Bleomycin-Iron Complexes. Electron Spin Resonance Study, Ligand Effect, and Implication for Action Mechanism

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Received September 26, 1979

Abstract: From the viewpoint of iron coordination and oxygen activation, the iron complexes of bleomycin (BLM) were investigated by electron spin resonance (ESR) spectroscopy and the findings compared with iron complexes of *i*-BLM, depyruvamide (dep) BLM, and deamido BLM. The oxidation of the 1:1 BLM-Fe^{II} complex efficiently generated reactive oxygen radicals such as superoxide and hydroxyl, which were evident in ESR spin-trapping experiments. In various base adducts of the low-spin BLM-Fe^{III} complex, there was a good correlation between the g_z value and the g value splitting ($g_z - g_x$), which is related to the axial ligation mode to the iron. The ESR parameters ($g_x = 2.039$, $g_y = 1.969$, $g_z = 2.008$, and $A^N = 23.8$ G) of the deamido BLM-$Fe^{II,14}NO$ complex at pH 9.6 are closer to those ($g_x = 2.041$, $g_y = 1.976$, $g_z = 2.008$, and $A^N = 23.6$ G) of the corresponding BLM complex than those ($g_x = 2.052$, $g_y = 2.016$, $g_z = 1.999$, and $A^N = 17.5$ G) of the dep BLM-$Fe^{II,14}NO$ complex. At pH 6.2, the deamido BLM-$Fe^{II,14}NO$ complex presented ESR parameters ($g_x = 2.050$, $g_y = 2.013$, $g_z = 1.998$, and $A^N = 17.6$ G) similar to the corresponding dep BLM complex. Deamido BLM, which is a product of BLM inactivated by BLM hydrolase, clearly showed the pH-dependent axial donor change in its Fe(III) complex as well as in the Fe^{II}-NO adduct complex. Of interest is the roughly parallel relationship between the spin concentration of hydroxyl radical spin adduct and the biological activity of DNA breakage reaction in BLM and its related compounds. The present results show the important effects of the fifth axial nitrogen coordination to iron on oxygen activation of the BLM antibiotics and of site-specific free radical on DNA cleavage by BLM.

Introduction

The bleomycins (BLM), which were originally isolated as a Cu(II) complex from a culture of *Streptomyces verticillus*,[1] are a family of histidine-containing glycopeptide antibiotics and have been clinically prescribed for certain tumors.[2] Cleavage of cellular deoxyribonucleic acid (DNA) by BLM probably accounts for the antibiotic and antitumor activities of this drug. BLM binds to and cleaves DNA in a reaction that depends on the presence of ferrous ion and molecular oxygen.[3] Ferric ion cannot replace ferrous ion in the degradation of DNA with BLM, but in the presence of a reducing agent either Fe(II) or Fe(III) greatly stimulates DNA degradation by BLM.[4] Divalent metal ions such as Cu(II), Zn(II), and Co(II) inhibit the BLM-induced damage to DNA.[5] The BLM antibiotic has both DNA-interacting and metal-binding sites, and the biological activity is related to this bifunctionality. Fluorescence studies revealed that the bithiazole portion of BLM preferentially binds to the guanine base in nucleic acids, and the positive charge at the terminal amine portion facilitates BLM binding to DNA.[6] Recent experiments demonstrated that guanine(G)-cytosine(C) and guanine(G)-thymine(T) sequences are preferentially cleaved by BLM in the presence of ferrous ion.[7] On the other hand, we have clarified that naturally occurring BLM-Cu^{II} complex has substantially a square-pyramidal configuration with four chelate rings of 5-5-5-6 ring members and that equatorial Cu(II)-binding donors between BLM and human serum albumin are similar.[8] The comparison of several metal complexes of BLM and its biosynthetic intermediate (P-3A) also showed the importance of the β-aminoalanine-pyrimidine-histidine portion for the metalcoordination of BLM.[9] In fact, recent X-ray crystallographic analysis of the 1:1 P-3A-Cu^{II} complex supported a distorted square-pyramidal structure which involves the secondary amine nitrogen, pyrimidine ring nitrogen, deprotonated peptide nitrogen of histidine residue, histidine imidazole nitrogen, and α-amino nitrogen as the coordination donors.[10] In addition, in preliminary electron spin resonance (ESR) work, evidence was obtained that the 1:1 BLM-Co^{II} complex with a square-pyramidal geometry incorporates dioxygen molecule into the vacant sixth coordination site.[11] It has been reported that reducing agents[12] and the superoxide radical[13] as well as ferrous ions stimulate the DNA degradation reaction by BLM. Lown and Sim have speculated that the DNA breakage by BLM is prevented by free radical scavengers such as superoxide dismutase, catalase, and 2-propanol.[14] These observations strongly suggest that reactive free radicals such as $O_2^{-}\cdot$ and $\cdot OH$ are responsible for the oxidative cleavage of DNA by BLM. Indeed, we reported the detection of the oxygen radicals from the BLM-Fe^{II} complex system by using an ESR spin-trapping technique.[15] Oberley and Buettner also observed the production of hydroxyl radical from the BLM-Fe^{II} system by the similar spin trapping.[16] Therefore, the studies on iron complexes of BLM antibiotics and their oxygen activation are essential for the elucidation of the mechanism of action of the DNA breakage by BLM.

