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# Bonding in Nitrosyl Adducts of Manganese-Substituted Hemoglobin and Myoglobin Monitored by Resonance Raman Spectroscopy

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Resonance Raman spectroscopy and $^{15}N$ substitution have been used to determine vibrational mode frequencies associated with Mn–NO and N–O stretching and Mn–N–O bending for nitrosyl adducts of manganese-substituted hemoglobin (Hb) and myoglobin (Mb) and for manganese(II) protoporphyrin IX dimethyl ester ((Pip)$Mn^{II}$PPDME) in piperidine. The Mn–NO stretching frequency, ~625 $cm^{-1}$, is ~30 $cm^{-1}$ higher than the $Fe^{III}$–NO stretch reported for nitrosyl adducts of $Fe^{III}$ Hb and Mb and ~125 $cm^{-1}$ higher than the $Fe^{II}$–CO frequency of the CO adducts of Hb and Mb. All of these adducts are isoelectronic, and the large bond strength differences can be understood on the basis of trends in $\pi$ bonding. Back-donation is much greater for $Mn^{II}$–NO than for $Fe^{II}$–CO because of the greater polarity of the Mn–NO bond, the electron distribution formally being $Mn^{I}$–$(NO)^{+}$. The greater back-donation is evident both in the higher Mn–NO frequency and in the lower N–O frequency, ~1730 $cm^{-1}$, relative to the C–O frequency, ~1950 $cm^{-1}$, for the $Fe^{II}$–CO adducts. Back-donation is somewhat less for $Fe^{III}$–NO than for $Mn^{II}$–NO because of the higher effective charge on Fe. The bending frequencies are nearly the same, ~575 $cm^{-1}$, for all of these globin adducts. The bending frequency is slightly lower, 566 $cm^{-1}$, for the nitrosyl adduct of (Pip)$Mn^{II}$PPDME; the upshift in the proteins may reflect the influence of distal interactions on the nitrosyl group. The appearance of this band in the model complex indicates that its resonance enhancement does not require bending or tilting of the Mn–N–O linkage, as has been suggested for Fe–C–O. The close frequency correspondence for Mn–NO stretching between model and proteins indicates that there is little bending or tilting of the Mn–N–O linkage in the proteins. A time-dependent conformational change in the heme pocket is, however, indicated by the observation that the high laser power needed to observe the weak N–O stretching band is sufficient to photolyze freshly prepared NO adducts of the Mn-substituted proteins, but not adducts that have been aged for ~3 h.

## Introduction

Carbonyl adducts of heme proteins have been widely used in studying protein dynamics via absorption or resonance Raman (RR) spectroscopy, because of their efficient photodissociation. In addition, the frequencies and intensities of RR bands associated with Fe–CO stretching and Fe–C–O bending have emerged as useful probes of interactions of the bound CO with protein residues in the distal region of the heme pocket.[1-5] Another theme in heme protein studies is the substitution of other metal ions for Fe in the heme group, most notably Co[6] and Mn,[7] which retain qualitative features of the ligand-binding processes. In the case of Mn it is possible to mimic CO binding to heme rather closely with NO, since $Mn^{II}$–NO and $Fe^{II}$–CO units are isoelectronic. It has been shown that the NO adduct of manganese-substituted hemoglobin, MnHbNO, resembles the CO adduct of native hemoglobin, HbCO, in its photolability and in its rebinding kinetics and cooperativity.[7]

In this study we explore the use of the RR spectroscopy to probe the heme pocket of Mn-substituted Hb and Mb (myoglobin) via the Mn–NO stretching band, in extension of the Fe–CO studies. We find the Mn–NO and N–O stretching frequencies to be much higher and lower, respectively, than the Fe–CO and C–O stretching frequencies, consistent with greater back-bonding in the Mn–NO units. The Mn–N–O and Fe–C–O bending frequencies are, however, nearly the same. There is little evidence of a bent or tilted Mn–N–O linkage, in contrast to the situation in MbCO and HbCO. There is evidence, however, of a slow conformation change in MnMbNO and MnHbNO, which lowers the photolability, probably by increasing the recombination yield.

