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Resonance Raman Studies of HOO−Co(III)Bleomycin and Co(III)Bleomycin: Identification of Two Important Vibrational Modes, ν(Co−OOH) and ν(O−OH)

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Abstract: Bleomycin is an antitumor agent whose cytotoxicity is dependent on its ability to bind DNA in the nucleus and effect double-stranded DNA cleavage, which is difficult for the cell to repair. In order for this DNA cleavage to occur, bleomycin must, through a series of reactions, form a low-spin Fe(III) complex, the putative "activated" form of the drug, HOO−Fe(III)bleomycin. The relative strengths of the bonds in the Fe(III)−OOH linkage have not been determined due to the weakness of the hydroperoxo-to-iron(III) charge-transfer transition. The much more stable HOO−Co(III)bleomycin has often been studied as a structural analogue of HOO−Fe(III)bleomycin, and hence, an understanding of the relative bond strengths in the Co−OOH linkage may serve to enhance our understanding of the analogous Fe−OOH linkage. In this report, we present resonance Raman data that identify two important vibrational modes in the Co−OOH linkage, the stretching modes, ν(Co−OOH) and ν(O−OH). Both of these vibrational modes were found to be unperturbed by complexation of the drug with calf thymus DNA. Advantage was also taken of the isostructural realtionship between Fe−bleomycin and Co−bleomycin to analyze and assign the high-frequency modes for HOO−Co(III)bleomycin and Co(III)bleomycin (A_2 and B_2). These data could be useful for future studies of photoactivated Co−bleomycin and Co−bleomycin analogues in an attempt to characterize oxygen-independent DNA damage pathways.

Introduction

Bleomycin (Blm) is a glycopeptide antibiotic that is clinically employed as an antitumor agent.[1,2] The source of bleomycin's cytotoxicity is generally accepted to be its ability to effect both single- and double-strand breaks in DNA, the latter being more difficult for the cell to repair.[1,3,4a−f] It has been established in vitro that this reaction is dependent on Fe(II)Blm + O_2 and involves the formation of HOO−Fe(III)Blm according to the following reactions:[1,4a−g]

$$\text{Fe(II)Blm} + \text{O}_2 \rightarrow \text{O}_2\text{−Fe(II)Blm} \quad (1)$$

$$2\text{O}_2\text{−Fe(II)Blm} \rightarrow \text{BlmFe(II)−O}_2\text{−Fe(II)Blm} + \text{O}_2 \quad (2)$$

$$\text{BlmFe(II)−O}_2\text{−Fe(II)Blm} + \text{H}^+ \rightarrow \text{HOO−Fe(III)Blm} + \text{Fe(III)Blm} \quad (3)$$

Blm also complexes with Co(II) and subsequently reacts with O_2 in an analogous series of reactions.[1]

There exists some disagreement as to whether HOO−Fe(III)Blm represents the final, active intermediate that directly reacts with DNA or whether a mechanism involving Fe(III)−O−OH bond cleavage leading to a higher valence ferryl or perferryl species is responsible for initiating DNA strand scission.[1,5] Because of its diamagnetic, stable character, HOO−Co(III)Blm has been studied by NMR techniques in combination with molecular modeling as a structural analogue of HOO−Fe(III)Blm.[6a−e] On the basis of a variety of recent studies, the hypothesis of an isostructural relationship between Co- and FeBlm species has been supported.[7a−c]

Early studies, based on the reaction of Fe(III)Blm with 10-hydroperoxy-8,12-octadecadienoic acid and analysis of the reduction products, supported the idea of homolytic cleavage

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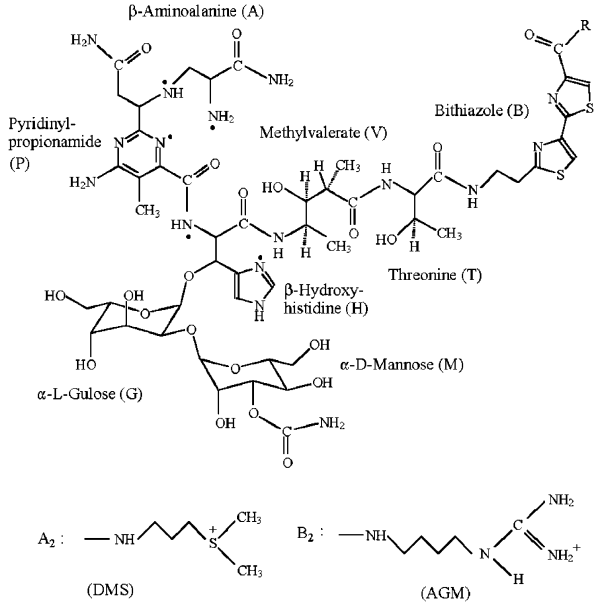

Figure 1. Structures of bleomycins A_2 and B_2. The atoms indicated with a dot (•) are the ligands for the transition metal, Co or Fe. The amino group on the carbon at position 2 and the N-atom at position 28 are axial ligands, and the others occupy equatorial positions about the metal center.

of the O−OH bond to form an •OH−Fe(III)Blm [O═Fe(IV)Blm] intermediate.[5a] More recently, extensive theoretical studies of the electronic structures of both Fe(III)Blm and HOO−Fe(III)Blm, based on analysis of absorption, resonance Raman, and optical and magnetic CD data, have argued against both heterolytic and homolytic cleavage of the O−OH bond and, instead, favored the idea that activated Blm chemistry proceeds via a direct, concerted attack on the DNA substrate C4′-H bond by the hydroperoxide group itself.[5b,c] However, these calculated results still require direct experimental validation.

The relative strengths of the bonds in the Fe(III)−OOH linkage for FeBlm have not been determined owing to the rapid turnover of the hydroperoxide and the weakness of the hydroperoxo-to-iron(III) charge-transfer transition,[4g,5b,c] but given the proposed isostructural relationship between the CoBlm and FeBlm species, an understanding of the relative bond strengths for the hydroperoxide ligand in the more stable HOO−Co(III)-Blm compound may contribute to our understanding of the analogous Fe(III)−OOH linkage. In this report, we present resonance Raman data that identify two important vibrational modes in the Co(III)−OOH linkage, ν(Co−OOH) and ν(O−OH), for the two major components, Blm A_2 and B_2, that are present in the clinically administered form of the drug (Figure 1). The effects of solvents, including H_2O, D_2O, and dimethyl sulfoxide (DMSO), as well as drug binding to DNA on the vibrational frequencies of these drug modes are also described.

