Influence of Heme-Surrounding Amino Acid Residues on the Manganese(V)–Nitrido Bond in Manganese-Substituted Hemoproteins: Resonance Raman Evidence for Porphyrin Core Expansion and Reduction of the Manganese(V)–Nitrido Stretching Force Constant†

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ABSTRACT: Nitridomanganese(V) protoporphyrin IX was prepared by hypochlorite oxidation of the corresponding manganese(III) protoporphyrin IX derivative in the presence of ammonium ion and by photolysis of the corresponding azidomanganese(III) complex. Myoglobin and horseradish peroxidase containing this novel protoporphyrin derivative were prepared for the first time. These remarkably stable species were examined by electronic absorption, electron paramagnetic resonance, and resonance Raman spectroscopies. The Mn^V–N stretching modes of the nitridomanganese(V)-substituted myoglobin and horseradish peroxidase were observed at 1010 and 1003 cm^{-1}, respectively, by resonance Raman spectroscopy, while the Mn^V–N stretching frequency for nitridomanganese(V) protoporphyrin IX in 0.1 N aqueous NaOH was found at 1046 cm^{-1}. The equilibrium dissociation energies of Mn^V–N bonds in these complexes were estimated from vibrational overtone spacings by introducing the Morse potential energy function, were found to be around 4.5 eV, and seemed independent of the surroundings of the manganese porphyrin, although its force constant decreased from 7.3 to 6.7 mdyn/Å upon incorporation into apoprotein. The porphyrin ring modes of these nitridomanganese(V) derivatives were influenced greatly upon incorporation into apoproteins, suggestive of the occurrence of porphyrin core expansion. Upon this core expansion the Mn^V center moves into the mean plane of porphyrin plane, but the access of nitrido (N) toward Mn^V is restricted due to a steric hindrance from porphyrin pyrrole nitrogens. The resulting stretched Mn^V–N bond might cause lowering of the Mn^V–N stretching frequency upon incorporation into apoprotein.

Recently, surprisingly stable complexes containing a manganese–nitrogen triple bond, Mn^VN(OEP)[1] and Mn^VN(TPP), were proposed, which were prepared by hypochlorite or iodosylbenzene oxidation of the corresponding manganese(III) porphyrins in the presence of ammonia (Hill & Hollander, 1982; Buchler et al., 1982, 1983a). Meanwhile, mononuclear pentacoordinated nitridochromium(V) porphyrins, Cr^VN-(OEP) and Cr^VN(TPP), were also prepared by hypochlorite oxidation of the corresponding chromium(III) porphyrins in the presence of ammonia, and these stable species were characterized by UV–visible, IR, EPR, ENDOR, and mass spectra (Buchler et al., 1983b). The identical complex was also obtained by photolysis of the corresponding azidochromium(III) porphyrin complex and structually characterized (Groves et al., 1983). These nitrido–metal complexes showed very short metal (M)–nitrogen bond lengths, suggesting M–N triple bonds. Neither nitridomanganese(V)–protoporphyrin IX, nitridochromium(V)–protoporphyrin IX, nor hemoproteins containing these highly oxidized nitrido–metal porphyrins have been prepared. We reported previously that, during the course of resonance Raman examination of the azide complex of Mn^{III}Mb, new additional Raman lines appeared in addition to bound azide modes (Yu & Tsubaki, 1980). These new Raman lines might be suggestive of the occurrence of photo-induced decomposition of bound azide to generate the nitridomanganese(V) derivative. Therefore, we have examined to prepare these nitrido–metal complexes of Mn–protoporphyrin IXs and Mn-substituted hemoproteins by using the same methods applied to nitrido–metal model systems.

Resonance Raman spectroscopy has been applied to various hemoproteins and was found to be sensitive to oxidation state, spin state, and anomalous structure of heme prosthetic group [see a review such as Spiro (1982)]. The most important interaction (or linkage) between heme prosthetic group and surrounding amino acid residues in hemoproteins is believed to be a bond between heme iron and its fifth ligand. Particularly, for the dioxygen-binding hemoproteins such as hemoglobins, myoglobins, peroxidases, and cytochromes P-450, this bond is the only covalent linkage between heme prosthetic group and apoprotein. Thus it is reasonable to assume that protein tertiary or quaternary structure regulates the biological

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[1] Abbreviations: OEP, octaethylporphyrin; $OEPMe_2$, 5,15-dimethyloctaethylporphyrin; TPP, tetraphenylporphyrin; TTP, tetra(*p*-tolyl)porphyrin; PPIXDME, protoporphyrin IX dimethyl ester; PPIX, protoporphyrin IX; DMF, *N*,*N*-dimethylformamide; Mb, myoglobin; HRP, horseradish peroxidase; MnMb, manganese protoporphyrin IX containing myoglobin; MnHRP, manganese protoporphyrin IX containing horseradish peroxidase; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance.

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function of hemoproteins by this linkage (Nagai et al., 1980; Nagai & Kitagawa, 1980; de Ropp et al., 1985). Most of the protein-induced shifts in high-frequency porphyrin resonance Raman bands have been interpreted by the changes in axial ligation through steric and/or electronic effects (Spiro, 1982). Another type of interaction, hydrogen bonding, between heme-bound exogenous ligand and distal amino acid residues has begun to draw our attention (Ikeda-Saito et al., 1977; Phillips & Schoenborn, 1981), and these interactions are considered to play an important role in the regulation of biological functions of hemoproteins (Sitter et al., 1985; Hashimoto et al., 1986). There are many other types of interactions, in addition, between the heme prosthetic group and surrounding amino acid residues as revealed by proton NMR and X-ray crystallographic studies for various hemoproteins (La Mar et al., 1983; Takano, 1977; Poulous et al., 1985). These amino acid residues were considered merely to provide the hydrophobic cavity to accommodate the heme prosthetic group since these residues are usually hydrophobic and make van der Waals type contacts with the heme. In this report, by utilizing manganese-substituted hemoproteins, we observed direct evidence of a new type of interaction between heme prosthetic group and surrounding amino acid residues other than proximal or distal amino residues, which may regulate the bonding strength of exogenuos ligand to the heme metal center.