The iron complexes of BLM were compared with those of related compounds such as *i*-BLM, depyruvamide (dep) BLM, and deamido BLM, and in particular the axial donor effect on iron coordination and oxygen activation by these BLM antibiotics is described. *i*-BLM[17] is the product of carbamoyl migration in the sugar of BLM molecule, dep BLM[18] lacks the α-amino group

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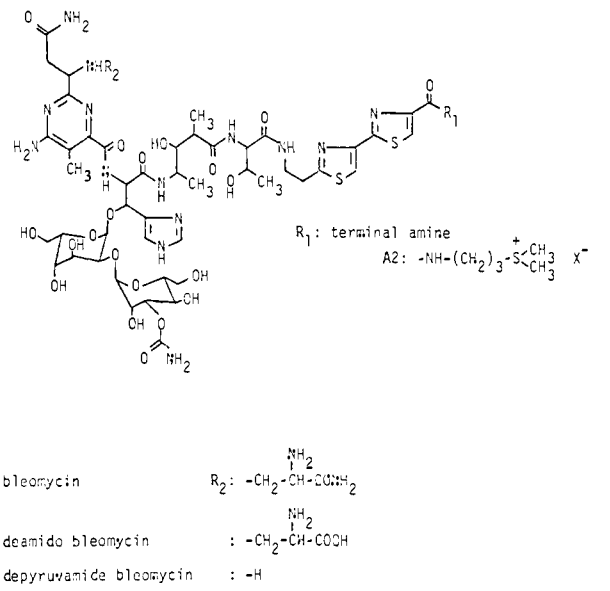

Figure 1. BLM and its related compounds.

of the β-aminoalanine portion in BLM ligand, and deamido BLM[19] is a product of BLM inactivated by BLM hydrolase (see Figure 1). The findings are most useful and should provide a better understanding of the molecular mechanism of this important therapeutic drug.

Experimental Section

Materials. Purified BLM-A_2, *i*-BLM-A_2, dep BLM-A_2, and deamido BLM-A_2, which contain (3-aminopropyl)dimethylsulfonium as terminal amine, were kindly provided Nippon Kayaku Co. Ltd. The standard Fe(II) solution was prepared from reagent grade material, $FeSO_4 \cdot 7H_2O$. The spin-trapping agents, *N-tert*-butyl-α-phenylnitrone (BPN) and 5,5-dimethyl-1-pyroline *N*-oxide (DMPO), were obtained from the Aldrich Chemical Co., and DMPO was purified by filtration with charcoal. Calf thymus DNA was purchased from P-L Biochemicals. The dialyzed solution of DNA was stored at 4 °C and the final concentration determined by phosphate analysis.[20] Catalase (bovine liver; 3000 units/mg) and superoxide dismutase (bovine blood; 2700 units/mg) were obtained from Sigma Chemical Co. All other chemicals were of the highest grade available. Distilled and deionized water was used throughout the experiments.

Sample Preparation. The 1:1 BLM-Fe^{II} complex was prepared by mixing BLM-A_2 and Fe(II) ion in aqueous solution (pH 6.8) under fully deaerated condition. Anaerobiosis was effected by using a septum-stoppered thunberg tube equipped with a side arm to permit anaerobic mixing of the reaction components. The 1:1 Fe(III) complexes of these antibiotics were produced by the oxidation of the corresponding Fe(II) complexes with oxygen or by adding 1 equiv of Fe(III) ion to the aqueous solution (pH 6.8) containing BLM-A_2 or dep BLM-A_2. The BLM-Fe^{III} complex is most stable and was isolated as a solid. The iron(II)-nitric oxide adduct complexes of dep BLM and deamido BLM were prepared by addition of a few milligrams of $Na^{14}NO_2$ (or $Na^{15}NO_2$, 99.2 atom % in ^{15}N, The British Oxygen Co.) and sodium borohydride to the solution of the 1:1 antibiotic Fe(II) complexes according to the previous procedure for the case of BLM-Fe^{II}·NO complex.[21] The technique was convenient because the oxygen was removed by the excess reducing agent and precautions to maintain the solution aerobic were not required.