Both HOO−Co(III)Blm and Co(III)Blm are capable of cleaving DNA when the drug, bound to DNA, is irradiated with UV light.[8a−e] Evidently, the hydroperoxide ligand plays no necessary role in these reactions. Initial studies of photoactivated CoBlm DNA cleavage revealed that the most effective wavelengths for DNA nicking are most likely those that excite ligand to metal charge-transfer transitions in Co(III)Blm, i.e., 330−450 nm.[8a,b] It was also found that, for CoBlm-induced DNA strand cleavage, O_2 was not required. The reaction involves little or no production of base propenals, results in pyrimidine base release, and exhibits the same DNA base sequence selectivity as that observed for FeBlm, i.e., reaction at 5′-GC(T)-3′ DNA sequences.[8b] The ability to detect a photoreduced CoBlm intermediate and to study its interaction with DNA by time-resolved resonance Raman experiments requires an initial, careful resonance Raman study of the vibrational modes in HOO−Co(III)Blm and Co(III)Blm that are sensitive to ligation and metal oxidation state. The hypothetical, isostructural relationship between FeBlm and CoBlm has allowed us to assign these modes through comparison with those previously reported for Fe(II)Blm, Fe(III)Blm, and CO−Fe(II)Blm species.[9a]

Experimental Procedures

Sample Preparation: Purification of Metal-Free Bleomycins. Metal-free bleomycins A_2 and B_2 (Calbiochem, La Jolla, CA) were separately purified by HPLC on a 250 × 4.8 cm C18 reversed-phase column (Vydac, Herpario, CA). A flow rate of 2.0 mL/min was employed and two solvents, 0.2% trifluoroacetic acid (A) and methanol (B) (Sigma−Aldrich, Milwaukee, WI), were used to make the following gradient: $T = 0$ min, 70% A and 30% B; $T = 10$ min, 65% A and 35% B. At $T = 15$ min, initial conditions were restored. The purified bleomycins were then lyophilized, reconstituted with deionized water, and relyophilized to dryness. The concentration of metal-free bleomycin was measured by UV−vis spectrophotometry; an extinction coefficient of $\epsilon_{290} = 14.0\ \text{cm}^{-1}\ \text{mM}^{-1}$ [10] was used.

HOO−Co(III)Bleomycin and Co(III)Bleomycin. HOO−Co(III)-Blm and Co(III)Blm (A_2 and B_2) were prepared in the following way. Metal-free Blm was dissolved in 0.02 M Tris buffer, pH 10.5 (Sigma T-6791 ultra grade)/0.1 M NaCl (Sigma S-3014). To this solution was added 0.9 equiv of Co^{2+} as $CoCl_2$ in deionized H_2O. The resulting solution of 50:50 HOO−Co(III)Blm:Co(III)Blm was mixed gently with a disposable pipet and allowed to incubate at 4 °C for 15−20 min. The final pH of this CoBlm drug solution was 7.4. Separation of the green HOO−Co(III)Blm from the brown Co(III)Blm was done by HPLC on a 250 × 4.8 cm reversed-phase C18 column (Bio-Rad Hi-Pore RP-318, Hercules, CA) at a flow rate of 4.70 mL/min. Two solvents, (A) 0.1 M ammonium acetate, pH 8.6, and (B) acetonitrile were used to form the following linear gradient: $T = 0$, 93.5% A and 6.5% (B); $T = 10$ min, 87.5% A and 12.5% B; and at $T = 20$ min, initial conditions were restored. Co(III)Blm eluted first at about 9.6 min followed by HOO−Co(III)Blm at about 11.8 min. After collection of the CoBlm eluents, they were lyophilized separately, reconstituted with deionized H_2O, and relyophilized until completely dry.

The ^{18}O isotopomer, $H^{18}O^{18}O$−Co(III)Blm, was prepared by placing the solution of metal-free Blm (0.02 M Tris/0.1 M NaCl) in an NMR

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tube equipped with a PFTE valve (Wilmad). The tube was then linked to a diffusion-pumped vacuum line and all of the oxygen was evacuated from the drug solution with subsequent replacement by $^{18}O_2$ gas (ICON Stable Isotopes, Summit, NJ). The metal-free drug solution/$^{18}O_2$ was tightly sealed, removed from the vacuum line, and allowed to sit on ice for 10 min. The tightly sealed NMR tube was then transferred to an anaerobic chamber where addition of anaerobically prepared $CoCl_2$ was performed. The contents of the tube were mixed and allowed to incubate in the anaerobic chamber for 15 min to allow complete transformation of the sample into a 50:50 mixture of $H^{18}O_2$–Co(III)Blm:Co(III)Blm. Purification of the products was done the same way as described above for the aerobically prepared sample.

Preparation of Samples for Resonance Raman Measurements. The lyophilized samples of both oxygen isotopomers of HOO−Co(III)Blm were reconstituted with 0.02 Tris/0.1 M NaCl buffer, pH 7.4, to yield a final drug concentration of 18−20 mM ($\epsilon_{290} = 18.0$ mM^{-1} cm^{-1}).[8b] A portion (130 μL) of this solution was transferred to an NMR tube and 10 μL of 0.5−1.0 M sodium sulfate decahydrate (Sigma, S-9413) was added as an internal standard. The amount of internal standard remained constant for a given set of samples. Samples measured in D_2O were reconstituted with D_2O buffer and a D_2O sodium sulfate (0.5−1.0 M) solution. The same procedure was used for the Co(III)Blm samples. Samples were prepared in DMSO by simply reconstituting the lyophilized CoBlm samples and adding sodium sulfate (final concentration, 1.0 M) that was also prepared in DMSO.