Materials and Methods

Manganese(III) protoporphyrin IX was prepared according to the method of Yonetani and Asakura (1969) and was purified on a column of silica gel (Merck 7734, 70–230 mesh) developed with methanol. The nitridomanganese(V) porphyrins prepared by hypochlorite oxidation of the corresponding methoxomanganese(III) porphyrins in the presence of ammonia were first reported by Buchler et al. (1982, 1983a). Similar but somewhat modified methods were applied to prepare $Mn^{V}N$(PPIX). In the presence of aqueous ammonia, Mn^{III}(PPIX) reacted with hypochlorite to form a crimson species as illustrated in Figure 1A. This new complex (**1A**) was surprisingly stable as reported previously (Buchler et al., 1982). Therefore, the complex (**1A**) was isolated by column chromatography on silica gel with methanol. The same compound could be obtained from the reaction of Mn^{III}(PPIX) in 0.1 M NaOH with hypochlorite in the presence of ammonia.

Groves et al. (1983) suggested that a nitridomanganese(V) porphyrin complex might be prepared by photolysis of the corresponding azidomanganese(III) derivative. Manganese(III) protoporphyrin IX in DMF reacted with sodium azide to form an azide complex as shown in Figure 1B. Photolysis of the azide complex in DMF with a 50-W xenon lamp for 2 h, in a water-cooled vessel at 15 °C with vigorous agitation with a magnetic stirrer, caused the smooth conversion of the yellowish brown complex to a crimson solution. This complex (**2A**) was quite stable and was purified by column chromatography on silica gel with DMF. Methanol solution containing **2A** gave an identical electronic absorption spectrum with that of **1A** in methanol.

Lyophilized preparations of sperm whale metmyoglobin and horseradish peroxidase were purchased from Calbiochem and Toyobo Co., Ltd. (I-C, R_z = 3.41), respectively, and used without further purification. Apoproteins were prepared in ferric form according to a modification of Teale's acid–butanone method (Teale, 1959), as described in detail elsewhere (Yonetani, 1967). The incorporation of Mn^{III}(PPIX) into these apoproteins was carried out by the method of Yonetani and Asakura (1969). Manganese-substituted myoglobin was

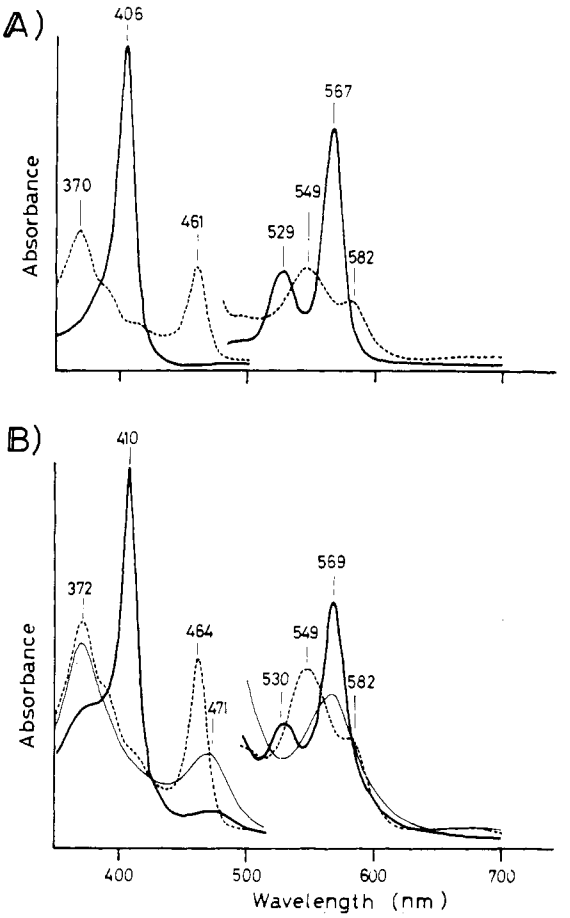

FIGURE 1: (A) Electronic absorption spectra of Mn^{III}(PPIX) (~0.01 mM) in methanol (3 mL) (broken line) and of $Mn^{V}N$(PPIX) (**1A**) in methanol, obtained 4 h after starting the reaction by adding 3–5 μL of aqueous sodium hypochlorite (Wako Pure Chemicals) in the presence of 0.1 mL of 28% aqueous ammonia (bold line). (b) Electronic absorption spectra of Mn^{III}(PPIX) in DMF in the presence of sodium azide (10 mg) (broken line) and without sodium azide (solid line) and of $Mn^{V}N$(PPIX) (**2A**) in DMF (bold line), obtained 2 h after starting the irradiation of the solution containing azide complex with a 50-W xenon lamp at 15 °C. All spectra were recorded at 15 °C.

crystallized from 75% saturated ammonium sulfate–phosphate solution at pH 6.0 (Hori et al., 1984).

Measurements of electronic absorption spectra were carried out with a Shimadzu Model UV-240 spectrophotometer at 15 °C. EPR measurements were carried out at X-band (9.35-GHz) microwave frequency by using a Varian X-band cavity with a home-built EPR spectrometer with 100-kHz field modulation. An immersion Deware flask was used for measurements at 77 K. A home-built two-circle Teflon goniometer was used for single-crystal measurements to rotate sample crystals in 5° or 10° steps. Resonance Raman spectra were obtained by using a Jasco Model R-800D Rman spectrophotometer with exciting wavelength at 441.6 nm from a He–Cd laser (Kimmon) or by using a highly sensitive multichannel laser Raman system (Yu & Srivastava, 1980) with exciting wavelength at 406.7 nm from a Kr ion laser (Spectra Physics, Model 171-01). All the wavenumbers reported herein were accurate within ±1 cm^{-1}. The temperature of the sample was kept at 25 °C, and local heating was avoided by rotating the cell.