## Experimental Section

Manganese(III) protoporphin IX dimethyl ester acetate and manganese(III) protoporphyrin IX acetate were obtained from Mid Century Chemicals and used without further purification. Lyophilized myoglobin was obtained from Sigma Chemicals and purified by isoelectrophoresis.[8] Hemoglobin was purified from packed cells by the procedure of Scholler et al.[9] Apoglobins were prepared and reconstituted with manganese hemes by the methods described in ref 9 and 10 for Hb and in ref 11 and 12 for Mb.

The nitrosyl derivatives of both proteins were prepared as follows: A 1–2-mg sample of sodium dithionite (BDH Chemicals) was added anaerobically to 150 $\mu$L of a 1–2 mM solution of the protein in the appropriate buffer. After the solution was deaerated for 10 min (by flushing with nitrogen gas (Matheson)), dry nitric oxide gas (Matheson) was passed, with periodic shaking, over the solution, which was kept on ice for 10–15 min. A color change from red to deep pink marked the formation of the nitrosyl protein; the absorption spectra showed Soret, $\beta$, and $\alpha$ bands at 433, 538, and 580 nm. The nitrosyl model compound was prepared as described in ref 13 by reductive nitrosylation of $(OAc^{-})Mn^{III}$PPDME in deaerated piperidine; the solution was flushed with dry nitric oxide gas for 20–30 min, until the color changed to deep pink.

Resonance Raman spectra were obtained with $Kr^{+}$ laser excitation at 413.1 nm. The laser power at the source was less than 10 mW for the fresh nitrosyl proteins, to keep the photodissociation to a minimum. For the (NO)(Pip)$Mn^{II}$PPDME complex, and for the aged nitrosyl protein solutions (see text), laser powers of 30–50 mW were used. The samples were spun in an NMR tube positioned in a backscattering 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 $cm^{-1}$. All spectra shown are unsmoothed. UV–visible absorption spectra were recorded with a Hewlett-Packard 8450 Diode Array spectrophotometer. Isotopically substituted $^{15}NO$ (99%) was obtained from Cambridge Isotopes.

## Results

Figure 1 displays portions of the resonance Raman spectra of nitrosyl adducts of MnHb, MnMb, and (Pip)MnPPDME showing the Mn–NO and N–O stretching and Mn–N–O bending modes. These modes are readily identified via their $^{15}NO$ isotope shifts. The largest shift, ~35 $cm^{-1}$, is seen for the N–O stretch near 1730

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(1) Tsubaki, M.; Srivastava, R. B.; Yu, N.-T. *Biochemistry* **1982**, *21*, 1132.
(2) Armstrong, R. S.; Irwin, M. G.; Wright, P. E. *J. Am. Chem. Soc.* **1982**, *104*, 626.
(3) Argade, P. B.; Ching, Y. C.; Rousseau, D. *Science (Washington, D.C.)* **1984**, *225*, 329.
(4) Smulevich, G.; Evangelista-Kirkup, R.; English, A.; Spiro, T. G. *Biochemistry* **1986**, *25*, 4426.
(5) Evangelista-Kirkup, R.; Smulevich, G.; Spiro, T. G. *Biochemistry* **1986**, *25*, 4420.
(6) Yonetani, T.; Yamamoto, H.; Woodrow, G. V. *J. Biol. Chem.* **1974**, *249*, 682.
(7) Hoffman, B. M.; Gibson, Q. H. *J. Biol. Chem.* **1979**, *254*, 4691.
(8) Manrique, A.; Lasky, M. *Electrophoresis* **1981**, *2*, 315.
(9) Scholler, D. M.; Wang, M.-Y. R.; Hoffman, B. M. *Methods Enzymol.* **1978**, *52*, 487.
(10) Rossi Fanelli, A. *Biochim. Biophys. Acta* **1958**, *30*, 608.
(11) Scholler, D. M.; Wang, M.-Y. R.; Hoffman, B. M. *Methods Enzymol.* **1978**, *52*, 490.
(12) Teale, F. W. J. *Biochim. Biophys. Acta* **1959**, *35*, 543.
(13) Piciulo, P. L.; Scheidt, W. R. *Inorg. Nucl. Chem. Lett.* **1975**, *11*, 309.