Purification of Calf Thymus DNA. DNA (100 mg; ICN Biomedical, Aurora, OH) was added to a 50 mL Erlenmeyer flask (previously soaked in 10% nitric acid and rinsed with deionized H_2O) containing 20 mL of freshly made, filter-sterilized, pH 8.5 Tris/0.1 M NaCl buffer. The DNA solution was incubated overnight at 30 °C. A 10 mg/mL solution of proteinase K (Sigma, P-0390) was prepared in 1.0 mL of pH 8.5 Tris/0.1 M NaCl buffer, and 50 μL (0.5 mg) was added to the DNA sample. The resulting solution was gently stirred for 15 min at room temperature and then incubated for 45 min in a 30 °C incubator.

After digestion of the protein in the DNA sample was completed, half was transferred to a 50 mL separatory funnel and 30 mL of extraction solvent was added (phenol/$CHCl_3$/isoamyl alcohol, 24:24:1, preequilibrated with 10 mL of Tris buffer, pH 8.0, and 1 mM EDTA, for 4 h or overnight at 4 °C) (Sigma P-2069). Extraction of the purified DNA was performed two times by shaking and allowing the phases to separate. In each extraction, the top, aqueous layer was saved, transferred to a gel separator tube (Sigma, P-2348), and centrifuged in a tabletop centrifuge (5000 rpm) for 5 min to completely separate the aqueous from the solvent phase. All aqueous portions were combined in a 1 L beaker previously washed in 10% nitric acid. Cold ethanol (Sigma, E−2703) was added until no more precipitation of DNA was observed. The purified DNA was removed with a glass rod and as much of the ethanol as possible was expressed by pressing the DNA against the side of a clean tube. The DNA was centrifuged at 8000 rpm in order to exclude additional ethanol. The DNA pellet was then redissolved in fresh 0.02 M Tris buffer, pH 7.5.

The DNA solution was concentrated by use of Amicon Centricon YM-3 tubes (Amicon). The resulting DNA solution was then sonicated in an ice bath for 45 min and filtered through a 0.2 μm syringe filter.[11a−c] Finally, the DNA concentration was determined by UV spectrophotometry with $\epsilon_{258} = 13.2$ cm^{-1} mM^{-1}.[10]

Preparation of HOO−Co(III)Blm/DNA Samples for Resonance Raman Measurements. Two 100 μL aliquots of purified DNA solution (pH 7.5) were transferred to NMR tubes. To each of the NMR tubes were added 10 μL of 1.0 M sodium sulfate and 10 μL of a concentrated solution of HOO−Co(III)Blm/0.02 M Tris, pH 7.5. A sample was prepared for each of the drug isotopomers, i.e., $H^{16}O_2$–Co(III)Blm and $H^{18}O_2$–Co(III)Blm, such that the final concentration of the drug was 1:4 drug:DNA base pair ratio with a DNA concentration of 20 mM base pairs.

Raman Measurements. Resonance Raman measurements were made on samples of ^{18}O and ^{16}O isotopomers of HOO−Co(III)Blm (A_2 and B_2) and Co(III)Blm (A_2 and B_2) in H_2O and D_2O buffer solutions; both ^{18}O and ^{16}O isotopomers of HOO−Co(III)Blm A_2 in DMSO solution; and the ^{18}O and ^{16}O isotopomers of HOO−Co(III)-Blm (A_2 and B_2) bound to DNA samples in H_2O buffer. Experiments were accomplished on a Spex Model 1269 spectrometer equipped with a UV-enhanced charge-coupled device (CCD) detector (Princeton Instruments) and appropriate notch filters (Kaiser Optical). The 406.7 nm excitation from a Coherent Model Innova 100 Kr^+ laser was used for all the samples. A cylindrical lens was employed and power was kept at 12 mW at the sample for all solutions of drugs not bound to DNA and 3 mW for the CoBlm−DNA samples. Multiple scans were taken of each sample, checked for discrepancies, and then added together to produce the final spectral result. Each sample was scanned for 6 h (low-frequency region) and 3 h (high-frequency region) in a spinning NMR tube to avoid localized heating of the sample. The CoBlm−DNA samples were scanned for 14 h (low-frequency region only). The scattered light was collected by use of a 135° backscattering geometry. The band at 981 cm^{-1}, assigned to sodium sulfate, was used as an internal intensity standard. All spectral files were corrected by use of the same baseline parameters, so that these corrections were consistent for each spectrum, before individual files from individual scans were added together by software in Spectrocalc (Galactic Industries). All samples were measured at ambient temperatures (22 °C).

Results and Discussion

Low-Frequency Raman Spectral Region, ν(O−OH) and ν(Co−OOH). The low-frequency Raman spectral region (300−1000 cm^{-1}) contained vibrational modes associated with the M−OOH linkage (Table 1). Figure 2 displays the low-frequency resonance Raman spectrum for HOO−Co(III)Blm A_2, the ^{18}O isotopomer, and the difference spectrum ($^{16}O - {}^{18}O$) derived from absolute spectra taken in H_2O solvent. The difference spectrum (bottom spectrum) identifies the two oxygen isotope-sensitive modes, ν(O−OH) and ν(Co−OOH). The band with the Raman shifted frequencies 545/518 cm^{-1} ($^{16}O/^{18}O$) has been assigned to ν(Co−OOH) on the basis of its 27 cm^{-1} isotopic shift, which is approximately that of a pure ν(Co−O) stretching frequency (25 cm^{-1}).[12] The low-frequency data obtained for both Blm A_2 and B_2 are summarized in Table 1.

