm^{-1} *meso*-D_4 shift. Both ν_9 and ν_{52} are allocated to porphyrin−substituent bending coordinates (C_β−C_1, Table 2), but their sensitivity to the available isotopomers is slight (Figure 6). The biggest shifts are seen in the 6,7-di(d-D_2) spectra, −7 cm^{-1} for ν_9 and +4 cm^{-1} for ν_{52}, suggesting that the C_β−propionate bends are involved along with the C_β−methyl bends of the adjacent 5- and 8-methyl groups (Figure 1). If the 1- and 3-methyl groups were major contributions, then larger shifts would have been expected, since in NiOEP, ν_9 shifts down 30 cm^{-1} when the ethyl C_1 atoms are deuterated.

2. Vinyl Influences. Vinyl groups are known to induce Raman activity in E_u modes and to remove their degeneracy.[7] These effects are confirmed in the spectra of metMb. In the high-frequency region (1450−1700 cm^{-1}), both ν_{37} and ν_{38} are seen with significant intensity (Figure 2). Moreover, ν_{38} is split into two components at 1511 and 1521 cm^{-1}. These two bands become well-resolved at 1496 and 1504 cm^{-1} in the spectrum for the *meso*-D_4 isotopomer. In this frequency region, the modes are mixtures of C_β−C_β and C_α−C_m coordinates, but the much larger *meso*-D_4 shift of ν_{38} (16 cm^{-1}) than that of ν_{37} (4 cm^{-1}) implies predominant $C_\beta C_\beta$ character for the former and $C_\alpha C_m$ character for the latter. As dicsussed in the preceding section, ν_{41} is now assigned to the 1341-cm^{-1} band; we note that this band is polarized with Soret excitation, but has been reported to be anomalously polarized when excited in the vicinity of the Q-bands.[11] This behavior suggests that the two E_u components are superimposed and have different polarization, one being enhanced in resonance with the Soret band and the other in resonance with the Q-bands. Additional E_u modes are assigned at 1007 (ν_{45}), 930 (ν_{46}), 584 (ν_{48}), and 252 (ν_{52}) cm^{-1} on the basis of their frequency and isotopic correspondences with E_u modes of NiOEP[4] and NiEPI.[5]

Vinyl coordinates are mixed into some of the skeletal modes. The ν_2 (1563 cm^{-1}) and ν_{11} (1544 cm^{-1}) modes are shifted down 2 cm^{-1} for the 2,4-di(a-D_1) isotopomer (Figure 3). More pronounced mixing is observed for ν_{28}, which shifts from 1426 *down* to 1421 cm^{-1} for C_a deuteration and *up* to 1434 cm^{-1} for C_b deuteration. This effect is due to interaction with the neighboring vinyl ═CH_2 scissor mode, 1451 cm^{-1}, which shift down to 1439 cm^{-1} upon C_a deuteration (see next section). When C_b is deuterated, the scissor mode shifts out of the region, thereby relieving the interaction and causing ν_{28} to shift up. A frequency upshift is also seen for ν_{29} (1401 cm^{-1}), which appears as a shoulder on the strong ν_4 band, but becomes a distinct band at 1404 cm^{-1} upon C_b deuterations (Figure 3).

The vinyl groups also induce a significant change in the normal mode composition of ν_2 and ν_{11}. These two modes are calculated to be nearly pure C_β−C_β stretching vibrations. They show no *meso*-D_4 sensitivity in NiOEP,[4] NiEPI,[5] or cytochrome *c*,[6] but shift down 5 and 11 cm^{-1} in metMb. This sensitivity implies a significant contribution of the C_α−C_m coordinate to the modes. The coordinate mixing cannot be ascribed to symmetry lowering alone, in view of the NiEPI and cytochrome *c* spectra,[6] but must arise as a specific effect of the vinyl substituents.

B. Substituent Modes. Protoheme possesses three sets of peripheral substituents, the vinyl, propionate, and methyl groups (Figure 1). In addition to the porphyrin−substituent stretches and bends described above, the internal vibrations of these groups are expected to exhibit RR activity inasmuch as ethyl and methyl vibrations have been shown to be enhanced in the RR spectra of NiOEP[4] and NiEPI.[5]

(11) Spiro, T. G.; Strekas, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 2622−2626.