0020-1669/87/1326-2280$01.50/0 

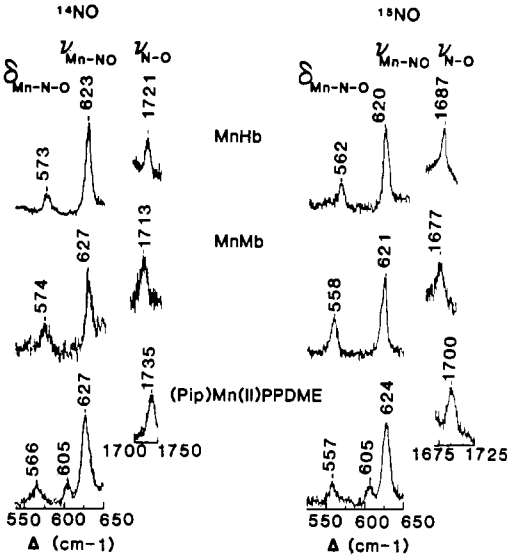


**Figure 1.** RR spectra of nitrosylmanganese hemoglobin, myoglobin, and (Pip)$Mn^{II}$PPDME, obtained with 413.1-nm excitation. Spectra obtained with natural-abundance NO are shown on the left, and those obtained with $^{15}NO$ on the right.

$cm^{-1}$. For an isolated N–O oscillator a 31-$cm^{-1}$ $^{15}NO$ downshift would be expected. The Mn–NO stretch is identified with the relatively strong band at ~625 $cm^{-1}$. The 3–6-$cm^{-1}$ $^{15}NO$ downshift is as expected for a linear Mn–N–O unit, the NO ligand acting essentially as a point mass; a 6.6-$cm^{-1}$ shift is expected for this model. The Mn–N–O bend is identified with the weaker band at ~570 $cm^{-1}$. This mode shows a larger $^{15}NO$ downshift, ~12 $cm^{-1}$, similar to the $^{13}CO$ shift seen[1] for the Fe–C–O bending mode of MbCO and HbCO. For a linear M–X–O unit, the largest displacement in the bending mode eigenvector is associated with the central X atom, and the frequency is therefore especially sensitive to its mass.

The ~1730-$cm^{-1}$ N–O stretching band is quite weak and required 30–50-mW laser power at the sample for its observation. This experiment could only be carried out, however, for protein samples, both Hb and Mb, that were aged for 3 h or more after preparation of the NO adduct. At earlier times the required laser power was sufficient to photodissociate the adducts (as evidenced by a decrease in intensity of the oxidation state marker band at ~1370 $cm^{-1}$ and an increase in intensity of the band at ~1357 $cm^{-1}$. The former is characteristic of ligated (NO)$Mn^{II}$, while the latter is typical of 5-coordinate $Mn^{II}$). No differences other than reduced photolability could be detected between fresh and aged samples. The low-frequency RR spectra, containing the Mn–NO stretching and Mn–N–O bending bands, which could be obtained on fresh samples at lower laser powers, 10 mW, showed no difference with respect to the aged samples, and the absorption spectra were unaltered.

Attempts to produce model complexes with imidazole (ImH) or *N*-methylimidazole (*N*-MeIm) failed. Additions of ImH or *N*-MeIm to methanolic solutions of dithionite-reduced manganese PPDME produces changes in the absorption and RR spectra indicative of complex formation, but exposure to NO, even for long periods, fails to produce NO adducts. This is consistent with the study of Piciulo and Scheidt[13] in which only aliphatic amines could be used to reductively nitrosylate manganese(III) porphyrins.

## Discussion

**A. Back-Bonding.** Although the $Mn^{II}$–NO and $Fe^{II}$–CO units are isoelectronic, the $Mn^{II}$–NO bond is much stronger than the $Fe^{II}$–CO bond, while the N–O bond is much weaker than the C–O bond. This is illustrated in Table I by the comparison between (NO)(Pip)$Mn^{II}$PPDME and (CO)(ImH)$Fe^{II}$PPDME. The M–X stretching and X–O stretching frequencies are ~25% higher and ~12% lower, respectively, for the Mn–NO than for the Fe–CO species. (Since force constants scale with $\nu^2$, the frequency shifts translate roughly to ~57% and ~22% differences in the force constants, neglecting mass differences and vibrational coupling.) Both differences imply much greater $\pi$ back-donation for Mn–NO than for Fe–CO from the metal $d_\pi$ to the XO $\pi^*$ orbitals. An orbital diagram for the back-bonding interaction is given in Figure 2, where it can be seen that the major difference stems from NO having one more electron than CO and $Mn^{II}$ having one fewer electron than $Fe^{II}$. The result is that Mn–NO bond formation is accompanied by transfer of an electron from NO to Mn, leading formally to a $Mn^{I}$–$(NO)^+$ electronic configuration.