Spin Trapping. The reaction mixture for spin trapping consisted of 1:1 antibiotic Fe(II) complex (1.0–0.02 mM) and BPN (0.08 M, ethanol solution) in buffered solution (pH 6.9). Oxygen was bubbled through the mixture for 5 s, and then an aliquot of the sample solution was rapidly transferred to a quartz cell for ESR measurements at 25 °C. BPN has the advantage of forming very stable spin adducts.

ESR Measurements. The Fe^{II}- and Fe^{II}·NO complexes formed were anaerobically transferred to an ESR sample tube and immediately frozen in liquid nitrogen (77 K). The time course of ESR spectral changes of the BLM-Fe(II) complex (1.0 mM) by oxygen bubbling was investigated at pH 6.9 and measured at 77 K. X-Band ESR spectra were recorded with a JES-FE-3X spectrometer operating with 100-kHz magnetic field modulation. The *g* values were determined by taking Li-TCNQ (g = 2.0026) as standard, and the magnetic fields were calculated by the splitting of Mn(II) in MgO (ΔH_{3-4} = 86.9 G) and Fremy's salt (a_{10}^N = 13.09 G).

Results and Discussion

ESR Spectral Change in Oxidation of BLM-Fe^{II} Complex by Molecular Oxygen. Although the pale pink-orange colored 1:1 BLM-Fe^{II} complex, which has a visible absorption maximum at 475 nm (ε 380), is ESR inactive at 77 K, the exposure of this complex to oxygen yielded ESR active species. Figure 2 shows the time course of the ESR spectral change of the BLM-Fe^{II} complex by oxygen bubbling at pH 6.9. At the initial reaction stage, the low-spin Fe(III) complex species with small rhombic splitting of the *g* values (g_z = 2.254, g_y = 2.171, and g_x = 1.937) was clearly observed together with a minor free-radical species near g = 2.005. However, the Fe(III) species is an unstable and transient species. At the final reaction stage, another stable BLM-Fe^{III} complex species was formed. This ESR feature is typical of low-spin Fe(III) type and characterized by the large rhombic splitting (g_z = 2.431, g_y = 2.185, and g_x = 1.893). The orange-yellow colored BLM-Fe^{III} complex was stable over a period of several days and presented a visible absorption shoulder near 370 nm (ε 2200). In addition, the Fe(III) complex species prepared from BLM and the ferric ion at pH 6.9 was identical with this species. The BLM-Fe^{III} complex generated by the oxidation of the corresponding Fe(II) complex or by the complexation of BLM and the Fe(III) ion easily underwent reduction by reducing agents such as sodium dithionite, sodium borohydride, and L-ascorbic acid.[22] During the recycle of the redox reaction of the BLM-Fe complex, reactive free radicals such as $O_2^{-\cdot}$ and ·OH are produced as demonstrated by the ESR spin-trapping experiment.[15] On the basis of the splitting of the *g* values in their ESR spectra, the coordination properties of the two BLM-Fe^{III} complex species are discussed in the following section. The radical ESR spectrum shows the 1:2:1 triplet signal with g = 2.0046 and a = 28.6 G, which may be attributed to the pyrimidine nitrogen radical in the BLM molecule. It has been reported that metal-free BLM is partly decomposed in the presence of Fe(II) and molecular oxygen and that the ^{13}C NMR spectrum of the product indicates a transformation of the pyrimidine chromophore in the BLM ligand.[23] However, a more definite assignment of the structure of the free-radical spectrum requires further investigation.

Of special interest is the oxidation of the BLM-Fe^{II} complex by molecular oxygen in the presence of DNA. As shown in Figure 3, in this case, the ESR signal of superoxide-like free radical was clearly detected together with the low-spin Fe(III) signal (g_z = 2.254, g_y = 2.171, and g_x = 1.937) at the initial reaction stage. Repeated experiments have established that the spectrum is reproducible, under similar conditions. The observed *g* values ($g_\perp$ = 2.006 and $g_\parallel$ = 2.030) of the free radical are close to those ($g_\perp$ = 2.006 and $g_\parallel$ = 2.080)[24] of the typical superoxide signal. The slight difference of the $g_\parallel$ value may be due to the difference of orientation of the superoxide molecule. In fact, the ESR $g_\parallel$ value of the superoxide radical has been known to change by variation of solvents.[25] We have already suggested that in the BLM-Fe^{II}·NO and BLM-Co^{II}·O_2 complexes, the orientation of the bound NO and O_2 molecules relative to the Fe(II) and Co(II) planes is somewhat different in the presence and absence of DNA.[11,21]