Prior studies of Co(II) vs Fe(II) compounds have consistently revealed that the metal−oxygen bond for the cobalt complexes was longer and weaker than that of corresponding iron complexes (Table 2).[12−14] A weaker Co−O bond relative to an Fe−O bond is attributed to the fact that the additional electron that resides in the $\pi^*(d_{xz}/\pi_g^*)$ antibonding orbital of Co^{2+} is more likely to reside in an antibonding π^* orbital localized on dioxygen in the complex.[12] Note that a density functional theory (DFT) calculation done by Solomon's group predicts a ν(Fe−O) stretching frequency of 575 cm^{-1} for HOO−Fe(III)Blm.[5c] This 30 cm^{-1} upshift from the value determined experimentally

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Table 1. Frequencies of the Resonance Raman Lines for HOO−Co(III)Blm, Co(III)Blm, Fe(III)Blm A_2, and CO−Fe(II)Blm A_2[a]

		HOO–Co(III)Blm		Co(III)Blm		
Fe(III)Blm A_2	CO–Fe(II) Blm A_2	A_2	B_2	A_2	B_2	proposed assignments
1608/1611	1607/1610	1613/1618	1613/1618	1613/1618	1613/1618	deprotonated amide I ν(C−O) of the β-hydroxyhistidine amide
1540/1540	1540/1540	1540/1541	1540/1541	1539/1540	1539/1541	bithiazole ring mode
1478/1481	1466/1470	1485/1485	1484/1485	1487/1487	1486/1486	δ(C−N−C) of the deprotonated amide
1388/1391	1390/1392	1401/1401	1400/1399	1399/1400	1400/1400	pyrimidine ring
1371/1375	1372/1377	1378/1382	1379/1380	1378/1381	1378/1382	pyrimidine ring
		1326/1321	1326/1321	1325/1322	1325/1321	pyrimidine δ(C−H) + ring
		1283/1274	1283/1275	1285/1275	1285/1275	pyrimidine ring
		828/828	826/827			ν(O−OH) for hydroperoxide (^{16}O)
		784/782	782/783			ν(O−OH) for hydroperoxide (^{18}O)
		545/544	545/544			ν(Co−OOH) for hydroperoxide (^{16}O)
		518/514	516/514			ν(Co−OOH) for hydroperoxide (^{18}O)
	511/498 (508)[b]					ν(Fe−CO)
	1980/1890 (1980)[b]					ν(C−O)
561/554 (551)						ν(Fe−OH)

[a] Frequencies are given in reciprocal centimeters for 406.7 nm excitation; the first number is for H_2O and the second is for D_2O. [b] Values in parentheses are those for heme proteins.

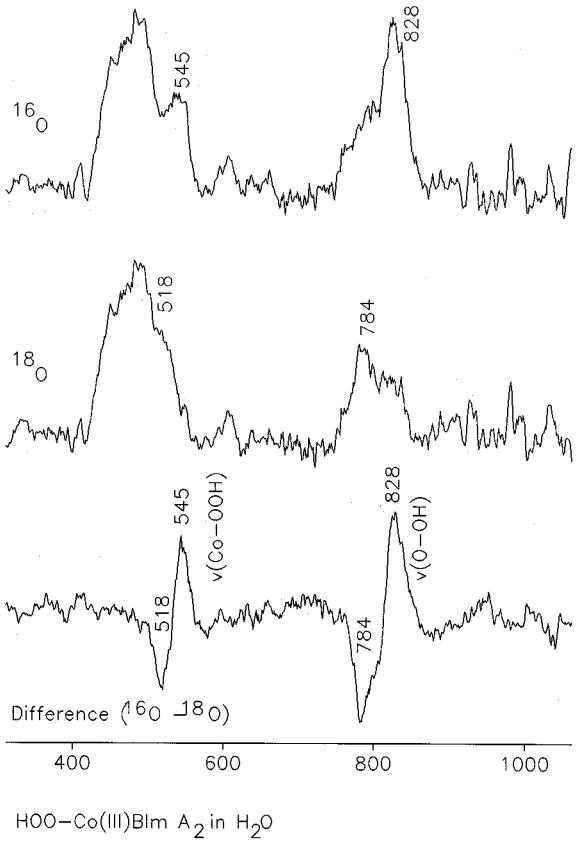

Figure 2. Low-frequency resonance Raman spectra of HOO−Co(III)Blm A_2: ^{16}O and ^{18}O isotopomers and their difference spectrum, $^{16}O - {}^{18}O$, in H_2O buffer.

in this work for HOO−Co(III)Blm for ν(Co−O) has also been seen for the ν(Fe−O) stretching frequency for O_2–Fe(II)hemoglobin vs O_2–Co(II)hemoglobin compounds.[12−14] Considering all of the above information, 545 cm^{-1} is a reasonable value for the ν(Co−OOH) vibrational mode obtained in this study.

The difference feature at 828/784 cm^{-1} (^{16}O/^{18}O) in Figure 2 is assigned to ν(O−OH). The isotopic shift of 44 cm^{-1} seen for this mode is very close to the predicted 46 cm^{-1} shift for a pure ν(O−OH) stretching mode. The 828 cm^{-1} frequency of ν(O−OH) also falls within the range reported for end-on transition metal-bound hydroperoxide ligands, i.e., 786−1146 cm^{-1}.[5b,c,15,16]

The data obtained in D_2O did not reveal any downshift in frequency for ν(O−OH) for HOO−Co(III)Blm, which was consistent with the lack of shift for ν(O−OH) seen in other studies of non-heme model compounds. Similarly, there was no significant downshift observed for ν(Co−OOH) in HOO−Co(III)Blm, while only a 5 cm^{-1} downshift in D_2O has been observed for the corresponding ν(Fe−O) vibrational mode in the aforementioned model compounds.[5b,c,15a−d] It should be mentioned that, for HOO−Co(III)Blm, NMR studies have shown moderate to strong NOE connectivities that suggest H-bonding interactions between the threonine NH and methylvalerate NH protons of the peptide linker with the hydroperoxide ligand.[6e,f,17] These H-bonding interactions may have an effect on H/D frequency shifts.[18]

The importance of H-bonding interactions involving water was ruled out by resonance Raman measurements of the ^{18}O/^{16}O isotopomers of HOO−Co(III)Blm A_2 in DMSO, an aprotic solvent (data not shown, Table 3). In DMSO, as was seen in D_2O, there were no significant deviations in the frequencies of ν(Co−OOH) and ν(O−OH) from those seen in H_2O buffers. This indicated that no H-bond interaction through a solvent molecule such as water was involved directly with the Co−OOH linkage. In turn, this result provided further, indirect support for the presence of H-bond interactions directly between the hydroperoxide ligand and the linker peptide region of HOO−Co(III)Blm.[17]

Figure 3 contains two difference spectra (^{16}O/^{18}O) obtained for both HOO−Co(III)Blm A_2 and B_2 bound to calf thymus DNA in a 1:4 ratio of drug:DNA base pairs. No changes in the