The photolysis experiments were performed with a 50-W xenon lamp, a 100-W mercury lamp (JES-UV-1, Japan Electron Optics Co., Ltd.), and a flashlight for photography without filters. The temperature of the sample was kept at 15 °C in a water-cooled vessel, and the sample was agitated

vigorously with a magnetic stirrer during the photolysis.

Triply labeled $Na^{15}N_3$ (99 atom %) and terminally labeled $Na^{15}N^{14}N^{14}N$ (99 atom %) were purchased from Stohler Isotope Chemicals and Icon Services, Inc. (Summit, NJ), respectively. $^{15}NH_4NO_3$ (96.5 atom %) was obtained from Shoko Tsusho Co. (Tokyo). *N*-Methylimidazole (Sigma) was distilled from KOH. Other chemicals used were purchased as reagent grade and used without further purification.

RESULTS

Reaction of MnMb with Hypochlorite. Mn^{III}(PPIX)-substituted myoglobin, MnMb, reacted with hypochlorite solution in the presence of ammonia (pH 10.0) to form a new red complex, which was characterized by a split band (peaks located at 583 and 574 nm), a band at 553 (sh) and 544 nm, and an intense Soret band (430 nm) as shown in Figure 2A. This new complex of MnMb (**3A**) was surprisingly stable and was not converted back to the original Mn^{III} derivative when left standing on ice for several days, so purification was readily achieved by the same procedure used for native myoglobin. The identical product was obtained by addition of hypochlorite in the presence of ammonium salts such as ammonium sulfate, ammonium carbonate, and ammonium acetate at alkaline pH (~10).

Photolysis of Azide Complex of MnMb. Mn^{III}Mb reacted with excess sodium azide to form an azide complex as reported previously (Yu & Tsubaki, 1980). Photolysis of the azide complex with 1000 times of flashing of a flashlight or with a UV lamp for 1 h at 15 °C resulted in spectroscopic changes indicative of formation of the same product as that formed by hypochlorite oxidation in the presence of ammonia, as illustrated in Figure 2B.

Reversible Reconstitution of the New Complex of MnMb. The manganese porphyrin was easily removed from the new complex of MnMb by the same procedure of Teale (1959) by using 2-butanone. The extracted manganese porphyrin derivative was then brought to dryness on a rotary evaporator. The solid material was redissolved in methanol or DMF. These solutions gave indistinguishable electronic absorption spectra from those of **1A** and **2A**. Furthermore, the incorporation of **1A** or **2A** into apo-Mb and the purification could be carried out by the same procedure for the reconstitution of Mn^{III}Mb (Yonetani & Asakura, 1969). The reconstituted MnMb containing **1A** or **2A** gave the same electronic absorption spectrum as that of **3A**.

Mn^{III}(PPIX)-substituted HRP, on the other hand, did not react with hypochlorite in the presence of ammonia. Without ammonia, Mn^{III}HRP reacted with hypochlorite to form a spectrally distinct Mn^{IV}HRP complex, confirming the previous report of Yonetani and Asakura (1969) as shown in Figure 2C. Addition of sodium azide had no appreciable effect on the absorption spectrum of MnHRP at any pH as reported previously (Yonetani & Asakura, 1969). Any new complex could be scarcely formed upon irradiation with UV light. Therefore, we examined recombination of **1A** or **2A** with apoperoxidase by the same method as for the Mn^{III}HRP (Yonetani & Asakura, 1969). The light absorption spectrum of this reconstituted peroxidase (**4A**) is shown in Figure 2C. However, the Soret band with shoulder indicated that this complex consisted of at least two components. These components could never be isolated by DEAE-cellulose column chromatography.

Electron Paramagnetic Resonance Spectra. We examined EPR spectra for the complexes **1A** and **3A** at 77K. No appreciable EPR absorption was detected in a wide range of magnetic field strength up to 0.8 T. Then, we prepared a single crystal of **3A**.[2] Magnetic field was applied to all orientations of the single crystal of **1C** in 5° or 10° steps. No conspicuous EPR absorption except a residual Mn^{III} center (Yonetani & Asakura, 1969; Hori et al., 1987) was observed when the magnetic field was applied to the *z* axis (porphyrin normal).