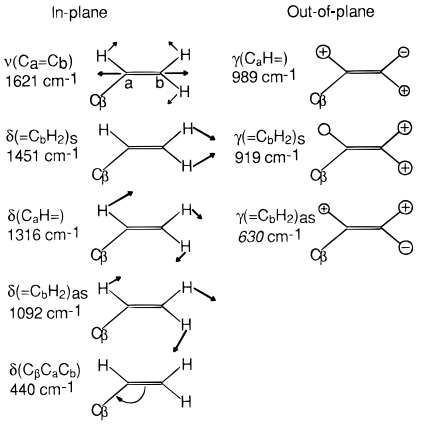

Figure 11. Diagram of the vinyl vibrations with frequencies assigned from metMb spectra. The 630-cm^{-1} $\gamma(=C_bH_2)_{as}$ is not seen. The 410-cm^{-1} $\delta(C_\beta C_a C_b)$ mode is formally included among the porphyrin skeletal modes ($\delta C_\beta - C_1$).

1. Vinyl Modes. Although most of the vinyl modes were previously identified in the RR spectrum and assigned via deuteration,[7] some of these assignments are revisited in connection with the available *meso*-D_4 and ^{15}N shifts. Figure 11 summarizes the observed frequencies of vinyl modes for metMb, along with a schematic description of their eigenvectors. Some notable aspects are discussed here with a view toward clarifying controversies in the literature.

***ν*(C$_a$=C$_b$).** The two $\nu(C_a{=}C_b)$ modes coincide in a single band at 1621 cm^{-1}. They are, however, revealed by selective labeling of the vinyl groups. When either group is deuterated at C_a, a split band is observed (Figure 4) with one component at 1621 cm^{-1} and the other at a lower frequency. However, the position of the lower component is different, 1612 cm^{-1} for 2-a-D_1 heme but 1606 cm^{-1} for 4-a-D_1 heme. The differing isotope shifts imply different mode compositions, even though the frequencies are the same in unlabeled protein.

***δ*(=C$_b$H$_2$)$_s$.** The vinyl $=CH_2$ scissor mode is reassigned to a spectral feature at 1451 cm^{-1}. (It was earlier assigned to the 1426-cm^{-1} band, now reassigned to ν_{28}, see above.) The 1451-cm^{-1} band shifts to 1439 cm^{-1} and disappears for 2,4-di(a-D_1) and 2,4-di(b-D_2) isotopomers, respectively. The $\delta(=CH_2)_s$ and ν_{28} modes are strongly coupled, as discussed above.

Inequivalent vinyl modes are again revealed by selective labeling. C_a deuteration of the 4-vinyl group (Figure 4) broadens the 1451-cm^{-1} band and shifts its center to 1447 cm^{-1}; ν_{28} is shifted only 2 cm^{-1}. However, C_a deuteration of the 2-vinyl group clearly splits the $\delta(=C_bH_2)_s$ band, with one component shifted down to 1428 cm^{-1}; ν_{28} is now shifted 7 cm^{-1}. Intriguingly, 2-vinyl deuteration has a greater effect than that of deuteration of both vinyl groups (Figure 3), while 4-vinyl deuteration has a much smaller effect.

The assignment of both $\delta(=C_bH_2)_s$ modes to the 1451-cm^{-1} band supplants the earlier assignment of one of these modes to the 1341-cm^{-1} band (now reassigned to ν_{41}, see above), an assignment that had been questioned by Kitagawa and coworkers[12] on the basis of a model normal mode calculation.

***δ*(C$_a$H=).** The in-plane C−H bending mode was previously identified at 1305 cm^{-1} for NiPPIX and at 1314 cm^{-1} for fluorometMb.[7] We detected two well-resolved spectral features at 1301 and 1316 cm^{-1} that can be assigned to $\delta(C_aH{=})$. These two features disappear in the spectra of 2,4-di(a-D_1) and collapse into a single band at 1311 cm^{-1} for 2,4-di(b-D_2) heme. Furthermore, we are able to assign the 1301- and 1316-cm^{-1} bands to the 2- and 4-vinyl group, respectively, because of their selective disappearance in the 2-a-D_1 and 4-a-D_1 spectrum. Thus the two vinyl groups have distinctly different frequencies for this mode.

***δ*(=C$_b$H$_2$)$_{as}$.** The vinyl CH_2 rocking modes are assigned to a pair of weak features at 1048 and 1092 cm^{-1}; the latter is close to the 1089-cm^{-1} RR band previously observed for NiPPIX.[7] These two bands shift slightly and intensify for 2,4-di(a-D_1) heme, but disappear for 2,4-di(b-D_2). The C_a deuteration shifts are too small to distinguish the vinyl groups by selective labeling.