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(23) (a) Takita, T.; Muraoka, Y.; Nakatani, T.; Fujii, A.; Umezawa, Y.; Naganawa, H.; Umezawa, H. *J. Antibiot.* **1978**, 31, 801–804. (b) Takita, T.; Muraoka, Y.; Nakatani, T.; Fujii, A.; Iitaka, Y.; Umezawa, H. *J. Antibiot.* **1978**, *31*, 1073–1077.
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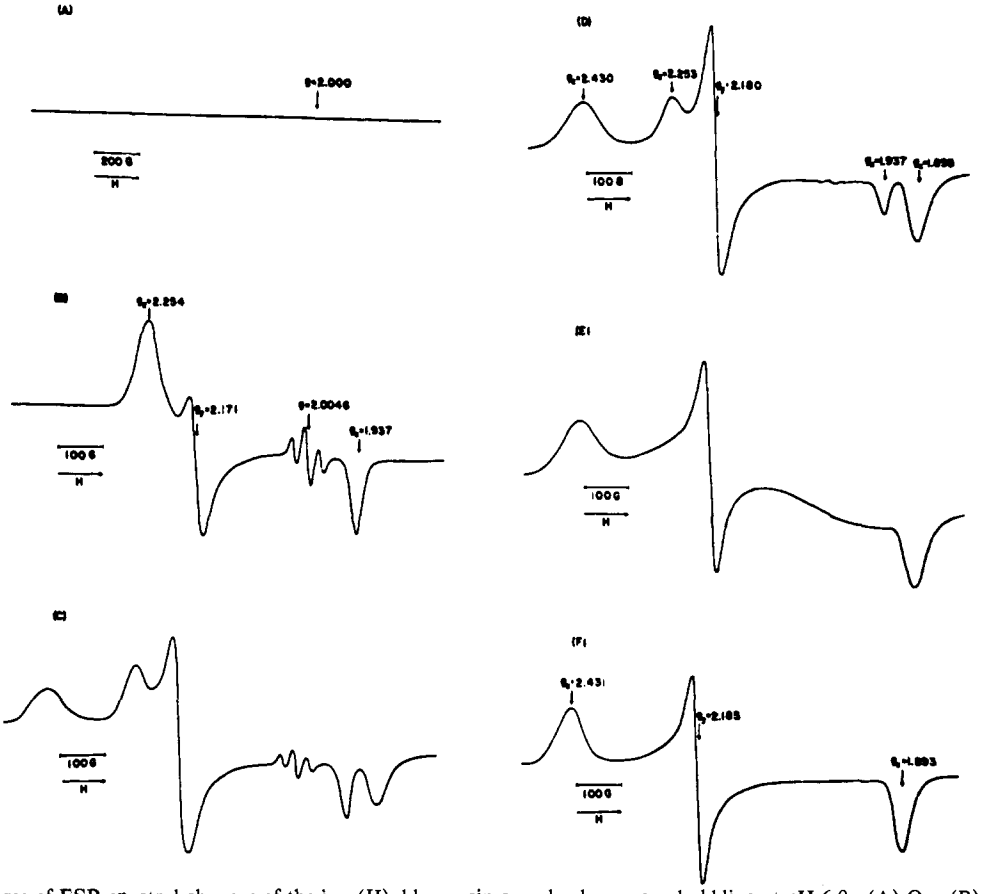

Figure 2. Time course of ESR spectral changes of the iron(II)–bleomycin complex by oxygen bubbling at pH 6.9: (A) O s; (B) 3 s; (C) 6 s; (D) 10 s; (E) 30 s; (F) 90 s. The concentration of the iron(II)–bleomycin complex was 1.0 mM.

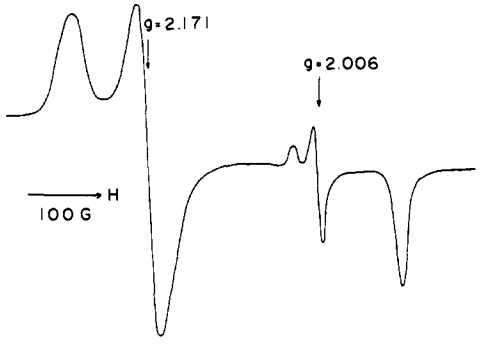

Figure 3. Intial ESR spectrum of the BLM–Fe^{II} complex system obtained by oxygen bubbling in the presence of DNA. The spectrum was measured after 0.5 s of the reaction, and the concentrations of the BLM–Fe^{II} complex and DNA were 1.0 and 0.02 mM, respectively.