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Table 2. Comparison of ν(O−O) and ν(M−O) for Some Representative Compounds Exhibiting Different Binding Geometries and Metals

compound	binding geometry	metal/spin state	ν(M–O)[a]	ν(O-O)[a]	ref
(a) $[Fe(III)(TPA)(OOH)]^{2+}$	end-on/hydroperoxide	Fe^{3+}/ls	626/nr	789/nr	15a
(b) $[Fe(III)(N4Py)(OOH)]^{2+}$	end-on/hydroperoxide	Fe^{3+}/ls	632/616 (627)[b]	790/746 (789)[b]	15a
(c) $[Cu(II)(HB(3\text{-}tBu\text{-}5\text{-}Prpz)_3(\ OOH)]$	end-on/hydroperoxide	Cu^{2+}/hs	624/607 (623)[b]	843/799 (842)[b]	15c
(d) $[Cu(II)(HB(3,5\text{-}Pr_2Pz)_3)(OO\ CMe_2Ph)]$	end-on/alkylperoxo	Cu^{2+}/hs	652/633	844/818	15c
(e) oxyhemerythrin	end-on/hydroperoxide (H-bonded to bridging oxo)	Fe^{3+}/hs	503/479	844/796	15d
(f) [Co(III)(Blm)(OOH)]	end-on/hydroperoxide	Co^{3+}/ls	545/518	828/784	this work
(g) [Fe(III)(Blm)(OOH)]	end-on/hydroperoxide	Fe^{3+}/ls	575[c]	879[c]	5c
(h) $[Co(II)(TPP)(pip)O_2]$	end-on/superoxo	Co^{2+}/ls	509	1142	15e
(i) $[Fe(II)(TPP)(pip)O_2]$	end-on/superoxo	Fe^{2+}/ls	575	1157	15e
(j) $[Co(II)(T_{piv}PP)(1\text{-}MeIm)O_2]$[d]	end-on/superoxo	Co^{2+}/ls	517	nr	15e
(k) $[Fe(II)(T_{piv}PP)(1\text{-}MeIm)O_2]$[c]	end-on/superoxo	Fe^{2+}/ls	568	nr	15e
(l) $[Co(II)(hemoglobin)(O_2)]$	end-on/superoxo	Co^{2+}/ls	539/516	1135/1065	12, 14
(m) $[Fe(II)(hemoglobin)(O_2)]$	end-on/superoxo	Fe^{2+}/ls	568/543	1130	13, 14

[a] Frequencies are given in reciprocal centimeters. The first value given is for ^{16}O; the second is for ^{18}O. [b] Values in parentheses represent values for −OOD complexes. [c] Values for these modes have been calculated but have not been observed experimentally. [d] $T_{piv}PP$, picket fence porphyrin.

Table 3. Comparison of the Frequencies for the Resonance Raman Bands of HOO−Co(III)Blm and Co(III)Blm in H_2O, D_2O, and DMSO Solvents[a]

HOO–Co(III)Blm		Co(III)Blm		
A_2	B_2	A_2	B_2	proposed assignments
1613/1618/1613	1613/1618	1613/1618/1614	1613/1618/1614	deprotonated amide I of the β-hydroxyhistidine amide
1540/1541/1537	1540/1541	1539/1540/1537	1539/1540/1537	bithiazole ring
1485/1485/1474	1484/1485	1487/1487/1476	1486/1486/1476	δ(C−N−C) of the deprotonated amide
1401/1401/1393	1400/1399	1399/1400/1393	1400/1400/1394	pyrimidine ring
1378/1382/1378	1379/1380	1378/1381/1378	1378/1382/1378	pyrimidine ring
1326/1321/1319	1326/1321	1325/1322/1317	1325/1321/1320	pyrimidine δ(C−H) + ring
1283/1274/1280	1283/1274	1285/1275/1281	1285/1275/1282	pyrimidine ring
828/828/831	826/827			ν(O−OH) for hydroperoxide ^{16}O
784/783/783	783/783			ν(O−OH) for hydroperoxide ^{18}O
545/544/542	545/544			ν(Co−OOH) for hydroperoxide ^{16}O
518/514/518	516/514			ν(Co−OOH) for hydroperoxide ^{18}O

[a] Frequencies are given in reciprocal centimeters for 406.7 nm excitation. The first number is for H_2O, the second is for D_2O, and the third, if listed, is for DMSO.

frequency of either ν(Co−OOH) or ν(O−OH) were observed upon drug binding to DNA. Thus, the orientation of the Co−OOH linkage was not measurably perturbed upon drug binding to DNA under conditions in which the hydroperoxide−Co(III) species was bound to a combination of sequence-specific (5′-GPy-3′) and nonspecific sites.[19] Previous studies of HOO−Co(III)Blm bound to calf thymus DNA at pH 7.5 have determined the K_d for the drug/DNA complex to be $\sim 10^{-7}$ M.[8a,b] The procedure used here was similar to that for a previous Raman/electron paramagnetic resonance (EPR) study of an Fe(III)Blm/calf thymus DNA complex with a larger dissociation constant, $K_d \sim 10^{-5}$ M.[9b] Nevertheless, considering the small base pair to drug ratio, the possibility that some unbound drug was present in the mixture cannot be excluded.

In a recently published study it was revealed that the hydroperoxide group in HOO−Co(III)Blm bound to a specific base sequence, 5′-GC-3′, and occupied a pocket composed of the drug structure and the DNA minor groove that was isolated from the bulk solvent and was protected from collisions with other polar molecules.[6f] Hydrogen bonds between guanine (G) and the drug's pyrimidinyl residue and intercalation of the bithiazole between DNA base pairs anchor the adduct.[6e,f] These studies also indicate that the hydroperoxide group occupies a sterically constained pocket in the DNA−drug adduct and is directed toward the C4′-H of one of the two cytosines of the GC site.[6e,f,20c] The lack of an effect of DNA binding on the vibrational properties of the hydroperoxide is consistent with previous recognition that the metal domain−linker conformation of HOO−Co(III)Blm is rigid and does not undergo significant change upon interaction with the DNA minor groove.[6d,20a,b] Considering the crucial need to direct the hydroperoxide of HOO−Fe(III)Blm toward the C4′-H of deoxyribose, the site of initial reaction during DNA strand scission, the present results are also consistent with the preorganized positioning of the hydroperoxide in the activated drug.