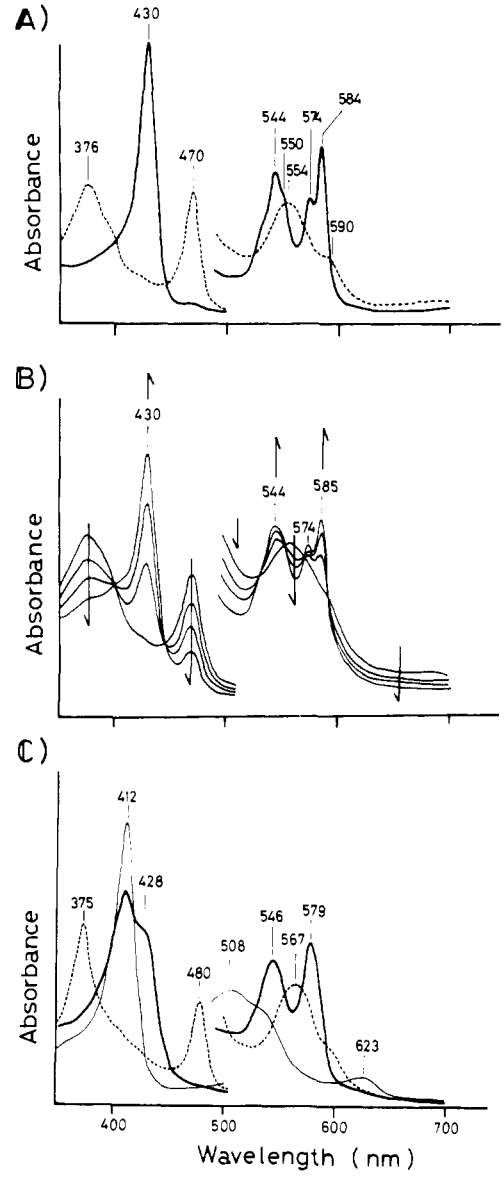

FIGURE 2: (A) Electronic absorption spectra of Mn^{III}Mb in the presence of 30 μL of 28% aqueous ammonia (broken line) and of the new complex of MnMb (**3A**) (bold line), obtained a few hours after staring the reaction by adding 10 μL of sodium hypochlorite solution. Upon addition of aqueous ammonia, the pH value of the buffered solution increased to pH ~10. (B) Electronic absorption spectra of the azide complex of MnMb and of the photolyzed product obtained after 0, 200, 500, and 1000 times of flashing of a flashlight at 15 °C. Upward arrows indicate the formation of the photolyzed product, and downward arrows indicate the concomitant decrease of the azide complex of Mn^{III}Mb. All spectra were recorded as ca. 0.01 mM MnMb solution in 0.1 M sodium phosphate buffer (pH 7.0) at 15 °C. (C) Electronic absorption spectra of Mn^{III}HRP (dotted line), the oxidized product with hypochlorite (solid line), and the reconstituted MnHRP (**4A**) with **1A** or **2A** (bold line).

[2] To a mother liquor (~3 mL) containing Mn^{III}Mb crystals was added 20 μL of aqueous NH_3 (28% solution) and 100 μL of aqueous NaOCl. The reaction mixture was kept standing overnight at room temperature under aerobic condition. The crystals of **1C** which were prepared by this treatment and the crystals of **3A** appeared to be isomorphous. A single crystal of **1C** was mounted on the sample holder and then immersed into liquid nitrogen.

In order to confirm the amount of the residual $Mn^{III}Mb$ in the crystal, the single crystal of **1C** was dissolved in 0.1 M sodium phosphate buffer (pH 7.0) after EPR measurements. The electronic absorption spectrum indicated that remaining $Mn^{III}Mb$ was about 5%.

Resonance Raman Spectra. We reported 6 years ago that, during resonance Raman examination of the azide ($^{14}N_3$) complex of $Mn^{III}Mb$, two new Raman bands appeared at 1010 and 2006 cm^{-1} in addition to bound azide modes (650 cm^{-1} for bending and 2039 cm^{-1} for antisymmetric stretching) with excitation wavelength at 413.1 and 406.7 nm (Yu & Tsubaki, 1980). Further, these two characteristic Raman bands showed shifts to 983 and 1953 cm^{-1} upon $^{15}N_3$ isotope substitution (Yu & Tsubaki, 1980). These observations were reconfirmed in the present study, and the results are shown in Figure 3. We used the 441.6-nm line for the photolysis instead of the 406.7- or 413.1-nm lines in the present study. Upon formation of the azide– ($^{14}N_3$–) $Mn^{III}Mb$ complex, the antisymmetric stretching frequency at 2039 cm^{-1} could be detected (Figure 3A,B). When laser irradiation was used for a longer period, the two characteristic Raman bands appeared simultaneously at 1010 and 2006 cm^{-1} (Figure 3C). When we used $^{15}N_3$ instead of $^{14}N_3$, these two characteristic bands shifted to 983 and 1953 cm^{-1}, respectively (Figure 3D; the 1969-cm^{-1} band was the antisymmetric stretching frequency of bound $^{15}N_3$). In Figure 3E, the resonance Raman spectrum of $Mn^{V}(^{14}N)$(Mb) (**3A**) is presented for comparison. When the hypochlorite oxidation was performed in the presence of $^{15}NH_4NO_3$ (60 mM at pH 10.0), the resultant complex showed identical Raman frequencies with those of the photolyzed product of $^{15}N_3$–$Mn^{III}Mb$ at 983 and 1953 cm^{-1} (Figure 3F). The resonance Raman spectrum of myoglobin reconstituted with $Mn^{V}N$-(PPIX) (**1A** or **2A**) also exhibited these two characteristic bands at 1010 and 2006 cm^{-1} (spectrum not shown). Thus, all three MnMb derivatives prepared by hypochlorite oxidation in the presence of ammonium ion, by photolysis of azide complex, and by reconstitution of apomyoglobin with $Mn^{V}N$-(PPIX) gave an identical species containing a Mn^{V}–N bond.

It is quite evident that the 1010-cm^{-1} band could be assignable to Mn^{V}–^{14}N stretching frequency on the basis of the frequency shift upon isotopic substitution. It is also very likely that 2006-cm^{-1} Raman band is the first overtone ($v = 0 \rightarrow v = 2$) of the fundamental Mn^{V}–^{14}N stretching mode ($v = 0 \rightarrow v = 1$) on the basis of the frequency shift (2006 to 1953 cm^{-1}, 53-cm^{-1} downshift), very close to the twice the frequency shift of the fundamental mode ($27 \times 2 = 54$ cm^{-1}). We failed to observe the Raman band corresponding to the second overtone ($v = 0 \rightarrow v = 3$) mode around 3000-cm^{-1} region.

The resonance Raman spectrum of the photolyzed complex of $^{14}N_3$–manganese(III) protoporphyrin IX in DMF (**2A**) employing the 406.7-nm line from a He–Cd laser gave a strong and sharp Raman band at 1049 cm^{-1} as shown in Figure 4. The resonance Raman spectra of **1A** in alkaline aqueous solution (0.1 N NaOH) or in methanol solution also gave sharp Raman bands at 1046 cm^{-1} for the former and at 1050 cm^{-1} for the latter. These frequencies showed a close resemblance to the reported values of 1049 and 1036 cm^{-1} for the Mn^{V}–N stretching frequencies of $Mn^{V}N$(OEP) (Buchler et al., 1982) and $Mn^{V}N$(TPP) (Hill & Hollander, 1982), identified by IR spectroscopy, respectively, indicating that this complex, **1A** or **2A**, was very likely to be the $Mn^{V}N$(PPIX). To confirm that the observed Raman bands were really Mn^{V}–N stretching frequencies, we carried out laser photolysis experiments of $^{15}N_3$–manganese(III) protoporphyrin IX and $^{15}N^{14}N^{14}N$–manganese(III) protoporphyrin IX in DMF using the 406.7-nm line from a Kr ion laser. A new Raman band appeared at 1021 cm^{-1} for the $^{15}N_3$ complex, and two Raman bands with equal intensities appeared at 1049 and 1021 cm^{-1} for the $^{15}N^{14}N^{14}N$ complex (Figure 4). These observations suggest that the terminal nitrogen atom of azide molecule, which was bound to metal center with a bent-on configuration before the photolysis, remains bound to the metal as an atomic ligand after the photodecomposition of azide.