Our previous potentiometric titration showed that the 1:1 BLM–Fe^{II} complex has equilibrium constants, log K = 7.43 and pK_c = −5.48, and that BLM behaves as at least a tetradentate ligand by the coordinations of its α-amino, secondary amine, deprotonated peptide, and histidine imidazole groups.[8] Recent results of ^{13}C NMR spectroscopy are consistent with the Fe(II) chelation by the amine–pyrimidine–imidazole portion.[26] A higher oxidation state of iron is favored in a strong ligand field. Accordingly, if the 1:1 BLM–Fe^{II} complex includes five nitrogen coordinations of α-amino, secondary amine, pyrimidine, deprotonated peptide, and imidazole groups, this complex should show a strong tendency to undergo oxidation via an oxygenated intermediate to the corresponding Fe(III) complex. Gupta et al. have also described its magnetic state in the BLM–Fe^{II} complex by NMR measurements which revealed the presence of a high-spin ferrous ion (S = 2), under anaerobic conditions.[26b,27] An S = 2 spin state has a relative short spin–lattice time, and such ESR spectra of high-spin Fe(II) complexes are difficult to obtain.

ESR Characteristics of BLM–Fe^{III} and Its Base Adduct Complexes. We previously reported that the ESR parameters of the stable BLM–Fe^{III} complex closely resemble that (g_z = 2.4, g_y = 2.2, and g_x = 1.9) of methemoglobin hydroxide and that the arrangement of ligands in the low-spin BLM–Fe^{III} complex mimics that in the hemoproteins.[21] In this study, various base adducts of the BLM–Fe^{III} complex were found to form when excess bases (10–100 times) were added. Some representative ESR spectra contrasting different ligation modes are shown in Figure 4. Although their spectra are of a rhombic low-spin type, the separated g values are largely different from the value of the parent BLM–Fe^{III} complex. Table I summarizes the ESR parameters and the g value splitting ($g_z - g_x$) of various BLM–Fe^{III}·base adducts, together with those of several base adducts of protoporphyrin IX dimethyl ester (PPIX DME)–Fe^{III}.[28] The estimated g values are effective g values, and the orientation of g values is tentatively assigned.[29] Three general types of axial ligation modes in the BLM–Fe^{III}·X complex as shown in Figure 5 were accessible by experimentation, and the g values were examined here in terms of their utility as empirical diagnostic indicators of axial coordination, an approach elaborated in crystal field terms by Peisach and Blumberg and embodied in their "truth" diagrams for low-spin ferric hemes.[30] Indeed, axial ligation modes of oxidized cyto-

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(27) The Mössbauer parameters of the BLM–Fe^{II} complex are typical of a high-spin S = 2 state (unpublished result).

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(29) On the basis of the similarity of crystal field parameters between the stable BLM–Fe^{III} complex and methemoglobin hydroxide, the notation of g values in the BLM–Fe^{III} complexes was employed according to that in the hemoproteins.

Table I. ESR Spectral Data of Low-Spin $Fe^{III}X$ Complexes of Bleomycin and Porphyrin

	Complex	Base(X)	g_z	g_y	g_x	g_{av}	g_z-g_x	pK_a
1	Fe(BLM)	(H_2O)	2.254	2.171	1.937	2.121	0.317	
2	Fe(BLM)	OH^-	2.431	2.185	1.893	2.170	0.538	
3	Fe(BLM)	H_2S	2.223	2.148	1.999	2.123	0.224	
4	Fe(BLM)	α-MPG	2.310	2.184	1.948	2.147	0.362	8.74
5	Fe(BLM)	$HO(CH_2)_2SH$	2.316	2.195	1.945	2.152	0.371	9.43
6	Fe(BLM)	$(H_3C)_2N(CH_2)_2SH$	2.330	2.203	1.937	2.157	0.393	10.71
7	Fe(BLM)	$HO(CH_2)_2OH$	2.417	2.192	1.896	2.168	0.521	
8	Fe(BLM)	C_6H_5OH	2.423	2.187	1.895	2.168	0.528	
9	Fe(BLM)	Im	2.456	2.183	1.890	2.176	0.566	6.95
10	Fe(BLM)	N-MeIm	2.460	2.180	1.888	2.175	0.572	7.25
11	Fe(BLM)	$(CH_3)_3CNH_2$	2.497	2.181	1.866	2.181	0.631	10.45
12	Fe(BLM)	$CH_3(CH_2)_3NH_2$	2.537	2.179	1.850	2.189	0.687	10.60
13	Fe(BLM)	CH_3NH_2	2.540	2.179	1.847	2.189	0.693	10.62
14	Fe(BLM)	NH_3	2.545	2.178	1.837	2.187	0.708	
15	Fe(PPIXDME)(N-MeIm)	$HSC_6H_4NO_2$	2.42	2.26	1.91	2.20	0.51	
16	Fe(PPIXDME)(N-MeIm)	$HOC_6H_4NO_2$	2.61	2.21	1.84	2.22	0.77	
17	Fe(PPIXDME)(N-MeIm)	N-MeIm	2.90	2.29	1.57	2.25	1.33	

