Contribution from the Department of Chemistry,
Princeton University, Princeton, New Jersey 08544

Axial Ligation of Manganese(III/II) Heme Complexes and Manganese-Substituted Myoglobin and Hemoglobin from Resonance Raman Spectroscopy

Niraja Parthasarathi and Thomas G. Spiro*

Received March 24, 1987

Resonance Raman spectra are reported for manganese-substituted myoglobin (Mb) and hemoglobin (Hb) and analogous complexes. The porphyrin skeletal mode frequencies for 5- and 6-coordinated Mn^{III} protoporphyrin (PP) complexes are in good accord with the expected core size expansion for the 6-coordinate species. In aqueous solution, $Mn^{III}PP$ gives evidence for being weakly 6-coordinated, as it does in $Mn^{III}Mb$ and $Mn^{III}Hb$. $Mn^{II}Mb$ and $Mn^{II}Hb$, as well as the 2-methylimidazole adduct of $Mn^{II}PP$, show the expected core size marker frequencies for 5-coordinate Mn^{II}, but in the absence of added ligand, aqueous $Mn^{II}PP$ shows much lower frequencies, indicating a large core expansion. This is attributed to a 6-coordinate species with the large Mn^{II} ion in the porphyrin plane; the protein spectra show a minority population of 6-coordinate Mn^{II} heme. The Mn^{II}–imidazole stretching band is readily identified in the low-frequency RR spectra. The frequency is 219 and 195 cm^{-1} for the 2-methylimidazole and 1,2-dimethylimidazole adducts of $Mn^{II}PP$. For $Mn^{II}Hb$, a broad band is seen, with a central frequency of 216 cm^{-1}, just as in $Fe^{II}Hb$, and the breadth is attributed to chain heterogeneity. The $Mn^{II}Mb$ frequency, 215 cm^{-1}, is lower than that in $Fe^{II}Mb$, 220 cm^{-1}, indicating a weakening of the Mn–imidazole bond by protein forces.

Introduction

Metal substitution is a useful method of exploring the active sites of metalloproteins via the altered electronic and steric requirements of different metal ions. Manganese substitution in hemoglobin has been shown to produce a protein that is functional in the sense that its binding of NO ligands is cooperative.[1] The MnNO adduct is isoelectronic with the FeCO adduct of native hemoglobin. Nevertheless, the Mn–NO bond is appreciably stronger than the Fe–CO bond, as demonstrated recently by resonance Raman (RR) spectroscopy, which provided a useful probe of the interactions of the Mn–NO heme adduct in the binding pocket.[2]

The crystal structure of $Mn^{III}Hb$[3] is similar to that of $Fe^{III}Hb$[4] but shows slight differences. A point of some interest is that the electron density indicates a water molecule bound to the Mn in the α-chains, but not in the β-chains, in contrast to $Fe^{III}Hb$, in which water is firmly bound to all Fe ions. For Fe^{III} (d^5) all of the d orbitals are half-filled, while for Mn^{III} (d^4) the most antibonding d orbital, $d_{x^2-y^2}$, is vacant. Consequently the M–N(pyrrole) bonds are shorter for Mn^{III} than for Fe^{III}, and the Mn atom is closer to being in the heme plane.[5] The axial ligand bonds, on the other hand, are longer for Mn^{III}, partly because its half-filled d_{z^2} orbital is more extended than that of Fe^{III}, the Mn^{III} nuclear charge being lower, and partly because the shorter Mn–N(pyrrole) bonds lead to larger nonbonded repulsions between the pyrrole N atoms and the axial ligand(s). No crystal structure has been reported for $Mn^{II}Hb$, but there may again be subtle differences relative to $Fe^{II}Hb$, since Mn^{II} is larger, and Mn^{II} porphyrins have more expanded cores.[5]

In this study we examine RR spectra of $Mn^{III/II}Mb(Hb)$ and analogous complexes with respect to the frequency of porphyrin skeletal modes, which are known to be markers of the porphyrin core size. We find evidence that Mn^{III} does indeed interact weakly with water, both in the proteins and in aqueous solution. In the case of manganese(II) protoporphyrin, the RR spectra suggest the formation of a 6-coordinate in-plane species with a greatly expanded porphyrin core in the absence of a strong fifth ligand; the proteins may contain a minority population of a species of this character. In the low-frequency region, the Mn^{II}–imidazole stretching band is readily observed, at a frequency similar to that shown by Fe^{II}. It is lower for $Mn^{II}Mb$ (215 cm^{-1}) than $Fe^{II}Mb$ (220 cm^{-1}) suggesting a protein-induced weakening of the Mn–imidazole bond. RR spectra of $Mn^{II}Mb$, $Mn^{III}Mb$, and (N_3)-$Mn^{III}Mb$ have previously been reported by Yu and Tsubaki,[6] who paid special attention to the modes of bound N_3^- and the charge-transfer character of the electronic transitions.