Previous DNA Raman studies had shown both that O_2−Co(II)Blm A_2 and B_2 induce B−A and B−Z conformational transitions in calf thymus DNA and that they differ subtly in their interactions with the polymer.[19] Nevertheless, in none of the above studies were any differences detected between Blm A_2 and B_2 for either ν(Co−OOH) or ν(O−OH). This shows that the bithiazole-bound R group (Figure 1) does not perturb the Co−OOH linkage in the free drug. Furthermore, under the conditions of the experiments shown here, changes in DNA structure that are anticipated to occur upon binding of HOO−Co(III)Blm species (unpublished data), apparently do not alter the vibrational properties of the hydroperoxide ligand.

High-Frequency Raman Spectral Region (1000−1700 cm^{-1}). The high-frequency Raman spectral region of HOO−

(19) (a) Rajani, C.; Kincaid, J. R.; Petering, D. H. *Biopolymers: Nucleic Acid Sci.* **1999**, *52*, 110−128. (b) Rajani, C.; Kincaid, J. R.; Petering, D. H.; *Biopolymers: Nucleic Acid Sci.* **1999**, *52*, 129−146. (c) Rajani, C.; Kincaid, J. R.; Petering, D. H. *Biophys. Chem.* **2001**, *94*, 219−236.

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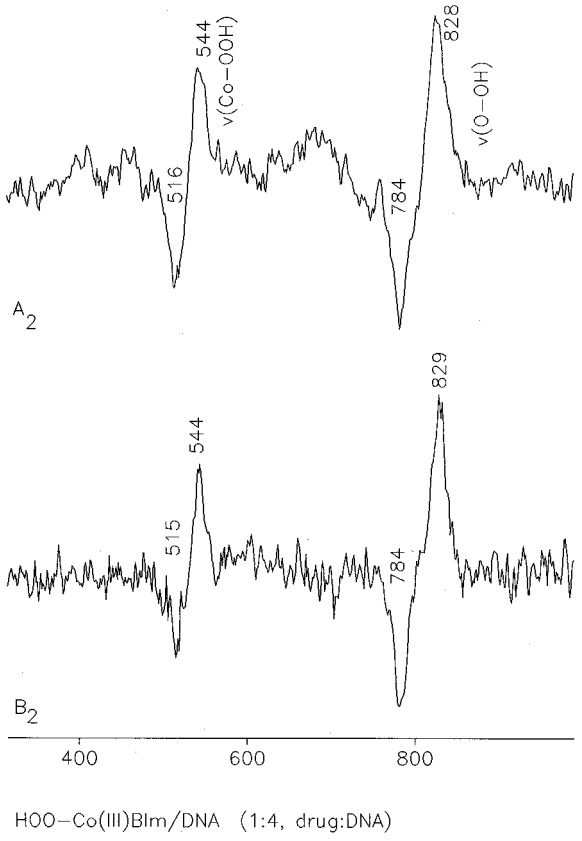

Figure 3. Two difference spectra, Blm A_2 and B_2, obtained by subtracting the spectrum of the ^{18}O isotopomer from that of the ^{16}O isotopomer of HOO−Co(III)Blm bound to calf thymus DNA in a 1:4 ratio of drug:DNA base pairs.

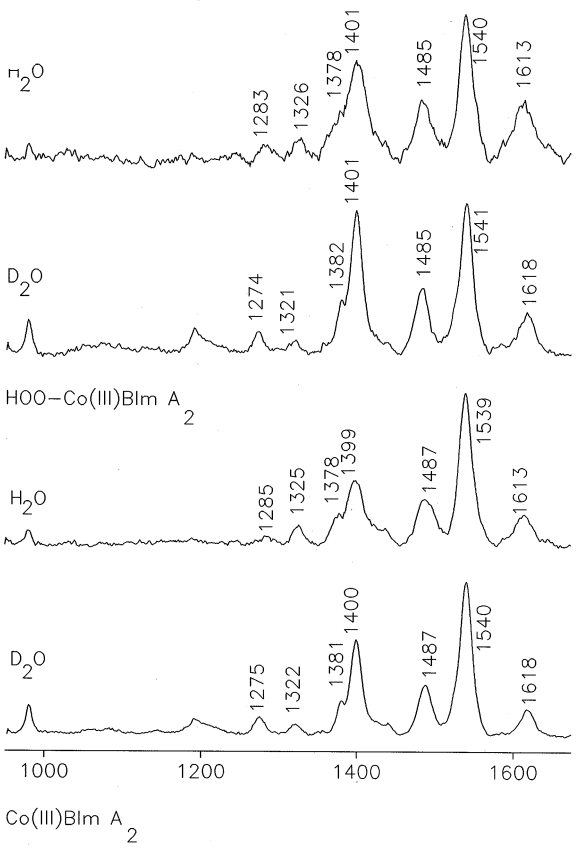

Figure 4. High-frequency resonance Raman spectra for HOO−Co(III)-Blm A_2 and Co(III)Blm A_2 in both H_2O and D_2O buffers.

Co(III)Blm A_2 and Co(III)Blm A_2 in both H_2O and D_2O is shown in Figure 4. Corresponding values obtained experimentally for Blm B_2 as well as the proposed assignments for these modes are summarized in Table 1. In this region the vibrational modes were predominantly associated with the pyrimidine, the deprotonated amide moiety, and the bithiazole rings.[5]

HOO−Co(III)Blm vs Co(III)Blm. The vibrational spectra of HOO−Co(III)Blm and Co(III)Blm were almost identical. Any differences seen between HOO−Co(III)Blm and HO−/H_2O−Co(III)Blm were small (2 cm^{-1}). As such, these results agree with a study of FeBlm that indicated only slight changes in the ligand field upon exchanging −OOH for −OH along with the retention of very similar geometries for these two species.[5b]

Deprotonated Amide Mode (δC−N−C). The 1487 cm^{-1} band of HOO−Co(II)Blm was not altered by a replacement of H_2O with D_2O, indicating that hydrogen bonding is not a significant factor in the strength of this vibration. In DMSO, this vibrational mode appeared at 1474 cm^{-1} for HOO−Co(III)-Blm A_2 and at 1476 cm^{-1} for both Co(III)Blm A_2 and B_2 (Table 3). Thus, the 11 cm^{-1} downshift in DMSO vs H_2O/D_2O for this mode cannot be attributed to a disruption of hydrogen bonding between the bulk solvent and the drug (Table 3). The basis for this perturbation in DMSO is unknown.