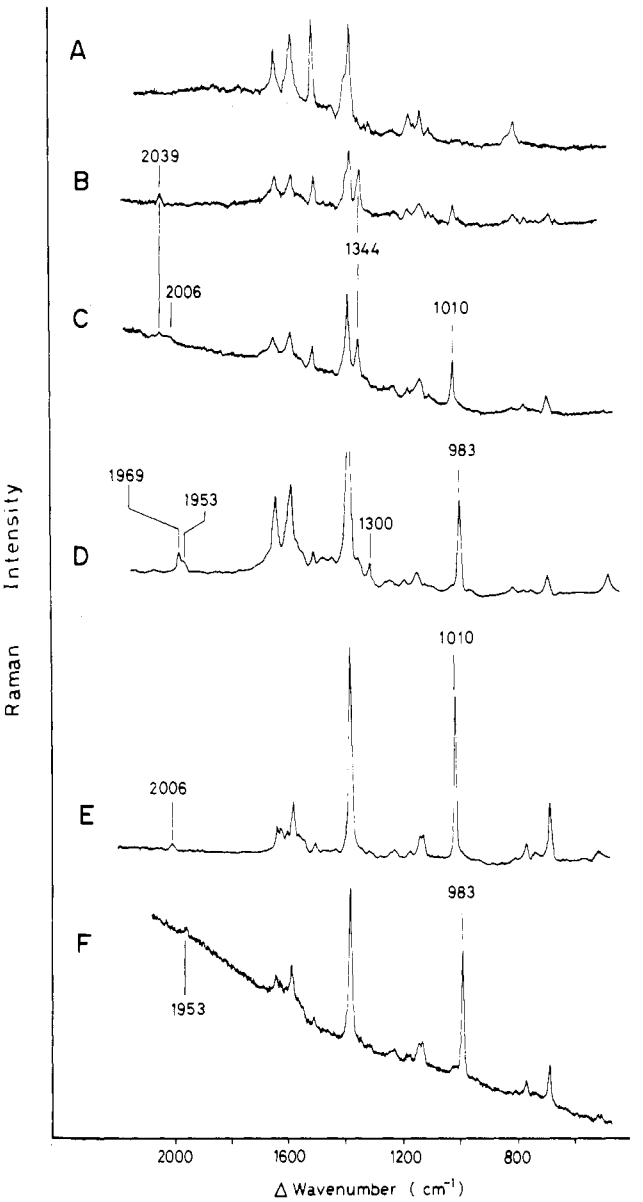

FIGURE 3: Resonance Raman spectra of $Mn^{III}Mb$ (A), the [^{14}N]azide complex of $Mn^{III}Mb$ (B), photoinduced product of the [^{14}N]azide complex of $Mn^{III}Mb$ (C), photoinduced product of the [^{15}N]azide complex of $Mn^{III}Mb$ (D), $Mn^{V}(^{14}N)$(PPIX)-containing Mb (**3A**), prepared by hypochlorite oxidation of the corresponding $Mn^{III}Mb$ in the presence of ammonia (E), and $Mn^{V}(^{15}N)$(PPIX)-containing Mb prepared by hypochlorite oxidation of the corresponding $Mn^{III}Mb$ in the presence of $^{15}NH_4OH$ (F). The 2039- and 1969-cm^{-1} bands are from manganese porphyrin bound azide (for $^{14}N_3$ and $^{15}N_3$, respectively), whereas the 1344- and 1300-cm^{-1} bands are from free azide (for $^{14}N_3$ and $^{15}N_3$, respectively) ion (Yu & Tsubaki, 1980). Azide concentration is ca. 0.25 M. The excitation wavelength was 441.6 nm. Spectra were obtained by a conventional Raman system. Slit height and width were 200 μm and 6 mm, respectively.

The resonance Raman spectrum of the reconstituted HRP (**4A**) gave a sharp Raman band at 1003 cm^{-1} and a weak Raman band at 1991 cm^{-1} (Figure 5B); those bands were corresponding to the 1010- and 2006-cm^{-1} bands of $Mn^{V}N$-

Table I: Observed and Expected Frequencies of the Porphyrin Skeletal Ring Modes of $Mn^{V}N$(PPIX) and $Mn^{V}N$(PPIX)-Containing Hemoproteins

mode	K^a	A^a	expected frequencies[a,b]	$Mn^{V}N$(PPIX)[b] in NaOH	in MeOH	in DMF	$Mn^{V}N$(Mb)[b]	$Mn^{V}N$(HRP)[b]
ν_{10} (B_{1g})	517.2	5.16	1655	1653	1650	1649	1640	1638
ν_3 (A_{1g})	448.3	5.35	1520	1516	1511	1511	1505	1510
ν_2 (A_{1g})	390.8	6.03	1590.5	1590	1587	1584	1585	1584
ν(Mn–N)			(1050)[c]	1046	1050	1049	1010	1003

[a] K ($cm^{-1}/Å$) and A (Å) are parameters for the relation $\nu = K[A - l(C_t–N_p)]$ obtained from Choi et al. (1982). Expected frequencies were calculated on the assumption that $l(C_t–N_p) = 1.960$ Å. [b] Numbers are the Raman shifts in cm^{-1} relative to the laser excitation frequency (this study). [c] This number is obtained by IR for $MnN(OEPMe_2)$ (Buchler et al., 1983a).