Experimental Section

(Cl)MnPP (PP = protoporphyrin IX) was prepared from free base PP (Mid Century) as described in ref 8, while (Ac)MnPP was obtained from Mid Century Chemicals and used without further purification. Lyophilized myoglobin was obtained from Sigma Chemicals and purified by isoelectrophoresis.[7] Apomyoglobin was prepared by the 2-butanone method[6] and reconstituted within 2–3 days after the apoprotein preparation. Reconstitution was carried out as described by Yonetani and Asakura,[9a] with a slight modification. The Mn heme was dissolved in a mixture of dimethyl sulfoxide (DMSO) and water (the volume of DMSO not exceeding 30% of the total apoprotein–heme solution mixture), and the pH was adjusted to 8.0.[9b] The reconstituted protein was purified[9a] on a CM-52 ion exchange column at pH 7.0 at 4 °C, by elution with 10 mM phosphate buffer, pH 7.0, to wash off excess hemes. Then the $Mn^{III}Mb$ was eluted off the column with 0.5 M phosphate buffer, pH 7.0, and stored in liquid nitrogen. Hemoglobin was purified from packed cells as described by Scholler et al.[10] The apoglobin was prepared from the purified hemoglobin by the acid–acetone technique.[10] The reconstitution and purification of the protein were carried out as described in ref 11. The Mn^{II} forms of both proteins were prepared by adding 1–2 mg of sodium dithionite anaerobically to 150 μL of a 1–2 mM solution of the protein in the appropriate buffer. All protein manipulations were carried out at 0–5 °C, to prevent denaturation, although the spectra were taken at room temperature.

$Mn^{II}PP$ complexes were prepared by adding a 2–3-fold excess of the ligand to $(Ac)Mn^{III}PP$ dissolved in a few drops of 0.1 N NaOH and diluted with distilled water. The solution was flushed with nitrogen for a few minutes and a few milligrams of sodium dithionite was added anaerobically. Formation of the complex was confirmed by a change in the UV–visible absorption spectrum from a split ($\lambda \simeq 465$ nm and 370 nm) to a single Soret band ($\lambda = 428$ nm for $(2\text{-MeImH})Mn^{II}PP$).

Resonance Raman spectra were obtained with excitation at 476.5 nm (Ar^+ laser) for Mn^{III} species and 413.1 nm (Kr^+ laser) for Mn^{II} species; Q-band excitation for both species was carried out at 568.2 nm (Kr^+) or 520.8 nm (Kr^+). The samples were spun in an NMR tube positioned in a back-scattering geometry, and spectra were obtained with a Spex 1401 double monochromator equipped with a cooled photomultiplier and photon-counting electronics. The data were collected digitally with a MINC(DEC) computer. Spectral slit widths were 5 cm^{-1}, and the laser power at the source was between 50 and 100 mW, unless otherwise

(1) Gibson, Q. H.; Hoffman, B. M. *J. Biol. Chem.* **1979**, *254*, 4691.
(2) Parthasarathi, N.; Spiro, T. G. *Inorg. Chem.* **1987**, *26*, 2280.
(3) Moffat, K.; Loe, R. S.; Hoffman, B. M. *J. Mol. Biol.* **1976**, *104*, 669.
(4) Perutz, M. F.; Muirhead, H.; Cox, J. M.; Goaman, L. C. G. *Nature (London)* **1968**, *219*, 131.
(5) Gonzalez, B.; Kouba, J.; Yee, S.; Reed, C. A.; Kirner, J. F.; Scheidt, W. R. *J. Am. Chem. Soc.* **1975**, *97*, 3247.
(6) Yu, N.-T.; Tsubaki, M. *Biochemistry* **1980**, *19*, 4647.
(7) Manrique, A.; Lasky, M. *Electrophoresis* **1981**, *2*, 315.
(8) Fuhrhop, J. H.; Smith, K. M. *Laboratory Methods in Porphyrin and Metalloporphyrin Research*; Elsevier Scientific: Amsterdam, 1975; p 41.
(9) (a) Yonetani, T.; Asakura, T. *J. Biol. Chem.* **1969**, *244*, 4580. (b) Yonetani, T., personal communication.
(10) Scholler, D. M.; Wang, M.-Y. R.; Hoffman, B. M. *Methods Enzymol.* **1978**, *52*, Part C, 487–490.
(11) Rossi Fanelli, A. *Biochem. Biophys. Acta* **1958**, *30*, 608.

* To whom correspondence should be addressed.

0020-1669/87/1326-3792$01.50/0

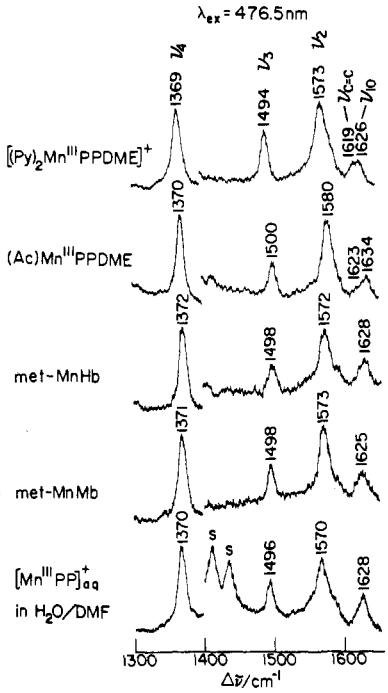

Figure 1. High-frequency Raman spectra excited at 476.5 nm, near resonance with the lower component of the Mn^{III} split Soret band, for $[Mn^{III}PP]^+$ in H_2O/dimethylformamide (1:1) solution, for Mn^{III}-reconstituted Mb and Hb, and for $Mn^{III}PP$ dimethylester in CH_2Cl_2 with acetate or (bis) pyridine ligands.