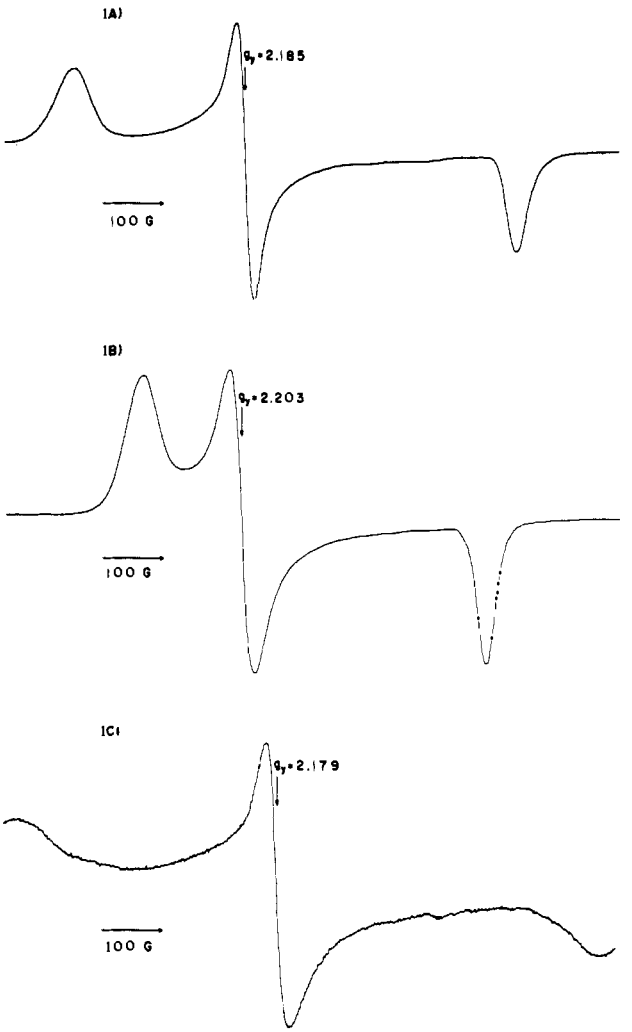

Figure 4. ESR spectra of the (BLM)Fe(OH) (A) (BLM)Fe-$((CH_3)_2NCH_2CH_2S)$ (B), and (BLM)Fe(CH_3NH_2) (C) complexes at 77 K.

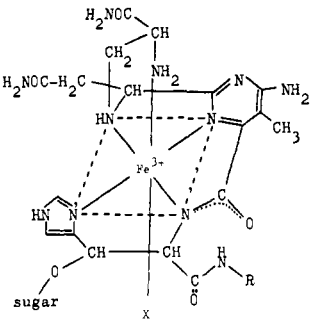

Figure 5. Bleomycin-iron(III) complex.

chrome P-450 have been drawn from the three g values of model low-spin iron(III) porphyrins,[28,31] which can be analyzed in terms of two crystal field parameters V/Δ and Δ/λ, expressions for rhombicity and tetragonality, respectively. The g value splitting ($g_z - g_x$) seems to be related to the axial ligation mode to the iron. Of the three categories of the axial ligation examined, BLM-Fe-N, BLM-Fe-O, and BLM-Fe-S, the BLM-Fe-N ligation type produced the most widely separated g values. In general, the g value splitting of the BLM-Fe^{III}-X complexes decreases in the order of BLM-Fe-N > BLM-Fe-O > BLM-Fe-S ligation modes, consistent with that (N-Fe-N > N-Fe-O > N-Fe-S) of the Fe(porphyrin)LL′ complexes. In addition, there is a good correlation between the ($g_z - g_x$) splitting and the ionization constants (pK_a) of the thiol or nitrogen-base ligands (see Table I). This behavior implies that the g value splitting is closely related to the strength of σ chemical bond from the axial ligands to the iron. Of interest is the linear correlation between the g_z value and the g value splitting in the present (BLM)Fe(X) complexes (see Figure 6). The method employed in this work allows for a clear identification of axial ligands in the above-mentioned two BLM-Fe^{III} complex species. The ESR spectral data provide strong