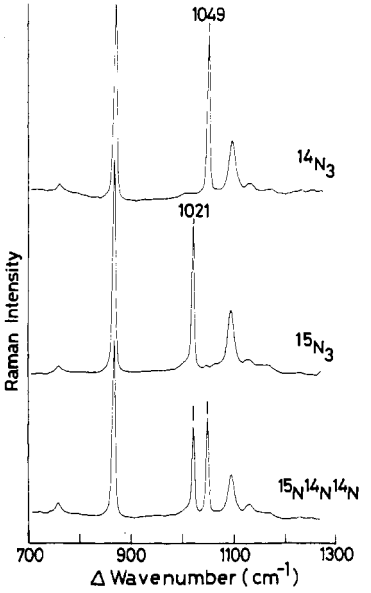

FIGURE 4: Resonance Raman spectra of the photolyzed complexes $^{14}N_3$–Mn^{III}(PPIX) (upper spectrum), $^{15}N_3$–Mn^{III}(PPIX) (middle spectrum), and $^{15}N^{14}N^{14}N$–Mn^{III}(PPIX) (lower spectrum) upon excitation at 406.7 nm from a Kr ion laser (45 mW). The spectra were obtained 60 min after staring the irradiation of the sample by laser light (406.7 nm) in the spinning Raman cell by using a multichannel vidicon detector Raman system. Slit height and width were 50 μm and 2 mm, respectively. Mn^{III}(PPIX) concentration in DMF was about 50 μM.

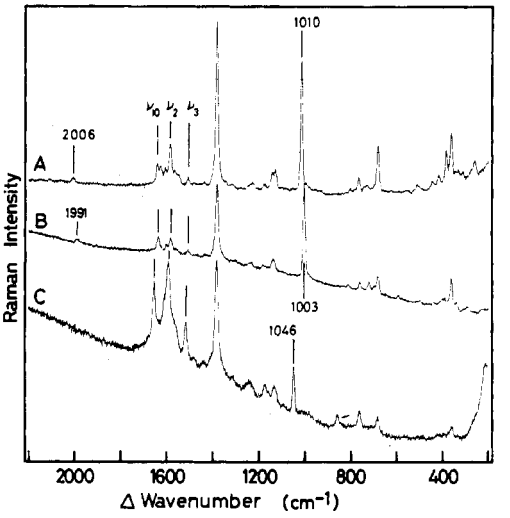

FIGURE 5: Resonance Raman spectra of $Mn^{V}N$(PPIX)-containing Mb (**3A**) in 0.1 M sodium phosphate buffer, pH 7.0 (A), $Mn^{V}N$-(PPIX)-containing HRP (**4A**) in 0.1 M sodium phosphate buffer, pH 7.0 (B), and Mn^{V}(PPIX) (**1A** or **2A**) in 0.1 M NaOH (C). Excitation wavelength, 441.6 nm.

(Mb), respectively, as shown in Figure 5A. The resonance Raman spectrum of $Mn^{V}N$(PPIX) in 0.1 N NaOH is also shown in Figure 5C. The Mn^{V}–^{14}N stretching frequency and its first overtone frequency were observed at 1046 and 2007 cm^{-1}, respectively. But their relative intensities were much weaker than corresponding bands in proteins, since the Soret maximum of $Mn^{V}N$(PPIX) in 0.1 N NaOH is located at much shorter wavelength than those in proteins. Corresponding frequencies to these modes observed for $Mn^{V}N$-(PPIX) in MeOH and in DMF were 1050 and 2084 cm^{-1} and 1049 and 2081 cm^{-1}, respectively (spectra not shown).

We added *N*-methylimidazole or pyridine to $Mn^{V}N$(PPIX) in DMF to see the effect of base ligand on the Mn^{V}–N stretching frequency. However, we could not detect any frequency change of the stretching mode nor any evidence for the formation of a new species, even in the presence of an excess (more than 1 M) of base ligand. Further, by spectroscopic examination of the visible absorption spectra, we could not find any evidence of base ligand binding to $Mn^{V}N$-(PPIX) in DMF.

The porphyrin ring modes, sensitive to the porphyrin core length, (e.g., ν_2, ν_3, and ν_{10})[3] were observed at 1585, 1505, and 1640 cm^{-1} for $Mn^{V}N$(Mb), at 1584, 1510, and 1638 cm^{-1} for $Mn^{V}N$(HRP), and at 1590, 1516, and 1653 cm^{-1} for $Mn^{V}N$-(PPIX) in 0.1 N NaOH, respectively (Figure 5). The observed frequencies are summarized in Table I, which includes the frequencies for $Mn^{V}N$(PPIX) in MeOH and DMF also.

Discussion

We found that the reaction of sodium hypochlorite with Mn^{III}(PPIX) in the presence of ammonia yielded a stable new complex as isolatable product and that the photolysis of the azide complexes of Mn^{III}(PPIX) also generated similar isolatable complexes. Furthermore, it was our surprise that similar kinds of new complexes could be prepared in metal-substituted Mb by hypochlorite oxidation of Mn^{III}Mb in the presence of ammonium ion or by photolysis of the azide complexes of Mn^{III}Mb. These new complexes were highly stable and appeared to be nitridometalloporphyrin derivatives on the basis of their spectral similarities to $Mn^{V}N$(OEP) (Buchler et al., 1982) synthesized under similar conditions. Nitridomanganese(V) porphyrin has been reported to be diamagnetic because of the d^2 low-spin ($S = 0$) Mn^{V}–porphyrin ground electronic state (Buchler et al., 1982). The manganese porphyrin derivatives in divalent (Mn^{II}), trivalent (Mn^{III}), and tetravalent (Mn^{IV}) states are EPR-observable under normal experimental conditions. No EPR absorption for nitridomanganese(V) complexes **1A** and **2A** and for single-crystal Mb containing $Mn^{V}N$(PPIX) is consistent with the diamagnetic nature of $Mn^{V}N$(OEP).