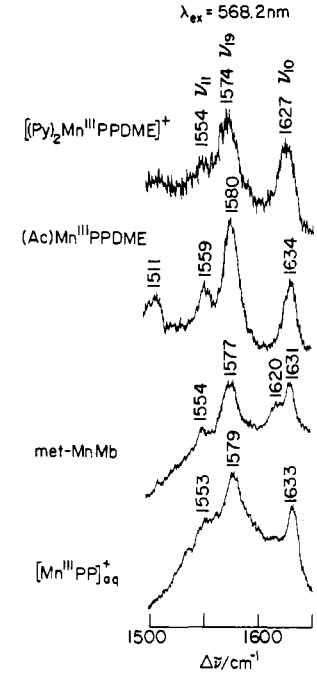

Figure 2. As for Figure 1, but with 568.2-nm excitation near resonance with the Q absorption bands.

mentioned. UV–visible absorption spectra were recorded in 1-mm quartz cells, with a Hewlett-Packard 8450 diode array spectrophotometer.

Results and Discussion

A. Mn^{III} and Mn^{II} Heme Spectra Suggest Weak H_2O Coordination. 1. Manganese(III) 5- and 6-Coordinate Hemes. Figure 1 shows RR spectra in the high-frequency porphyrin skeletal mode region for Mn^{III}Mb and Mn^{III}Hb, as well as manganese(III) protoporphyrin complexes, obtained with 476.5-nm excitation. This wavelength is in resonance with the lower energy component of the split Soret absorption band, characteristic of manganese(III) porphyrins.[12] The RR spectra are dominated by totally symmetric ring breathing modes,[13,14] ν_2, ν_3, and ν_4 at ~1580, ~1500, and ~1370 cm^{-1}. The vinyl mode[14] $\nu_{C=C}$ at ~1625 cm^{-1} can also be seen. There is variable activation of the non totally symmetric (B_{1g}) ring mode ν_{10} at ~1634 cm^{-1}, probably via a Jahn–Teller effect in the resonant excited state.[15] Overlap with the $\nu_{C=C}$ mode produces an apparent variation in the band frequency. Excitation at 568.2 nm (Figure 2), in resonance with the Q absorption bands,[12] which yield selective enhancement of non totally symmetric modes,[16] gives a more accurate value for ν_{10}, and also reveals ν_{11} (B_{1g}) at ~1555 cm^{-1}, and ν_{19} (A_{2g}) at ~1575 cm^{-1}, which is identifiable via its anomalous polarization (data not shown).

All of the skeletal mode frequencies (but not the vinyl mode frequencies) are sensitive to the core size of the porphyrin ring (measured by C_t–N, the porphyrin center to pyrrole N distance), as it adjusts to the steric requirements of the central metal ion and the axial ligand(s).[17,18] The complex (Ac)Mn^{III}PPDME (Ac = acetate, PPDME = protoporphyrin IX dimethyl ester), dissolved in methylene chloride, is 5-coordinate, and its skeletal mode frequencies are known[18] to be in good agreement with the expected frequencies for a core size of 1.99 Å, the measured value for the 5-coordinate (Cl)Mn^{III}TPP.[20] The complex $[(py)_2Mn^{III}PPDME]^+$ (py = pyridine) gives noticeably lower core size marker frequencies, consistent with a slight expansion of the core in this 6-coordinate high-spin complex.[12] A core size of 2.005 Å has been determined for the 6-coordinate (py)(Cl)Mn^{III}TPP.[19] If the expansion relative to (Cl)Mn^{III}TPP (0.015 Å) is used to calculate[18] the expected frequency shifts relative to those of (Ac)Mn^{III}PPDME, excellent agreement is obtained with the observed frequencies for $[(py)_2Mn^{III}PP]^+$, as shown in Table I. Thus, the skeletal mode frequencies for these two complexes are well behaved with respect to the expected core size expansion between 5- and 6-coordinate Mn^{III} hemes.

Table I. Mn^{III} Heme Core Size Marker Frequencies (cm^{-1})

	ν[(Ac)-Mn^{III}PPDME]	ν[$[(py)_2$-$Mn^{III}PP]^+$]	Δ^a	$\Delta_{calcd}{}^b$	$\nu(Mn^{III}Mb)$	Δ^c
ν_{10}	1634	1627	7	8	1631	3
ν_{19}	1580	1574	6	7	1577	3
ν_2	1580	1573	7	5	1573	7
ν_{11}	1559	1554	5	5	1554	5
ν_3	1500	1494	6	6	1498	2
ν_4	1370	1369	1	2	1371	2

[a] Frequency shift ν[(Ac)Mn^{III}PPDME] − $\nu[[(py)_2Mn^{III}PPDME]^+]$. [b] Calculated difference for 5- vs. 6-coordinate Mn^{III} hemes, using[16] $\Delta\nu = -K\Delta d$ with Δd = 0.015 Å (see text) and the following values[18] of K for ν_{10}, ν_{19}, ν_2, ν_{11}, ν_3, and ν_4: 564.9, 452.0, 329.6, 353.1, 423.7, and 136.7. [c] Frequency shift ν[(Ac)Mn^{III}PPDME] − Mn^{III}Mb).