***γ*(C$_a$H=).** The out-of-plane $C_aH{=}$ wag is assigned to a spectral feature at 989 cm^{-1}, which disappears for both 2,4-di(a-D_1) and 2,4-di(b-D_2) hemes. The previously allocated band at 1007 cm^{-1} is reassigned to ν_{45} on the basis of its expected *meso*-D_4 and ^{15}N shifts. Interestingly, the 989-cm^{-1} band seems to derive its intensity solely from the 2-vinyl group. The spectrum of 2-a-D_1 heme exhibits complete loss of the 989-cm^{-1} band and the occurrence of a new feature at 838 cm^{-1}, assignable to $\gamma(CD{=})$, while the 989-cm^{-1} intensity is unaffected in the spectrum of 4-a-D_1 heme and no new feature is discernible in the 800−1000-cm^{-1} spectral region.

***γ*(=C$_b$H$_2$)$_s$.** The symmetric $=CH_2$ wag is located at 919 cm^{-1} for metMb. It disappears for 2,4-di(b-D_2) heme and shifts up to 922 cm^{-1} upon C_a deuteration in either vinyl group (Figure 4). This mode was assigned to the 903-cm^{-1} IR band for NiPPIX[7] and has not been detected in other RR spectra. It seems to derive its intensity from the nearby ν_{46} (930 cm^{-1}); the 919-cm^{-1} band vanishes when ν_{46} loses its intensity in *meso*-D_4 heme (Figure 2).

***δ*(C$_\beta$C$_a$C$_b$).** Two vinyl bending modes are assigned, at 405−412 and 435−440 cm^{-1}, on the basis of their 8−15-cm^{-1} C_bD_2 downshifts (Figures 6, 8, and 9). Deuteration at C_a produces only slight shifts (Figure 7) because C_a is the central atom of the bending coordinate and its motion is small. However, selective labeling shows these shifts to be localized to the 4-vinyl group for the lower frequency band and the 2-vinyl group for the higher frequency band (Figure 7). Thus the two vinyl groups have quite different frequencies for the bending mode. Some of this difference is associated with the substantial coupling to the 1- and 3-methyl group coordinates that are evident in the 6,7-di(d-D_2) spectra (Figures 6, 8, and 9). Relative to the 2,4-di(b-D_2) spectra (the vinyl C_b atoms are deuterated in both isotopomers), the 6,7-di(d-D_2) shifts are ∼12 cm^{-1} for $\delta(C_\beta C_a C_b)_4$ and ∼20 cm^{-1} for $\delta(C_\beta C_a C_b)_2$. Thus these modes should really be viewed as coupled vinyl and methyl bends on the two vinyl-bearing pyrrole rings (Figure 1).

We note that Gersonde et al.[13] assigned vinyl bending modes at 412 and 591 cm^{-1} in a vinyl-labeled monomeric insect hemoglobin. The latter frequency corresponds to the 584-cm^{-1} band in Mb, which we assign to the skeletal mode ν_{48}, on the basis of its substantial ^{15}N and *meso*-D_4 shifts. We confirm, however, that vinyl deuteration shifts are large, 9 cm^{-1} for $C_a{-}D$ (Figure 7) and 23 cm^{-1} for $C_b{-}D_2$ (Figures 6 and 8), implying strong coupling with vinyl bending. Interestingly the effect appears to be localized to the 4-vinyl group, as judged by selective labeling (Figure 7).

2. Propionate Modes. The propionate groups at the 6,7 positions resemble the ethyl groups in NiOEP. In view of the significant RR activity of these aliphatic groups in the RR spectra of NiOEP,[4] the propionate modes are expected to show up in the Mb spectra. The propionate assignments were suggested for cytochrome *c* on the basis of small isotope shifts

(12) Lee, H.; Kitagawa, T.; Abe, M.; Pandey, P. K.; Leung, H.-K.; Smith, K. M. *J. Mol. Struct.* **1986**, *146*, 329−347.

(13) Gersonde, K.; Yu, N.-T.; Lin, S. H.; Smith, K. M.; Parish, D. W. *Biochemistry* **1989**, *28*, 8197−8201.

and by analogy to the ethyl modes of NiOEP.[4] They are confirmed for Mb by comparing the spectra of 2,4-di(b-D_2) and 6,7-di(d-D_2) (Figure 5).