There is some discussion regarding the assignment for this vibrational mode. The initial resonance Raman study of Fe(III)Blm and Fe(II)Blm regarded this band as an amide II mode of the metal-coordinated β-hydroxyhistidinyl amide.[9a] The deprotonated amide in FeBlm and CoBlm was thought to have mostly ν(C−N) stretching character in the initial assignment by Takahashi et al.[9a] Subsequently, another resonance Raman study in combination with DFT calculations of a set of model compounds assigned this band to a C−N−C bending mode of the same deprotonated amide moiety. According to this work, the amide II, ν(C−N) mode would occur in a much lower frequency range, i.e., 1150−1250 cm^{-1}.[5b]

Amide I, ν(C═O) Mode of the β-Hydroxyhistidinyl Amide. Two different frequencies have been reported for the amide I band, ν(C═O), of the deprotonated amide moiety in Fe(III)Blm. Using 406.7 nm laser excitation, Takahashi et al.[9a] indicated that, for Fe(III)Blm, this mode occurred at ∼1608 cm^{-1} and was sensitive to the oxidation state of Fe because it downshifted to 1595 cm^{-1} for Fe(II)Blm. This slight weakening of the equatorial metal−ligand bonds upon a decrease in oxidation state from low-spin Fe^{3+} to low-spin Fe^{2+} is reminiscent of heme compounds.[21,22] This mode was also found to be sensitive to ligation at the sixth axial position as it shifted back to 1608 cm^{-1} in CO−Fe(II)Blm.[9]

Neese et al.[5b] used 386 nm excitation and determined a frequency of 1627 cm^{-1} for this same mode in Fe(III)Blm. This difference in frequency could be the result of the quite different solvent conditions employed in the two investigations. Another possibility that cannot be ruled out is that two distinct vibrational

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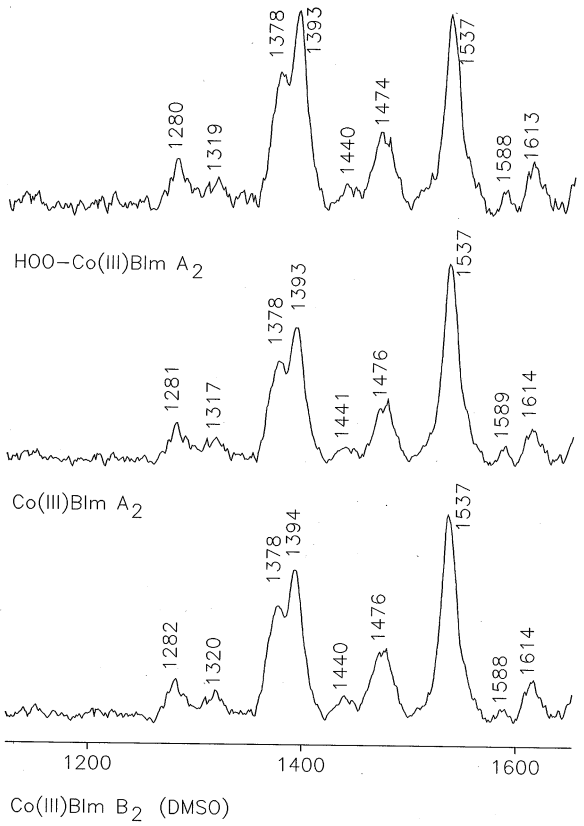

Figure 5. High-frequency resonance Raman spectra for HOO−Co(III)Blm A_2,

modes are being observed in this region by use of different excitation laser lines. In the present study, with 406.7 nm excitation and the solvent conditions employed by Takahashi et al., this mode was observed at 1613 cm^{-1} for both HOO−Co(III)Blm and Co(III)Blm. Evidently, the change in axial ligation did not affect the frequency of the amide I band. In contrast, in D_2O this mode was upshifted by 3 cm^{-1} for FeBlm and by 5 cm^{-1} for CoBlm, indicating the possibility of an H-bonding interaction or vibrational coupling with another mode having exchangeable protons.[9a] In DMSO, an aprotic solvent, this mode was also located at 1613 cm^{-1}, exactly the same position as observed in H_2O buffer (Table 3, Figure 5). Thus, the D_2O effect did not involve the bulk water solvent.

Examination of the original NMR structure of HOO−Co(III)Blm for possible hydrogen-bond partners for the C12 carbonyl oxygen failed to locate a candidate (Figure 1).[6a] However, an unpublished recalculation of the structure based on additional information gleaned from $^1H-^{15}N$ heteronuclear single quantum coherence (HSQC) NMR spectroscopy suggests the possibility that the mannose C-hydroxyl group might be able to form a hydrogen bond with the C12 carbonyl.[23]

Pyrimidine Ring Modes. Four other vibrational modes occurred in the high-frequency resonance Raman spectra (1280−1401 cm^{-1}) of HOO−Co(III)Blm, Co(III)Blm, and the previously examined FeBlm compounds. All have all been assigned to pyrimidine ring stretching modes (Figures 4 and 5 and Tables 1 and 3).[5] Some of them displayed a definite downshift in frequency in D_2O solvent. Thus, with HOO−Co(III)Blm the pyrimidine ring mode at ∼1326 cm^{-1} downshifted by 5 cm^{-1} to 1321 cm^{-1} in D_2O. In DMSO solvent, the same mode was downshifted to 1319 cm^{-1} for HOO−Co(III)Blm A_2. Co(III)Blm A_2 exhibited similar behavior (Table 3). Another pyrimidine ring vibrational mode of HOO−Co(III)Blm and Co(III)Blm that was centered at 1283 cm^{-1} showed a 9−10 cm^{-1} downshift in D_2O and a 3−4 cm^{-1} downshift in frequency in DMSO.