The situation is less clear cut when Mn^{III}Mb is compared with (Ac)Mn^{III}PPDME, as shown in Table I. The modes ν_2 and ν_{11} show downshifts fully as large as those observed for $[(py)_2Mn^{III}PP]^+$, but the shifts for ν_{10}, ν_{19}, and ν_3 are less than half as large. The same results are obtained for Mn^{III}Hb. The crystal structure of the latter protein has been determined,[3] and a water molecule has been located within binding distance of the Mn ion in the α-chains but not in the β-chains, whose Mn heme appears to be 5-coordinate. The RR spectra do not support the existence of a 5-/6-coordinate mixture, at least in solution, since

(12) Boucher, L. J. *J. Am. Chem. Soc.* **1968**, *90*, 6640.
(13) Abe, M.; Kitagawa, T.; Kyogoku, Y. *J. Chem. Phys.* **1978**, *69*, 4516.
(14) Choi, S.; Spiro, T. G.; Langry, K. C.; Smith, K. M. *J. Am. Chem. Soc.* **1982**, *104*, 4337.
(15) Cheung, L. D.; Yu, N.-T.; Felton, R. H. *Chem. Phys. Lett.* **1978**, *55*, 527.
(16) Spiro, T. G. In *Iron Porphyrins, Part II*; Lever, A. B. P., Gray, H. B., Eds.; Addison-Wesley: Reading, MA, 1983; pp 89–160.
(17) Choi, S.; Spiro, T. G.; Langry, K. C.; Smith, K. M.; Budd, L. D.; LaMar, G. N. *J. Am. Chem. Soc.* **1982**, *104*, 4345.
(18) Parthasarathi, N.; Hansen, C.; Yamaguchi, S.; Spiro, T. G. *J. Am. Chem. Soc.* **1987**, *109*, 3865.
(19) Kirner, J. F.; Scheidt, W. R. *Inorg. Chem.* **1975**, *14*, 2081.
(20) Spiro, T. G.; Stong, J. D.; Stein, P. J. *J. Am. Chem. Soc.* **1979**, *101*, 2648.
(21) Terner, J.; Reed, D. *Biochim. Biophys. Acta* **1984**, *789*, 80.

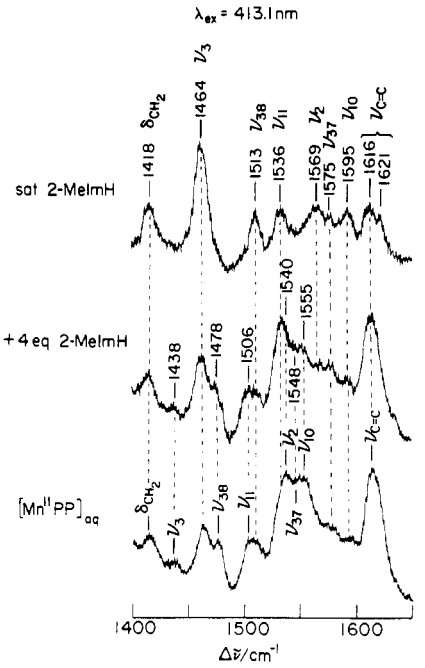

Figure 3. Raman spectra excited at 413.1 nm (Soret band resonance) for $Mn^{II}PP$ in water and in the presence of excess 2-MeImH ligand.

a superposition of 5- and 6-coordinate bands would then be expected. In particular the ν_2 band, which shifts from 1580 to 1573 cm^{-1} between (Ac)Mn^{III}PPDME and $[(py)_2Mn^{III}PPDME]^+$ should show a doublet character. However, the Mn^{III}Hb ν_2 band, at 1572 cm^{-1}, is at least as sharp as those of the model complexes. Nevertheless, it is apparent that the binding of H_2O is significantly weaker for Mn^{III}Hb than for Fe^{III}Hb, for which both hemes show bound H_2O,[4] whose RR frequencies are fully consistent with the expected values for 6-coordinate high-spin Fe^{III}.[22] We infer that the RR frequency differences between Mn^{III}Mb(Hb) and (Ac)-Mn^{III}PPDME on the one hand and $[(py)_2Mn^{III}PPDME]^+$ on the other are attributable to a relatively weak axial interaction with a water molecule in the heme pocket that pulls the Mn ion part way toward the heme plane, producing a slight core expansion. It is curious, however, that the character of the frequency shifts are not uniform for all the core size markers, the ν_2 and ν_{11} shifts being appreciably larger than the others. We have no explanation for this effect.

When dissolved in water, (Ac)Mn^{III}PP gives essentially the same frequencies as does Mn^{III}Mb. Especially notable is the ν_2 lowering relative to (Ac)Mn^{III}PPDME (see Figure 1). We infer that the complex is at least weakly 6-coordinate when dissolved in water. Water is expected to replace the acetate ion in the Mn^{III} coordination sphere, but symmetrical binding by two water molecules appears to be exluded by the frequency differences in ν_{10}, ν_{19}, and ν_{11} relative to $(py)_2Mn^{III}$PP, a symmetrical 6-coordinate complex. Perhaps the Mn ion is displaced slightly from the heme plane in the aquo complex.