The present Raman data for nitridomanganese(V) porphyrin complexes revealed Mn^{V}–N stretching frequencies at 1049 cm^{-1} for complex **2A** and at 1010 cm^{-1} for **3A**, in the region

[3] The designation of porphyrin ring modes are based on Kitagawa et al. (1978) and Abe et al. (1978).

characteristic for the metal–nitrogen triple bond (Dehnicke & Strahle, 1981). The excellent agreement of the frequency shifts of 28 cm^{-1} from 1049 (^{14}N) to 1021 cm^{-1} (^{15}N) for **2A** in DMF and 27 cm^{-1} from 1010 (^{14}N) to 983 cm^{-1} (^{15}N) for **3A** with predictions of a diatomic harmonic oscillation model (28.3 cm^{-1} for **2A**, 27.2 cm^{-1} for **3A**, respectively) indicated that these Raman modes were pure metal(V)–N stretching modes and these bonds could be treated as simple diatomic molecules, as the first approximation.

One of important findings in the present study was the observation of the first overtone modes of the Mn^V–N stretching vibrations. The observation of the overtone mode, particularly for the metal–ligand stretching frequency, is very rare. As far as we know at present, only the observation of the overtone mode of Fe^{IV}–O stretching vibration has been reported by Nakamoto and his co-workers (Proniewicz et al., 1986). The actual frequencies of those first overtones ($v = 0 \rightarrow v = 2$) were observed at 2006 cm^{-1} (^{14}N) and at 1953 cm^{-1} (^{15}N) for Mn^VN(Mb), at 1991 cm^{-1} for Mn^V(^{14}N)-(HRP), and at 2077 cm^{-1} for Mn^V(^{14}N)(PPIX) in 0.1 N NaOH. The assignments of these new Raman lines to the first overtone modes ($v = 0 \rightarrow v = 2$) were based on the frequency shift observed for Mn^VMb (2006 → 1953 cm^{-1}, 53 cm^{-1} downshift) upon isotopic substitution from ^{14}N to ^{15}N, very close to the expected frequency shift (27.2 $cm^{-1} \times 2 = 54.4$ cm^{-1}). However, if a diatomic harmonic oscillator model holds, the first overtone frequencies for the Mn^V–N stretching mode should be located at 2020 (1010 × 2) (^{14}N) and 1966 (983 × 2) cm^{-1} (^{15}N) for Mn^VMb, at 2006 (1003 × 2) cm^{-1} for Mn^VHRP, and at 2092 (1046 × 2 cm^{-1} for Mn^VN(PPIX) in 0.1 N NaOH. These discrepancies of frequencies might be attributable to the anharmonicity of the potential energy function of the Mn^V–N bond.

The Morse function is generally accepted as a better approximation to the potential function of a diatomic molecule. The vibration energy E_v for the Morse function can be expressed by the equation (Karplus & Porter, 1970):

$$E_v = (v + 1/2)\omega_e hc - (v + 1/2)^2 \omega_e x_e hc \qquad (1)$$

where the quantity ω_e is the spacing of the energy levels, the positive constant $\omega_e x_e$ is the first anharmonicity constant, and h, c, and v are Planck's constant, the velocity of light, and the vibrational quantum number, respectively. From eq 1, we can obtain the following expressions for the vibrational Raman lines from the ground state ($v = 0$) to the excited states ($v = 1, 2$):

$$\text{fundamental } (v = 0 \rightarrow 1): \quad \nu_1 = \omega_e - 2\omega_e x_e \qquad (2)$$

$$\text{first overtone } (v = 0 \rightarrow 2): \quad \nu_2 = 2\omega_e - 6\omega_e x_e \qquad (3)$$

From these two equations, ω_e and $\omega_e x_e$ can be calculated easily. Then, the value of D_e, the equilibrium dissociation energy, can be obtained (Karplus & Porter, 1970):

$$D_e = hc\omega_e^2/4\omega_e x_e - \omega_e x_e hc/4 \cong hc\omega_e^2/4\omega_e x_e \quad (\text{since } \omega_e \gg \omega_e x_e) \qquad (4)$$

We plotted the relation between D_e and k_e for these complexes, as shown in Figure 6. It is very interesting to note that incorporation of nitridomanganese(V) protoporphyrin IX into apo-Mb or apoperoxidase caused a decrease of the force constant, k_e, by 0.5–0.6 mdyn/Å (from 7.3 to 6.7 mdyn/Å). The decrease of the force constant is manifested as a reduction of the Mn^V–N stretching frequency as large as about 40 cm^{-1} upon incorporation of Mn^VN(PPIX) into apoprotein. This is the most important observation in the present study. But the equilibrium dissociation energy, D_e, did not change so much upon incorporation into apoprotein (D_e = 4.5 eV). These

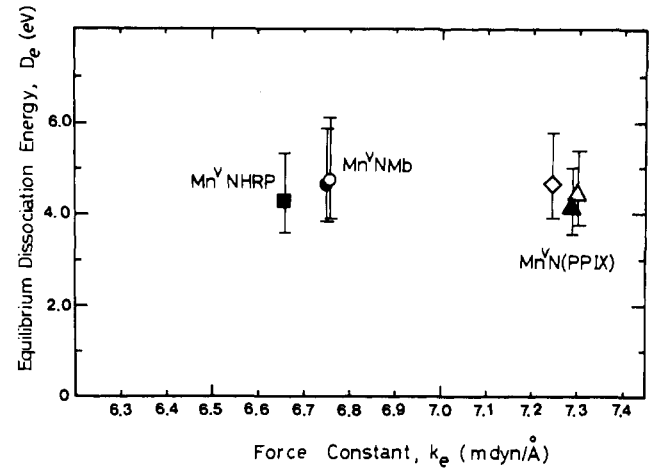

FIGURE 6: Relationship between the equilibrium dissociation energies (D_e) and force constants (k_e) of Mn^V–N bonds. The bars indicate the maximum error assuming that the readings of the frequencies in the Raman spectra were within an accuracy of ±1 cm^{-1}. (■) Mn^V-(^{14}N)(HRP); (●) Mn^V(^{14}N)(Mb); (○) Mn^V(^{15}N)(Mb); and Mn^V-(^{14}N)(PPIX) (◇) in 0.1 N NaOH, (▲) in DMF, and (△) in MeOH.

results mean that the curvature of the Morse function of the Mn–N system changed upon incorporation into apoprotein so as to keep the dissociation energy remaining almost the same.