**Table I.** Vibrational Frequencies ($cm^{-1}$) of the M–X–O Unit in Isoelectronic Nitrosyl and Carbonyl Hemes

| MXO | M–X–O bend | M–XO str | X–O str |
|---|---|---|---|
| $HbMn^{II}NO^a$ | 573 | 623 | 1721 |
| $MbMn^{II}NO^a$ | 574 | 627 | 1713 |
| (Pip)(PPDME)$Mn^{II}NO^a$ | 566 | 627 | 1735 |
| $HbFe^{III}NO^b$ | | 594 | |
| $MbFe^{III}NO^b$ | 573 | 595 | |
| $HbFe^{II}CO^c$ | 578 | 507 | 1951 |
| $MbFe^{II}CO^c$ | 577 | 512 | 1944 |
| (ImH)(PPDME)$Fe^{II}CO^d$ | | 495 | 1960 |

[a] This work. [b] From ref 14. [c] From ref 1. [d] From ref 5.

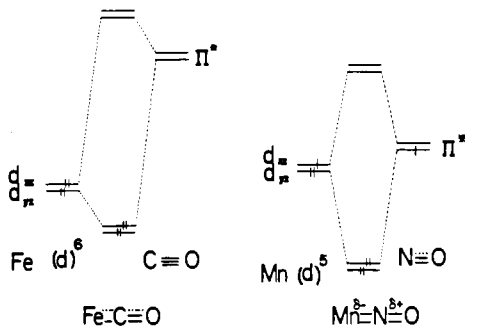


**Figure 2.** Schematic molecular orbital diagram of $Fe^{II}$–CO and $Mn^{II}$–NO porphyrins.

Back-bonding is a dominant feature of the $Fe^{II}$–CO bond, the $\sigma$ donation of the carbon lone pair inducing back-donation via the $\pi$ system and lowering the C≡O bond order, in the well-known synergic bonding of metal carbonyls. The polarity of the $Mn^{I}$–$(NO)^+$ bond enhances the back-donation: $NO^+$ is a better $\pi$ acceptor than CO, and $Mn^{I}$ is a better $\pi$ donor than $Fe^{II}$. These relationships are further illustrated by the $Fe^{III}$–NO stretching frequency of the NO adduct of metMb or metHb[14] (Table I), which is about three-fourths of the way between the $Fe^{II}$–CO and $Mn^{II}$–NO frequencies. The $Fe^{III}$–NO unit is formally $Fe^{II}$–$(NO)^+$, so that the $\pi$ acceptor is the same as in $Mn^{II}$–NO while the $\pi$ donor is the same as in $Fe^{II}$–CO. Evidently the enhanced $\pi$ acidity of $NO^+$ accounts for about three-fourths of the bond strengthening of $Mn^{II}$–NO relative to $Fe^{II}$–CO.

Interestingly, these marked changes in back-bonding are essentially without effect on the M–X–O bending frequency, which is close to 575 $cm^{-1}$ for all three units, $Fe^{II}CO$, $Fe^{III}NO$, and $Mn^{II}NO$, at least in the Mb and Hb complexes (data being unavailable for protein-free $Fe^{II}CO$ or $Fe^{III}NO$ porphyrins—vide infra). At first sight, this is a surprising result, since enhanced back-bonding might have been expected to stiffen the M–X–O linkage and increase resistance to bending. However, the bending force constant is determined by the dependence of the overall potential energy on the bending coordinate. When the M–X–O linkage is bent, back-bonding in the bending plane is expected to diminish, leading to a decrease in the M–X bond order but an increase in the X–O bond order. These countervailing effects may render the bending potential relatively insensitive to the extent of back-bonding.