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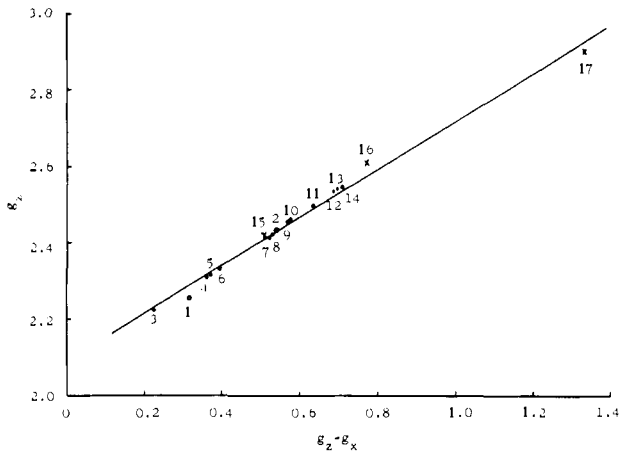

Figure 6. Correlation between the g_z value and splitting of the g values $(g_z - g_x)$.

evidence for the (BLM)Fe(OH^-) coordination mode in the finally stable BLM–Fe^{III} complex with $g_z = 2.431$, $g_y = 2.185$, and $g_x = 1.893$. If the initially unstable BLM–Fe^{III} complex has a pentacoordinated square-pyramidal configuration without a sixth axial ligand, the complex should give less widely separated g values than the (BLM)Fe(H_2S) coordination complex. Most of the five-coordinated Fe(III) complexes are not of a low spin but rather of a high-spin one. Therefore, the most probable axial ligation mode is the (BLM)Fe(H_2O) coordination type for the other unstable species having $g_z = 2.254$, $g_y = 2.171$, and $g_x = 1.937$. Since this assignment assumes a slow proton dissociation, further investigation would be required for the definitive assignment. The present results show that the correlation between the ESR g values and the axial ligation mode can also apply to four different nitrogen ligands instead of the four pyrrols in porphyrins. In ferric complexes of macrocyclic N_4 ligands, the macrocycle ring size has an important effect on the ground-state electronic features.[32] In the BLM–Fe^{III} complexes, it is presumed that its four chelate rings with 5-5-5-6 ring members result in a strong ligand field and present an electronic property similar to that seen with porphyrins. Actually, the dep BLM–Fe^{III} complex which does not have the α-amino group of the β-aminoalanine portion in BLM exhibits the ESR signal at $g = 4.28$, which is typical of high-spin ferric type. The previous potentiometric result indicated that the divalent metal complexes of BLM have substantially a similar coordination core, though their stabilities are in the order Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II). Recent spectroscopic and X-ray crystallographic studies on the Cu(II), Co(II), and Ni(III) complexes of BLM and its biosynthetic intermediate (P-3A) showed the secondary amine nitrogen, pyrimidine ring nitrogen, deprotonated peptide nitrogen of histidine residue, and histidine imidazole nitrogen coordinate as the basal planar donor and the α-amino nitrogen as axial donor and that the metal site is fundamentally a square-pyramidal structure with four chelate rings of 5-5-5-6 ring members. In addition, it has been clarified by ESR spectroscopy that the oxygen molecule is incorporated into the vacant sixth axial coordination site of the 1:1 low-spin BLM–Co^{II} complex. On the basis of these observations and the present results, a similar coordination environment is proposed for the BLM–Fe^{III} complexes (see Figure 5). In the iron(III) complexes of the BLM-related ligands as well as ferric porphyrins, the spin state of the Fe(III) center has been significantly controlled by the nature of the axial ligands (see the following section).

Fe(III) Complex Species of Dep BLM and Deamido BLM. In contrast with the low-spin ($S = {}^1/_2$) Fe^{III}–BLM complex which presents a rhombic ESR signal at near $g = 2$, the 1:1 dep BLM–Fe^{III} complex showed a strong ESR absorption at $g = 4.28$, which is assigned to high-spin ferric ion ($S = {}^5/_2$). The dep BLM ligand differs from the BLM ligand with regard to the fifth axial nitrogen