Normal coordinate analysis of simple pyrimidine bases such as uracil have revealed that these modes are complicated combinations of C−H and N−H bending modes as well as C−C and C−N stretching modes.[24] This extensive mixing of ring skeletal with N−H or C−H bending coordinates makes direct H−D exchange correlations difficult because the normal mode composition of a particular vibration may be altered upon deuteration. As a result, the change in frequency of this mode may not be predictable.[24] Nevertheless, a downshift in frequency commonly indicates participation of an H-bond donor atom, probably a N atom, with exchangeable protons.[9,18] Considered in combination with the DMSO effects, these vibrations appear to involve hydrogen bonding with the bulk water.

The vibrational mode at 1401 cm^{-1} for either HOO−Co(III)Blm or Co(III)Blm did not undergo a significant change in frequency in D_2O buffers and, therefore, could not be associated with any vibration involving exchangeable protons. Thus, it was attributed to ν(C−C═C). In the DMSO solvent, however, this vibrational mode downshifted by 5−8 cm^{-1} (Table 3), a change that can only be attributed to some type of solvent−drug interaction. This band, centered at 1388 cm^{-1} in Fe(III)Blm, was sensitive to the oxidation state of Fe such that it occurred at 1392 cm^{-1} for Fe(II)Blm (Table 1).[9a] Unlike heme compounds in which there is a significant difference in the displacement of the metal relative to the plane of the porphyrin rings that occurs with a change in oxidation/spin state of the Fe atom, no such displacement has been documented for the metal center in either FeBlm or CoBlm.[5b,21,22] Thus, the basis for the oxidation state effect on this resonance remains to be defined.

Last, the band centered at ∼1378 cm^{-1} showed a small upshift to 1382 cm^{-1} in D_2O buffers. A possible explanation for the upshift in frequency may be that the mode is a mixture of C−N−C stretching and N−H bending modes, which has been shown to produce upshifts in frequency upon deuteration in other amine-substituted pyrimidine bases.[9,24] The frequency of this mode was unchanged in DMSO from what was observed in H_2O buffer. This could mean that any H-bond interactions associated with this vibration involve another part of the CoBlm molecule and not the water solvent. In particular, in the newly recalculated structure, either the carbonyl or the NH_2 group of the pyridinyl−proponamide region of the structure (C4 and N4) is in the vicinity of the pyrimidinylamine or adjacent ring nitrogen, respectively.[23] Thus, either might make the hydrogen-bonding interaction that is detected in this experiment.

Blm A_2 vs Blm B_2. There were no significant differences in the high-frequency Raman spectral region between Blm A_2 and Blm B_2 for either HOO−Co(III)Blm or Co(III)Blm in terms of band position (Table 1). The intensity of the two overlapping bands centered at 1400 and 1378 cm^{-1} is slightly more intense for CoBlm B_2 vs A_2 complexes. This indicated that the structure

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of the R group (Figure 1) bonded to the bithiazole rings has a minimal impact on the structures of the pyrimidine ring, deprotonated amide, and the metal center−OH/−OOH linkage. This finding is consistent with the NMR structures of HOO−Co(III)Blm and Co(III)Blm, which show that the R group is directed away from and does not make contact with the metal domain−linker region of either molecule.[4,6]

Comparison of Co/FeBlms with Co/Fe Heme Systems. According to the results described here, the vibrational modes of CoBlms display close similarities in frequency, relative intensities, and behavior in D_2O solvent to those of related FeBlms.[5−7] The congruence between the vibrational properties of these structures provides additional support for the hypothesized isostructural relationship between Co- and FeBlms. Three modes have been identified as being sensitive to the oxidation state of the metal center and at least one, centered at 1485 cm^{-1} in HOO−Co(III)Blm, is sensitive to the nature of the axial ligand, i.e., OH vs OOH. The frequencies for the CoBlm vibrational modes are somewhat higher than those observed for FeBlm, a fact that was also found in the comparison of low-spin Co and low-spin Fe heme compounds.[14,15e,22] Because the frequency differences are small, i.e., 5−12 cm^{-1}, the Raman information provided another indication that the metal−ligand structure and metal domain conformation are quite similar in CoBlm and FeBlm species.

The magnitudes of these shifts compare very well with those seen for Co−heme vs Fe−heme species that are low-spin, six-coordinate, and where the metal is not significantly displaced from the plane of the porphyrin rings.[22] Whereas previous analysis of the electronic structure of Fe(III)Blm and HOO−Fe(III)Blm has revealed the metal center to be close to the spin-crossover point between high- and low-spin Fe^{3+}, Co(III)Blm and HOO−Co(III)Blm are low-spin due to the greater value of −10dq associated with Co^{3+} vs Fe^{3+} species.[5b,24] The same situation exists in the comparison of many Fe and Co heme compounds.[14,15e]

In heme complexes, it is well documented that there can be electronic communication between axial ligands, the porphyrin ring, and the metal center.[9,22] In particular, the heme macrocycle supplies a delocalized π-electron buffer for the metal center.[9,21,22] It has been proposed that a similar situation may exist for the metal center in Blm via a conjugated system involving the pyrimidine ring and deprotonated amide.[9] The pervasive effect of metal ligation and oxidation state on the vibrational properties of the deprotonated amide and the pyrimidinyl ligands of FeBlm described above and in ref 9 argue strongly for a significant electronic interaction between these parts of the Blm structure and the iron center.

Conclusion

Resonance Raman studies of HOO−Co(III)Blm and Co(III)Blm (both A_2 and B_2) were performed. Two key vibrational modes, ν(O−OH) and ν(Co−OOH), were identified by ^{18}O substitution in the hydroperoxide ligand. Identification of these two stretches will be helpful in future, time-resolved resonance Raman investigations of the HOO−Fe(III)Blm species, a much more unstable compound, in the continuing effort to fully characterize the mechanism of oxygen activation responsible for sequence-selective DNA strand cleavage. The assignment of the high-frequency resonance Raman vibrational modes for HOO−Co(III)Blm and Co(III)Blm strongly supports an isostructural relationship between FeBlm and CoBlm. Overall, the vibrational behavior of Co- and FeBlms parallels that of Co− and Fe−porphyrin systems.

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