**B. Resonance Enhancement.** Heme adducts with $\pi$-acid ligands X–O (X = C, N, O) show enhancement of the Fe–ligand stretching mode in Raman spectra excited in resonance with the

(14) Benko, B.; Yu, N.-T. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 7042.

porphyrin Soret absorption band.[15] This has been demonstrated for the Fe–$O_2$,[16] Fe–NO,[14] and Fe–CO[1,17] stretches in $O_2$, NO, and CO adducts. The required coupling of the metal–ligand stretch to the electronic transition is provided by the $\pi$ system. The empty $\pi^*$ orbitals of the axial ligand and of the porphyrin ring compete for the Fe $d_\pi$ electrons. Excitation into the $\pi$–$\pi^*$ state of the porphyrin is therefore expected to lengthen the metal–ligand bond, accounting for the origin shift along the Fe–ligand stretching coordinate, which is required for resonance Raman enhancement via the dominant *A*-term mechanism.[18] By the same token, the X–O bond is expected to contract in the excited state, due to the complementary effects of $\pi$ back-donation on the M–X and X–O bonds, and resonance enhancement of the X–O stretching mode can also be expected. The X–O displacement is, however, smaller, and the resonance enhancement is appreciably weaker. Thus the ~1730-$cm^{-1}$ stretching bands in Figure 1 are much weaker than the ~625-$cm^{-1}$ Mn–NO stretching bands. C–O[1] and N–O[17] stretches have been detected, albeit weakly, in RR spectra of CO and NO adducts of Hb and Mb, but the O–O stretch cannot be observed in RR spectra of $HbO_2$ or $MbO_2$.[19]

Enhancement of the M–X–O bending mode is a more subtle matter. This mode has been studied extensively for $Fe^{II}$ CO adducts by Yu and co-workers, who were able to locate it in RR spectra of MbCO and HbCO via its characteristic $^{13}CO$ and $C^{18}O$ isotope shifts.[1] They were unable to locate it, however, in RR spectra of protein-free heme-CO, except in the case of a porphyrin with a covalently linked strap across its face,[20] which lowers the CO affinity, presumably by steric interference with the mode of binding. Construction of the strapped porphyrin[21] was inspired by the neutron-diffraction structure of MbCO,[22] showing the Fe–C–O linkage to be bent or tilted due to steric interference by distal residues. Yu et al.[20] proposed that Raman enhancement of the Fe–C–O bend required bending or tilting of the Fe–C–O unit. The resulting increase in overlap between CO and porphyrin $\pi^*$ orbitals might provide direct coupling of the bending mode with the resonant $\pi$–$\pi^*$ transition.

It has since been found,[23] however, that the Ru–C–O bend is enhanced in the RR spectrum of $(CO)Ru^{II}OEP$ (OEP = octaethylporphyrin), in which there is no steric interference with a linear Ru–C–O linkage. And the present study shows the Mn–N–O bend to be enhanced in the RR spectrum of (NO)(Pip)$Mn^{II}$PPDME, again without any perturbation of the linear Mn–N–O linkage.[24]

**C. Protein Effects.** When one compares the CO adducts of Mb and Hb with the NO adducts of MnMb (Table I), there is a satisfying parallelism in the M–X and X–O stretching frequencies. In each case the X–O frequency is 7–8 $cm^{-1}$ higher and the M–X frequency is 3–4 $cm^{-1}$ lower for Hb than for Mb. Evidently back-bonding is slightly greater for Mb than for Hb, presumably due to differential polarizing effects of residues in the heme pocket. Moreover, the X–O frequency is 9–14 $cm^{-1}$ higher for the model compounds, (CO)(ImH)$Fe^{II}$PPDME and (NO)(Pip)$Mn^{II}$PPDME, than for the Hb adducts, again reflecting the heme pocket polarizing effects.

The parallelism breaks down, however, when one compares the M–X frequency for model and protein. (NO)(Pip)$Mn^{II}$PPDME has the same Mn–NO frequency, 627 $cm^{-1}$, as MnMbNO, while for (CO)(ImH)$Fe^{II}$PPDME, the Fe–CO frequency, 495 $cm^{-1}$, is 17 $cm^{-1}$ lower than in MbCO. This frequency difference has been attributed[20] to bending or tilting of the Fe–C–O linkage, as seen in the MbCO crystal structure. (We note that the crystal structure has been refined in terms of a bent structure, but while the electron density clearly shows the O atom to be off the heme normal, it is uncertain whether the C atom might also be displaced sufficiently to allow for a tilted but linear Fe–C–O linkage.) This interpretation was supported by the observation of an increase in the Fe–CO frequency, to 514 $cm^{-1}$, for the CO adduct of imidazole-bound "strapped" porphyrin,[20] in which there is steric hindrance to a perpendicular Fe–CO linkage.