Other remarkable effects of reconstitution into apoproteins were seen in the porphyrin ring mode region of resonance Raman spectra. It is well-known that the frequencies of some of the porphyrin ring modes (e.g., ν_2, ν_3, and ν_{10}) are related to the size of porphyrin core (Spaulding et al., 1975; Choi et al., 1982). Buchler et al. (1983a) have demonstrated the stereochemistry of the nitridomanganese(V) porphyrin, showing that the average distance from the center of the four pyrrole nitrogen atoms (C_t) to pyrrole nitrogen (N_p) is 1.960 Å. If this is also the case for Mn^VN(PPIX), the expected frequencies of the ν_2, ν_3, and ν_{10} modes should be 1590.5, 1520, and 1655 cm^{-1}, respectively, using porphyrin core size correlation parameters (Choi et al., 1982). Of the five nitridomanganese(V) porphyrin derivatives summerized in Table I, the modes of Mn^VN(PPIX) in 0.1 M NaOH were the closest to the expected values, whereas those of Mn^VN(Mb) and Mn^VN(HRP) were shifted to lower energy by ~5–15 cm^{-1} (see Table I). This means that the incorporation of nitridomanganese(V) porphyrin into apoprotein causes an increase of the C_t–N_p distance to 1.990 Å.

It is generally accepted that hydrogen bonding to the ligand causes weakening of the metal–ligand bond, decreasing the force constant (Sitter et al., 1985; Hashimoto et al., 1986). However, the observed decrease in the Mn^V/N bond force constant is a reverse direction of that expected, since the ligated nitrogen atom of **1A** or **2A** in 0.1 M NaOH should be much more hydrogen bonded than those of **3A** and **4A**, if any. Thus it seems unlikely to ascribe the observed reduction of the Mn^V–N force constant to hydrogen bonding to nitrogen from the distal site.

Recently, an Fe^{IV}=O stretching frequency of the "base-free" heme model compound Fe(TPP) at 15 K in an O_2 matrix at 852 cm^{-1} was reported by resonance Raman spectroscopy (Bajdor & Nakamoto, 1984; Proniewicz et al., 1986). This Fe^{IV}=O stretching frequency was much higher than those in HRP compound II [779 cm^{-1} (Terner et al., 1985); 787 cm^{-1} (Hashimoto et al., 1984)]. Since, in HRP compound II, Fe^{IV}=O has the imidazole group of the proximal histidine residue as a trans ligand, and various metal oxide stretching frequencies are known to vary substantially with differences in the ligand at the trans position (Buchler et al., 1978), the

observed large differences (as much as 70 cm^{-1}) were ascribed to the trans ligand (Terner et al., 1985; Proniewicz et al., 1986). Thus, one may suggest that, upon incorporation of nitridomanganese(V) protoporphyrin IX into apoproteins, bonding interactions from amino acid residues in the proximal side, e.g., the imidazole group of the proximal histidine residue, to Mn^V may cause the formation of a 6-coordinated species with a weaker Mn^V–N bond, which results in the manganese(V) atom moving slightly toward the mean plane of the pyrrole nitrogens, leading to core expansion. However, this explanation seemd erroneous in the present case, since addition of *N*-methylimidazole or pyridine to Mn^VN(PPIX) in DMF caused no shift of the Mn^V–N stretching frequencies and there was no evidence of N–base ligand bonding to Mn^VN(PPIX). If bonding between Mn^V and the imidazole group occurs, the Mn^V–Im stretching vibration couples to the Mn^V–N(nitrido) stretching mode considerably and the excellent agreement with the calculation will take place no longer.

Therefore, we propose the importance of the interactions between porphyrin peripheral groups and surrounding amino acid residues as the cause of the present observations. The low-spin state ($S = 0$) of the Mn^V atom could allow the metal atom to be centered in the porphinato plane (Scheidt, 1977). But since the Mn^V–N(nitrido) bond has a remarkably short distance (1.512 Å) (Buchler et al., 1983a), considerable nonbonded interactions between the nitrido and the nitrogen atoms of the porphyrin pyrroles would occur. Diminution of the nonbonded repulsion would be achieved only by stretching the Mn^V–N(nitrido) bond or by displacing the metal atom out of plane (Bright & Ibers, 1969; Scheidt et al., 1979). In the case of $Mn^VN(OEPMe_2)$ the diminution was achieved by displacement of the Mn^V atom by 0.603 Å from the mean plane of the porphodimethane core and by 0.426 Å from the N_4 plane (Buchler et al., 1983a). A similar situation is expected for Mn^VN(PPIX) in protein-free condition. Upon incorporation of Mn^VN(PPIX) into apoproteins numerous interactions between the porphyrin peripheral groups and amino acid residues may cause the stretching of the porphyrin ring toward the porphyrin periphery, leading to porphyrin core expansion. The expanded porphyrin core now can accommodate or pull back the Mn^V center closer to the mean plane of the porphyrin ring. As the result of structural adjustments to minimize the nonbonded interactions, a slightly stretched Mn^V–N(nitrido) bond, leading to the lowering of the stretching force constant, must take place. The difference of the force constants of the Mn^V–N(nitrido) bond between Mb and HRP might be attributable to the different heme–protein contacts in these hemoproteins (La Mar et al., 1983).

Although we think our proposal to be very likely, we should not neglect the possibility of nonbonding interactions between Mn^VN(PPIX) and the imidazole group of the proximal histidine residue. To reveal the contribution from the nonbonding interaction between Mn^VN(PPIX) and the proximal imidazole group, it is necessary to investigate the Mn^V–N stretching frequency for a heme model complex with the imidazole group being forced to face the Mn^V center from the proximal side. This kind of study is in progress in our laboratories.

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