2. Aquo-Mn^{II} Heme and Core Expansion. Figure 3 shows RR spectra in the high-frequency skeletal mode region for Mn^{II}PP, produced by dithionite reduction of (Ac)Mn^{III}PP in aqueous solution, to which successively larger amounts of 2-methylimidazole (2-MeImH) are added. The spectra were obtained with 413.1-nm excitation near resonance with the Soret band. Marked spectral changes are seen upon adding 2-MeImH. A limiting spectrum is obtained in saturated 2-MeImH solution, and is attributed to the 5-coordinate complex with 2-MeImH as axial ligand. The absorption spectrum in the presence of a large excess of 2-MeImH is very similar to those of Mn^{II}Mb and Mn^{II}Hb, although the peaks are red-shifted in the proteins (λ_{max} = 438, 560, and 598 nm for Mn^{II}Mb, and 444, 557, and 600 nm for

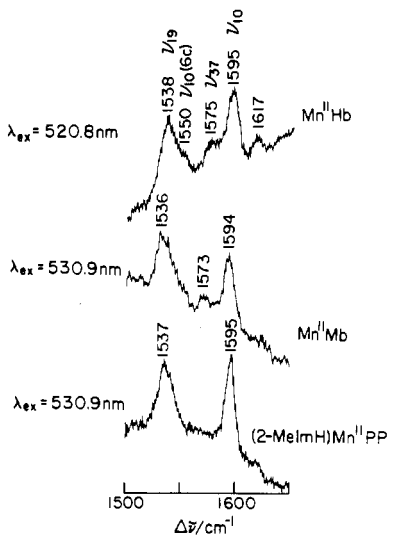

Figure 4. Raman spectra, near Q-band resonance, for Mn^{II}Hb, Mn^{II}Mb, and aqueous (2-MeImH)Mn^{II}PP. The 1550-cm^{-1} band in Mn^{II}Hb is attributed to a minority 6-coordinate (6c) component.

Mn^{II}Hb vs. 428, 556, and 588 nm for (2-MeImH)Mn^{II}PP).

The skeletal mode frequencies of the 2-MeImH complex have been shown[18] to be consistent with the expected values for a core size of 2.065 Å, as observed in the complex (1-MeIm)Mn^{II}TPP,[5] although significant low-frequency deviations are observed for ν_4 and ν_{19} (the latter band seen with Q-band excitation, Figure 4). Electronic explanations have been offered for these deviations.[18] The spectrum of (2-MeImH)Mn^{II}PP (top of Figure 3) appears more complex than those seen for the Mn^{III} hemes, because several skeletal modes are comparably activated. For example ν_2 at 1569 cm^{-1} is anomalously weak and has intensity similar to that of the non totally symmetric modes ν_{10} and ν_{11}. In addition, the E_u-derived modes, ν_{38} and ν_{37}, which are activated by virtue of the asymmetric displacement of the peripheral vinyl substituents in protoheme,[14] are seen with comparable intensity (1513 and 1575 cm^{-1}); usually these bands are much weaker than ν_2 and appear only as shoulders. The vinyl $\delta_{=CH_2}$ (1418 cm^{-1}) and $\nu_{C=C}$ modes are likewise comparably activated, and two $\nu_{C=C}$ modes are observed, at 1616 and 1621 cm^{-1}. The existence of two $\nu_{C=C}$ modes, corresponding to in-phase and out-of-phase stretching of the two vinyl C=C bonds, was detected in NiPP by the noncoincidence of the infrared and Raman bands attributable to these modes via vinyl deuterium isotope shifts.[14] Usually only one mode appears with significant intensity in the RR spectrum, but occasionally two $\nu_{C=C}$ bands are seen. A particularly clear example has been reported by Terner and co-workers in RR spectra of horseradish peroxidase.[21]

The crystal structure of 4-coordinate Mn^{II}TPP (toluene solvate) has been determined,[22] and the core size is 2.082, 0.017 Å *larger* than in the 5-coordinate (1-MeIm)Mn^{II}TPP. This expansion is in contrast to the slight *contraction* seen when 4-coordinate Zn^{II}TPP (2.036 Å)[23] is compared with 5-coordinate zinc porphyrin complexes (2.045 Å on average).[23] This contraction is attributable to the elimination of nonbonded interactions between the axial ligand and the pyrrole N atoms. The contrasting expansion for 4-coordinate Mn^{II}TPP reflects the large size of the high-spin Mn^{II} ion. When the bond to a fifth ligand is absent, the Mn^{II} ion sinks into the heme plane (but a large amplitude motion relative to the plane is seen in the X-ray data[22]) in order to maximize the bonding interactions with the pyrrole atoms, but this is at the expense of strain in the porphyrin ring, which is forced to expand. We note that the situation is quite different for 4-coordinate Fe^{II} porphyrins, which are intermediate spin, with a contracted core[24] and upshifted core size marker frequencies.[25]

(22) Kirner, J. F.; Reed, C. A.; Scheidt, W. R. *J. Am. Chem. Soc.* **1977**, *99*, 1093.
(23) Scheidt, W. R.; Kastner, M. E.; Hatano, K. *Inorg. Chem.* **1978**, *17*, 706.
(24) Collman, J. P.; Hoard, J. L.; Lang, G. J.; Radonovich, L. J.; Reed, C. A. *J. Am. Chem. Soc.* **1975**, *97*, 2676.