Methylene Deformations. Expected CH_2 bending modes include the scissors (~1440 cm^{-1}), wag (~1300 cm^{-1}), twist (~1250 cm^{-1}), and rock (~750 cm^{-1}). No RR candidates are found for the first two of these, but the 1223- and 1282-cm^{-1} bands can be assigned to methylene twist and wag deformations on the basis of the propionate labeling (Figure 5).

$\nu(C_c-C_d)$. In cytochrome *c*, a pair of bands at ~970 cm^{-1} was assigned to C_c-C_d stretching. A weak feature is also observed in Mb at 975 cm^{-1} (Figure 5), which is unaffected by isotope substitution except when the propionates are labeled.

$\delta(C_\beta C_c C_d)$. Particular interest attaches to the 376-cm^{-1} band, which is the strongest band below 600 cm^{-1} for metMb (Figure 6). This band is assigned to the porphyrin−propionate bending because its frequency is invariant for all isotopomers except 6,7-di(d-D_2), for which it shifts down 6 cm^{-1}. This modest shift is consistent with the effect of deuteration at the second propionate methylene group (C_d) on the bending frequency. This band is at the same frequency in metMb−CN^- (Figure 8) and at a slightly higher frequency in MbCO (379 cm^{-1}, Figure 9), but at a distinctly lower frequency in deoxyMb (Figure 7). In metMb and deoxyMb, its intensity is higher than that of ν_8, normally the strongest low-frequency band in metalloporphyrin spectra, but in metMb−CN^- and MbCO, ν_8 is stronger. Bands in this region have also been identified in cytochrome *c* and CCP and have moderate intensities. The frequency and intensity variations may be related to conformational changes of the propionate substituents.

3. Ring-Adjacent Methyl Groups. Although some methyl modes (rocking mode at ~1060 cm^{-1} and umbrella mode at 1362 cm^{-1}) were identified in the spectra of cytochrome *c*,[6] no candidate band is seen in the 406-nm excitation spectra of Mb derivatives.

C. Out-of-Plane Vibrations. In the low-frequency (100−900-cm^{-1}) region, the Mb derivatives exhibit a number of bands that can be assigned to out-of-plane modes by comparing their frequencies and isotope shifts to those of NiOEP[4] and cytochrome *c*.[6] Table 3 allocates these bands for metMb to the local coordinates and compares the assigned or calculated frequencies to those in NiOEP and cytochrome *c*.

The highest out-of-plane modes are expected at ~850 cm^{-1} and involve C_m-H wagging, $\gamma(C_m-H)$. These modes are not enhanced for metMb or deoxyMb, but bands are observed at 830 and 831 cm^{-1} for the CO and CN^- adducts, which disappear in the *meso*-D_4 spectra. They are assigned to γ_{10} on the basis of the interaction with γ_{12} (see below).

Three pyrrole folding modes can be assigned. A pair of bands at 715 and 721 cm^{-1} in the metMb spectrum exhibit 6- and 4-cm^{-1} ^{15}N downshifts and are assigned to γ_{11} and γ_5. These assignments are further supported by their large *meso*-D_4 upshifts, from 715 to 731 cm^{-1} for γ_{11} and from 721 to 774 cm^{-1} for γ_5 as observed in NiOEP. Interestingly, γ_5 is also found to be particularly sensitive to the heme structure, shifting from 721 cm^{-1} for metMb to 732 cm^{-1} for deoxyMb and to 733 cm^{-1} for Mb−CO. This large a frequency shift has not been seen for other low-frequency heme modes. Another pyrrole folding mode, γ_{21}, is assigned to a spectral feature at 547 cm^{-1}. The marked sensitivity of the pyrrole folding modes to vinyl, methyl, and propionate deuteration reflects substantial contributions from substituent bending coordinates.

One of the pyrrole swiveling modes (γ_{12}, B_{1u}) is seen at 502 cm^{-1} for metMb, exhibiting little ^{15}N, but a large (8 cm^{-1}) *meso*-D_4 shift. We note that the isotope shift pattern of γ_{12} is different from that in NiOEP, but similar to that seen in cytochrome *c*. The difference has been attributed[6] to an altered mixing pattern between $\gamma(C_m-H)$ (γ_{10}) and γ_{12}.