If this interpretation is correct, the lack of any frequency differences between MnMbNO and (NO)(Pip)$Mn^{II}$PPDME for the Mn–NO stretch suggests that the M–X–O linkage is not bent or tilted in MnMbNO, in contrast to that in MbCO.

We note that the Mn–N–O bending frequency is 7–8 $cm^{-1}$ higher in MnMbNO and MnHbNO than in (NO)(Pip)-$Mn^{II}$PPDME. Perhaps steric constraints of the distal residues increase the resistance to Mn–N–O bending slightly. Unfortunately, a similar comparison is not possible for MbCO and HbCO since unencumbered Fe–C–O bending modes have not been detected in RR spectra of heme-CO models.

An additional curiosity of MnMbNO and MnHbNO is the decrease in photolability with time noted under the Results. Samples left standing for ~3 h or more after NO adduct formation withstand the ~30 mW of laser power needed to detect the N–O stretching RR band, but fresh samples photolyze. Nevertheless, there is no difference in the Mn–NO frequency nor in the UV–visible absorption spectra between fresh and aged samples. We infer that the decrease in photolability is unlikely to involve a change in the primary photochemistry but is due instead to an increase in the recombination probability associated perhaps with some change in the disposition of residues in the heme pocket. This change might be induced by the polarity of the $Mn^{II}$–NO unit. It is unlikely, however, to involve a close interaction, e.g. an H bond, with the NO group since there is no change in the Mn–NO frequency.

## Conclusions

N–O and Mn–NO stretching and Mn–N–O bending vibrational modes are readily identified in RR spectra of the NO adducts of Mn-substituted Mb and Hb. Comparison of isoelectronic porphyrin complexes containing $Fe^{II}CO$, $Fe^{III}NO$, and $Mn^{II}NO$ shows metal to ligand back-bonding to increase strongly along this series, as reflected in compensating increases and decreases in the M–XO and X–O stretching frequencies. The M–X–O bending frequencies are nearly the same, however, for $Mn^{II}NO$ and $Fe^{II}CO$. The Mn–NO stretching frequency in Mb or Hb does not show the ~15-$cm^{-1}$ elevation, relative to a protein-free analogue complex, seen for Fe–CO stretching, which has been attributed to bending or tilting of the Fe–CO unit. The MnNO photolability decreases with time after preparation of the protein adducts, but without any change in the Mn–NO stretching and Mn–N–O bending frequencies, suggesting a change in the disposition of protein residues that leads to an increased recombination probability.

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**Registry No.** (Pip)(PPDME)$Mn^{II}NO$, 108473-61-6; $^{15}N$, 14390-96-6.

(15) Spiro, T. G. *Adv. Protein Chem.* **1985**, *37*, 111.
(16) Walters, M.; Spiro, T. G. *Biochemistry*, **1982**, *21*, 6989.
(17) Tsubaki, M.; Yu, N.-T. *Biochemistry* **1982**, *21*, 1140.
(18) Spiro, T. G.; Stein, P. *Annu. Rev. Phys. Chem.* **1977**, *28*, 501.
(19) Tsubaki, M.; Yu, N.-T. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 3581.
(20) Yu, N.-T; Kerr, E. A.; Ward, E. B.; Chang, C. K. *Biochemistry* **1983**, *22*, 4534.
(21) Ward, B.; Wang, C.-B.; Chang, C. K. *J. Am. Chem. Soc.* **1981**, *103*, 5236.
(22) Norvell, J. C.; Nunes, A. C.; Schoenborn, B. P. *Science (Washington, D.C)* **1975**, *190*, 568.
(23) Kim, D.; Su, O.-Y.; Spiro, T. G. *Inorg. Chem.* **1986**, *25*, 3993.
(24) Scheidt, W. R.; Hatano, K.; Rupprecht, G. A.; Piciulo, P. L. *Inorg. Chem.* **1979**, *18*, 292.