Table II. Assigned Core Size Frequencies for (2-MeImH)Mn^{II}PP and Aqueous Mn^{II}PP

	ν[(2-MeImH)Mn^{II}PP]	$\nu(Mn^{II}PP)$	Δ^a	$\Delta_{calcd}{}^b$
ν_{10}	1595	1555	40	40
ν_{37}	1575	1548	27	26
ν_2	1569	1540	29	23
ν_{11}	1536	1506	30	25
ν_{38}	1513	1478	35	32
ν_3	1464	1438	26	30

[a] Frequency difference. [b] Frequency difference calculated for a core size expansion of 0.070 Å from the K values[18] given in Table I.

If the RR spectral changes in Figure 3 were attributable to a transition from a 5-coordinate 2-MeImH complex (top) to a 4-coordinate complex (bottom) in the absence of added ligand, then 5–10-cm^{-1} downshifts in the core size marker frequencies would be expected on the basis of a 0.017-Å core expansion. However, the spectrum in the absence of added 2-MeImH can only be assigned (see Figure 3 and Table II) in a consistent fashion if much larger core size marker downshifts are assumed. Particularly striking are the bands at 1478 and 1506 cm^{-1}, for which no other skeletal mode assignments appear possible than ν_{38} and ν_{11}. These assignments, however, imply 32- and 25-cm^{-1} downshifts relative to the 2-MeImH adduct spectrum. In Table II the downshifts are shown to be calculated remarkably well, if a 0.07-Å core size expansion is assumed. Adding this to the 2.065-Å core size appropriate for the 5-coordinate adduct would produce a 2.135-Å core size, which is unprecedentedly large.[5] The calculation must be viewed as rather uncertain, in view of the fact that this core size is well beyond the range for which the RR frequency correlations have been established via known structures, and there is some indication of deviant values at the high end of this range.[18] Nevertheless, it seems apparent that there is a substantial core expansion, which is significantly larger than the 0.017-Å expansion expected for a 4-coordinate Mn^{II} species.

A possible explanation is that for aqueous Mn^{II}PP there is a symmetric interaction with two axial water molecules, which keeps the Mn^{II} anchored in the center of the ring. These water molecules would limit the Mn out-of-plane excursions (which are reflected in the high X-ray thermal parameter of the 4-coordinate Mn^{II}TPP[22]), thereby maximizing the tendency to expand the porphyrin ring; this tendency would also be assisted by the nonbonded interactions with the axial water molecules. In this view, the RR spectrum of aquo Mn^{II}PP should be seen as arising from a 6-coordinate high-spin species, although the bonds to the axial water molecules are expected to be quite weak. The core size expansion is analogous to, although much larger than, that observed between the 5-coordinate (2-MeImH)Fe^{II}TPP (C_t–N = 2.044 Å[30]) and the 6-coordinate high-spin $(THF)_2Fe^{II}$TPP (C_t–N = 2.057 Å[35]). There also appears to be a minority 5-coordinate aquo species since weak bands can be seen at the positions of the (2-MeImH)Mn^{II}PP RR features in the spectrum with no ligand added (see especially ν_3 in Figure 3).

3. Mn^{II}Mb (-Hb). Figures 4 and 5 compare RR spectra with Q and Soret band excitation for Mn^{II}Mb and Mn^{II}Hb with (2-MeImH)Mn^{II}PP. Very similar band frequencies and intensities are seen, confirming the expectation that Mn^{II} is 5-coordinate in the proteins, by virtue of binding to the proximal histidine. The protein spectra, however, contain extra bands at 1506 and 1556 cm^{-1}. These are the frequencies for prominent bands in the aquo–Mn^{II}PP spectrum, assigned to ν_{11} and ν_{10}, respectively; they are definitely not attributable to any 5-coordinate Mn^{II} heme band. We infer that these bands arise from a population of heme groups whose core is expanded by virtue of the Mn being in the plane. This could happen if the Mn–histidine bonds were weakened and/or there were distal ligand (or water) binding for this population of hemes. It cannot be excluded that these heme groups are actually nonspecifically bound to the exterior of the protein although precautions were taken to remove exogeneous heme in the purification step via ion exchange chromatography (see Experimental Section).

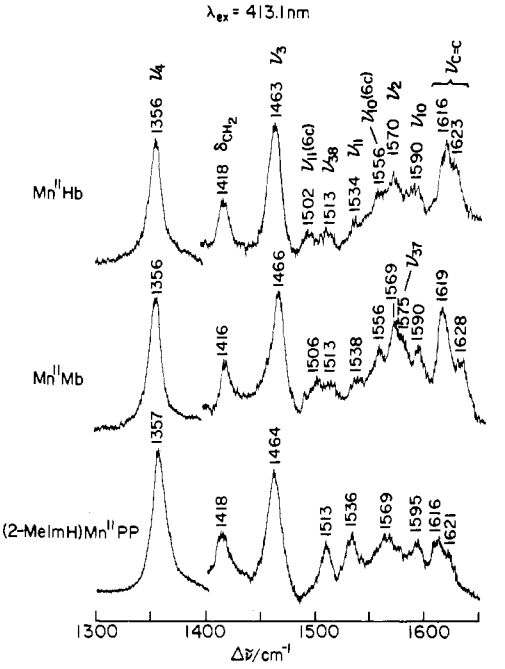

Figure 5. As for Figure 4, but with 413.1-nm excitation, in resonance with the Soret band. Bands labeled 6c are attributed to a minority 6-coordinate component.