Other observed out-of-plane modes include pyrrole tilting (γ_6 at 337 cm^{-1}) and methine wagging (γ_7 at 305 cm^{-1}). γ_6 is seen as a low-energy shoulder on a broad feature centered at 344 cm^{-1}, the main peak being assignable to ν_8. When *meso*-protons are exchanged with deuterium, the asymmetrical 344-cm^{-1} band becomes symmetric and a new feature emerges at 315 cm^{-1}. The large *meso*-D_4 shift (22 cm^{-1}) agrees with that expected for γ_6. The 307-cm^{-1} band had been assigned to vinyl bending[7] because of its sensitivity toward vinyl deuteration, but the observed *meso*-D_4 shift (9 cm^{-1}) clearly favors assignment to γ_7. The shift seen for the 2,4-di(b-D_2) and 6,7-di(d-D_2) isotopomers implies substantial contributions from vinyl and methyl and/or propionate substituents, as do the even larger isotope shift seen for the γ_{12} pyrrole swiveling mode at 502 cm^{-1} (Figure 6).

We note that the out-of-plane modes γ_6, γ_7, γ_{12}, and γ_{21} are active in the high-spin complexes metMb (Figure 6) and deoxyMb (Figure 7), but not in the low-spin adducts MbCO (Figure 9) and metMb−CN (Figure 10). This pattern is consistent with the out-of-plane intensity depending on an out-of-plane distortion of the heme group, since the Fe atoms are out of the heme plane for the high-spin complexes, but in the plane for the low-spin complexes. It is curious, however, that the $\gamma(C_m-H)$ mode γ_{10} (830 cm^{-1}) is seen for Mb−CO and metMb−CN, but not for metMb or deoxyMb. The metMb−CN and Mb−CO spectra contain additional bands arising from Fe−CN (453 cm^{-1})[14] and Fe−CO (509 cm^{-1}) stretching and Fe−C−O bending (575 cm^{-1}),[15] while deoxyMb displays the well-known Fe−His stretch[16] at 220 cm^{-1}.

D. Implications. The original assignments[7] by Choi et al. of vinyl modes and of vinyl effects on porphyrin modes from RR and IR spectra of isotopically labeled nickel protoporphyrin and myoglobin have been mostly confirmed in subsequent studies.[12] However, a few assignments were in error and are corrected in the present study, which has the benefit of high signal/noise and a larger range of isotopomers. These misassignments had suggested a strong coupling between the vinyl substituents leading to ~100 cm^{-1} separation between modes involving the same vinyl coordinate, e.g., $\delta(=C_bH_2)$ and $\delta(C_\beta C_a C_b)$. This idea was challenged by Kitagawa and coworkers[12] who found no evidence of large intervinyl coupling in their normal mode calculation on a model tetravinylporphyrin. The current reassignments support the absence of such an effect, although smaller differences between some nominally equivalent vinyl modes [$\delta(C_aH=)$, $\delta(=C_bH_2)_{as}$, and $\delta(C_\beta C_a C_b)$] are found.

The role of vinyl orientation has been investigated by Kalsbeck et al.[17] who observed two $\nu(C_a=C_b)$ RR bands, at 1620 and 1631 cm^{-1}, in solutions containing hemes that have either one or two vinyl substituents. The relative intensities were temperature dependent, indicating contributions from different conformers. Density functional calculations on model compounds gave two low-energy conformers with predicted $\nu(C_a=C_b)$ differing by 10−20 cm^{-1}. A number of heme proteins[18] are reported to have two $\nu(C_a=C_b)$ RR bands, at

(14) Henry, E. R.; Rousseau, D. L.; Hopfield, J. J.; Noble, R. W.; Simon, S. R. *Biochemistry* **1985**, *24*, 5907−5918.
(15) Tsubaki, M.; Srivastava, R. B.; Yu, N.-T. *Biochemistry* **1982**, *21*, 1132.
(16) Stein, P.; Mitchell, M.; Spiro, T. G. *J. Am. Chem. Soc.* **1980**, *102*, 7795−7797.
(17) Kalsbeck, W. A.; Ghosh, A.; Pandey, R. K.; Smith, K. M.; Bocian, D. F. *J. Am. Chem. Soc.* **1995**, *117*, 10959−10969.
(18) Smulevich, G.; Miller, M. A.; Gosztola, D.; Spiro, T. G. *Biochemistry* **1989**, *28*, 5058−5064.