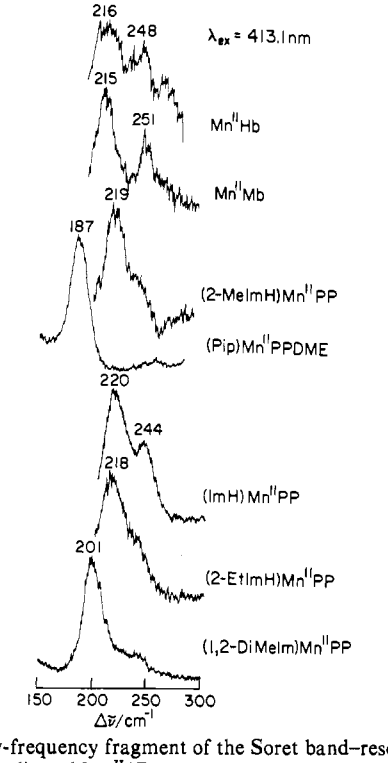

Figure 6. Low-frequency fragment of the Soret band–resonant Raman spectra of the indicated Mn^{II}PP species, showing the variable Mn^{II}–axial ligand stretching band.

B. The Mn^{II}–Imidazole Bond Is Similar to the Fe^{II}–Imidazole Bond in Strength. 1. Mn^{II} Heme. Figure 6 shows the 150–300-cm^{-1} region of the RR spectra of Mn^{II}Hb and Mn^{II}Mb and several Mn^{II} heme complexes. The prominent band near 220 cm^{-1} is entirely analogous to the band near this frequency seen for Fe^{II}Mb[26] and Fe^{II}Hb[27] and for model complexes.[27-29] It has been

(25) Spiro, T. G.; Burke, J. M. *J. Am. Chem. Soc.* **1976**, *98*, 5482.

(26) Kitagawa, T.; Nagai, K.; Tsubaki, M. *FEBS Lett.* **1979**, *104*, 376.
(27) Kincaid, J.; Stein, P.; Spiro, T. G. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 549, 4156.

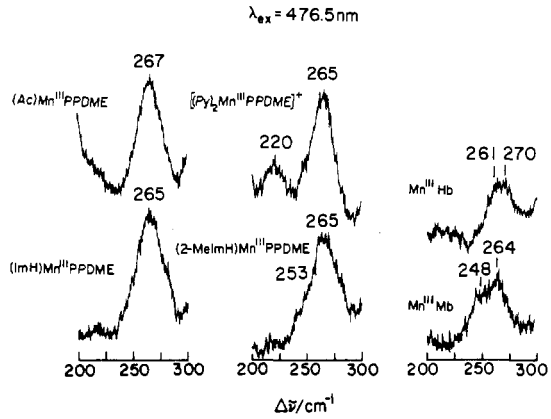

Figure 7. Low-frequency fragment of the Soret band–resonant Raman spectra of the indicated $Mn^{III}PP$ species, showing the insensitivity of the ~265-cm^{-1} band to the nature of the axial ligands.

assigned to Fe^{II}–imidazole stretching vibration via isotope shifts seen on perdeuteriation of 2-MeImH ligand[27,29] and ^{54}Fe substitution.[29] Yu and Tsubaki[6] suggested a similar assignment for the 215-cm^{-1} $Mn^{II}Mb$ band. As with Fe^{II} heme complexes,[27-29] the Mn–ligand stretching frequency is seen to depend on the nature of the fifth ligand (Figure 6, lower spectra). ImH, 2-MeImH, and 2-EtImH give nearly the same frequency, ~219 cm^{-1}, but 1,2-Me_2Im shows a large downshift to 201 cm^{-1}. This is again analogous to the Fe^{II} heme behavior, for which 1,2-Me_2Im gives 195 cm^{-1}.[28] The frequency for (2-MeImH)Fe^{II}PP is variable, depending on the H-bonding state of the N3 proton;[28,29] it is at 207 cm^{-1} in benzene but 220 cm^{-1} in water, a good H-bond acceptor. Deprotonation of the 2-MeImH ligand in dimethylformamide was found to increase the frequency to 239 cm^{-1}.[28] Hydrogen bonding to water can likewise account for the elevated frequencies for the ImH, 2-MeImH, and 2-EtImH ligands, relative to 1,2-Me_2Im in which the opportunity for such bonding is absent due to the methylation of the N3 atom. The frequency is even lower, 187 cm^{-1}, for the aliphatic cyclic amine ligand piperidine.

These data establish that the Mn–imidazole stretching band can be observed for Mn-substituted heme proteins, and is in the same frequency region as observed for Fe^{II} heme proteins. The frequency match for the 2-MeImH adducts of Fe^{II} and Mn^{II} heme indicates that the M–imidazole bond strength is very similar for the two metal ions. We note that the axial ligand bond lengths are nearly the same for (1-MeIm)Mn^{II}TPP[22] and (2-MeImH)-Fe^{II}TPP,[30] 2.192 vs. 2.161 Å.

Likewise in Mn^{II}Hb the M–imidazole stretching band (Figure 6) shows the same characteristics as in Fe^{II}Hb, namely a large width with a central frequency of ~215 cm^{-1}. For Fe^{II}Hb this large width has been demonstrated via valency hybrid[31] and Co and Fe hybrid[32] studies to result from chain heterogeneity, the β-chain and α-chain frequencies being ~220 and ~207 cm^{-1} in the normal T state of deoxyHb. There may well be similar chain heterogeneity in Mn^{II}Hb.

Surprisingly, the M–imidazole frequency in Mn^{II}Mb, 215 cm^{-1}, is lower than that in Fe^{II}Mb, 220 cm^{-1}.[26] Thus there appears to be a protein-induced weakening of the Mn–imidazole bond. A more marked instance of the same effect in myoglobin has recently been reported for Zn-substituted protein;[33] the Zn–imidazole frequency is 146 cm^{-1}, much lower than that in (2-MeImH)-ZnPPDME, 177 cm^{-1} (in methylene chloride). It was suggested[33] that the proximal imidazole group in myoglobin is not optimally oriented for binding to Zn, in contrast to Fe. The same argument may hold for the Mn-reconstituted protein. While the intrinsic M–imidazole bond strength may be quite similar for Fe^{II} and Mn^{II}, the latter ion is significantly larger and may not bind quite as well to the proximal imidazole due to geometric constraints in the myoglobin heme pocket.

2. Mn^{III} Heme. Yu and Tsubaki[6] suggested that a 267-cm^{-1} band of Mn^{III}Mb be assigned to Mn–imidazole stretching, corresponding to the 215-cm^{-1} band of Mn^{II}Mb. Figure 7 shows the relevant portion of the RR spectra, excited at 476.5 nm, for Mn^{III}Mb, Mn^{III}Hb, and several Mn^{III} heme complexes. All of these show a band very close to 267 cm^{-1}, despite the fact that (Ac)Mn^{III}PPDME and $[(py)_2Mn^{III}PPDME]^+$ do not contain an imidazole ligand. The Mn–Ac stretch should be at a distinctly different frequency from the Mn–imidazole stretch, and in the case of $[(py)_2Mn^{III}PPDME]^+$, the symmetric M–$(py)_2$ stretch is expected below 200 cm^{-1}; it is found at 185 cm^{-1} for $(py)_2Fe^{II}$–mesoporphyrin,[34] the low frequency being attributable to the large effective mass of the symmetrically disposed ligands. The bond length is expected to be greater, and the force constant lower, for high-spin Mn^{III} than for low-spin Fe^{II}; a frequency lower than 185 cm^{-1} is therefore expected.

Thus the Mn^{III}PPDME complexes show that there is a ~265 cm^{-1} RR band, probably the porphyrin mode ν_9,[13,14] which is not assignable to Mn^{III}–imidazole stretching. We cannot rule out that Mn–imidazole stretching contributes to the RR bands of the Mn^{III}-substituted proteins, but the presence of an interfering porphyrin band will have to be taken into account in efforts to pin down this assignment.

Acknowledgment. This work was supported by NIH Grant GM33576. We are grateful to Professor T. Yonetani for helpful advice on protein reconstitution.

Registry No. $[(py)_2Mn^{III}PPDME]^+$, 110433-17-5; (Ac)-Mn^{III}PPDME, 110433-23-3; $[Mn^{II}PP]_{aq}$, 110433-18-6; (2-MeImH)-Mn^{II}PP, 108149-39-9; (pip)Mn^{II}PPDME, 110433-19-7; (ImH)Mn^{II}PP, 110433-20-0; (2-EtImH)Mn^{II}PP, 110433-21-1; (1,2-DiMeIm)Mn^{II}PP, 110433-25-5; (ImH)Mn^{III}PPDME, 110433-22-2; (2-MeImH)-Mn^{III}PPDME, 110433-24-4.

(28) Stein, P.; Mitchell, M.; Spiro, T. G. *J. Am. Chem. Soc.* **1980**, *102*, 7795.
(29) Hori, H.; Kitagawa, T. *J. Am. Chem. Soc.* **1980**, *102*, 3608.
(30) Hoard, J. L.; Scheidt, W. R. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 3919; **1974** *71*, 1578.
(31) Nagai, K.; Kitagawa, T. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2033.
(32) Ondrias, M. R.; Rousseau, D. L.; Kitagawa, T.; Ikeda-Saito, M.; Inubushi, T.; Yonetani, T. *J. Biol. Chem.* **1982**, *257*, 8766.
(33) Feitelson, J.; Spiro, T. G. *Inorg. Chem.* **1986**, *25*, 861.
(34) Wright, P. G.; Stein, P.; Burke, J. M.; Spiro, T. G. *J. Am. Chem. Soc.* **1979**, *101*, 3531.
(35) Reed, C. A.; Mashiko, T.; Scheidt, W. R.; Spartalian, K.; Lang, G. *J. Am. Chem. Soc.* **1980**, *102*, 2302.