~1620 and 1630 cm^{-1}, suggesting that the two vinyl substituents have different orientations (or that a single substituent has enough flexibility in the protein to occupy alternative conformations). However, crystal structures are unavailable for these proteins. The fact that the two vinyl $\nu(C_a{=}C_b)$ modes are both at 1620 cm^{-1} in Mb, and also in the acid form of cytochrome *c* peroxidase,[19] is not inconsistent with the orientation hypothesis because the crystallographically determined $C_\beta C_\beta C_a{=}C_b$ dihedral angles are all essentially the same, 30−37°.[20]

However, the orientation may not be the only relevant variable, since the compositions of the two $\nu(C_a{=}C_b)$ modes are different even when these frequencies are the same. This difference is evident in the differing isotopic shifts upon selective labeling of the 2- and 4-vinyl groups in both Mb and cytochrome *c* peroxidase.[19] What determines these compositions is not clear. It is interesting that the 2- and 4-vinyl frequencies do diverge for other vinyl modes in Mb, i.e., $\delta(C_aH{=}) = 1316/1301$ cm^{-1}, $\delta({=}C_bH_2)_{as} = 1092/1048$ cm^{-1} and $\delta(C_\beta C_a C_b) = 440/409$ cm^{-1}. The vinyl bends are heavily mixed with methyl bends, and this mixing differs for the two vinyl-bearing pyrroles, as evidenced by different isotope shifts for the 2- and 4-vinyl-localized modes. A possible explanation of these complex effects is that out-of-plane distortions affect the pyrrole rings differently and modify the vinyl couplings to the porphyrin modes.

Effects of out-of-plane distortion are seen directly in the activation of out-of-plane porphyrin modes. The RR enhancement is derived from the coupling of heme vibrational modes to the $\pi-\pi^*$ excitation of the B-band, which is polarized in the porphyrin plane. For out-of-plane motions, such coupling requires a static out-of-plane distortion. Evidence for this mechanism is the activity of several out-of-plane modes, γ_6, γ_7, γ_{12}, and γ_{21}, only in the high-spin Mb derivatives metMb and deoxyMb in which the Fe atom lies out of the average heme plane. However, additional porphyrin distortion is implied by the activation even in the low-spin adducts of other out-of-plane modes, γ_5, γ_{10}, and γ_{11}.

It is interesting to contrast the influence of the protein on the RR spectrum of Mb and cytochrome *c*. The heme in cytochrome *c* is covalently bound to the protein and is subject to a pronounced propeller distortion of the porphyrin skeleton.[21] One consequence of this is activation of anomalously polarized bond-alternant A_{2g} modes with B-band excitation. Such modes are not normally seen in the B-band-excited RR spectra of metalloporphyrins and are not observed for Mb. Another effect was strong activation of the pyrrole folding mode γ_{12}, even though cytochrome *c* is low spin. In Mb, only the high-spin forms show γ_{12} activation. On the other hand, the pyrrole and methine wagging modes γ_6 and γ_7 were not seen in cytochrome *c*, but are activated in high-spin Mb derivative. These modes apparently require out-of-plane displacement of the metal for their activation. Thus, distinctive differences in protein-specific effects can be detected in the RR spectra.

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(19) Smulevich, G.; Hu, S.; Rodgers, K. R.; Goodin, D. B.; Smith, K. M.; Spiro, T. G. *Biospectroscopy*, in press.

(20) Reid, L. S.; Lim, A. R.; Mauk, A. G. *J. Am. Chem. Soc*. **1986**, *108*, 8197−8201.

(21) (a) Dickerson, R. E.; Kopka, M. L.; Borders, C. L., Jr.; Varnum, J. C.; Weinzierl, J. E.; Margoliash, E. *J. Mol. Biol*. **1967**, *29*, 77−95. (b) Dickerson, R. E.; Kopka, M. L.; Weinzierl, J. E.; Varnum, J. C.; Eisenberg, D.; Margoliash, E. *J. Biol. Chem*. **1967**, *242*, 3015−3018. (c) Takano, T.; Kallai, O. B.; Swanson, R.; Dickerson, R. E. *J. Biol. Chem*. **1973**, *248*, 5234−5255. (d) Takano, T.; Dickerson, R. E. *J. Mol. Biol*. **1981**, *153*, 79−94. (e) Takano, T.; Dickerson, R. E. *J. Mol. Biol*. **1981**, *153*, 95−115. (f) Louie, G.; Brayer, G. D. *J. Mol. Biol*. **1990**, *214*, 527−555. (g) Berghuis, A. M.; Brayer, G. D. *J. Mol. Biol*. **1992**, *223*, 959−976.

(22) Strekas, T. C.; Packer, A. J.; Spiro, T. G. *J. Raman Spectrosc*. **1973**, *1*, 197−206.