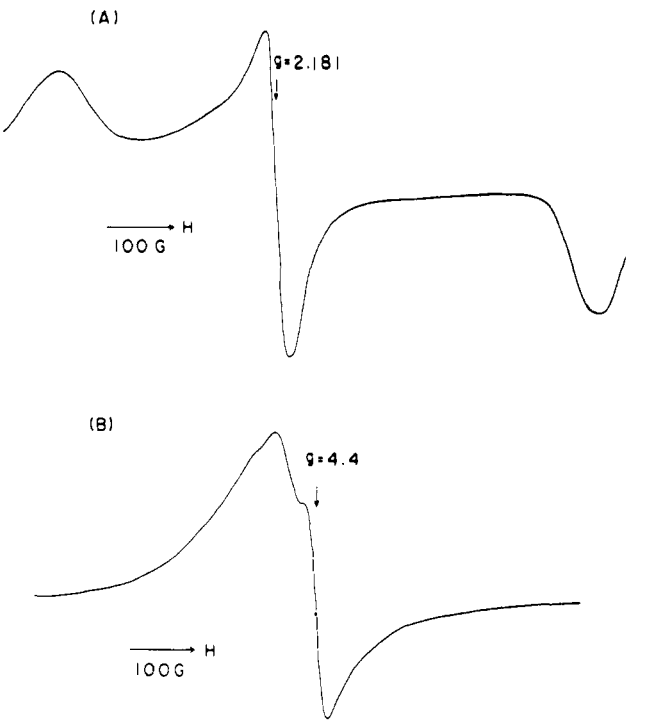

Figure 7. ESR spectra of the deamido BLM–Fe^{III} complex at (A) pH 9.6 and (B) pH 6.2.

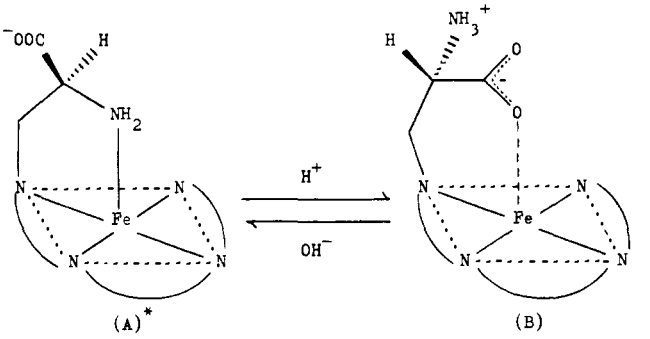

Figure 8. Change of axial donor in the deamido BLM–Fe complex. The structure with the asterisk is the same coordination structure as the BLM–Fe complex, taking no account of the sixth coordination site.

donor for metal coordination, because dep BLM is lacking in the β-aminoalanine portion of the BLM molecule. The result indicates an important effect of fifth axial nitrogen on iron coordination environment and iron spin state of the BLM antibiotics. Figure 7 shows the ESR spectra of the 1:1 deamido BLM–Fe^{III} complex at pH 9.6 and 6.2, respectively. At pH 9.6, the spectral feature is characteristic of the rhombic low-spin Fe(III) type and its ESR parameters ($g_x = 1.874$, $g_y = 2.181$, and $g_z = 2.472$) are close to those ($g_x = 1.893$, $g_y = 2.185$, and $g_z = 2.431$) of the BLM–Fe^{III} complex. The value (0.598) of the g value splitting $(g_z - g_x)$ in the present Fe(III) complex species is larger than that (0.538) in the corresponding, BLM–Fe^{III} complex. The basicity of the α-amino group is considerably higher in deamido BLM ($pK_a = 9.4$)[33] than in BLM ($pK_a = 7.7$).[8] Therefore, the larger splitting is reasonably explained by the higher basicity of the α-amino group in deamido BLM. In addition, the g values of the deamido BLM–Fe^{III} complex were also satisfied with the g_z–$(g_z - g_x)$ plot as shown in Figure 6. At pH 6.2, on the other hand, the deamido BLM–Fe^{III} complex exhibited the ESR absorption with slight rhombicity at near $g = 4.4$. This signal is similar to that of the dep BLM–Fe^{III} complex and is assigned to the high-spin ferric ion. Table II summarizes the g values of these Fe(III) complexes.

(32) Koch, S.; Tang, S. C.; Holm, R. H.; Frankel, R. B. *J. Am. Chem. Soc.* **1975**, *97*, 914–916.

(33) The potentiometric titration of deamido BLM with standard KOH solution showed $pK_a = 9.4$ as the ionization constant of its α-amino group.

Table II. ESR Parameters for Fe^{III} and Fe^{II}·NO Complexes of Bleomycin and Its Related Ligands

Complex	g_x	g_y	g_z	A^N,G	N-hfs (line)
BLM-Fe(III)	1.893	2.185	2.431		
dep BLM-Fe(III)		g=4.28			
iso-BLM-Fe(III)	1.892	2.185	2.430		
deamido BLM-Fe(III) (pH 6.2)		g=4.43			
deamido BLM-Fe(III) (pH 9.6)	1.874	2.181	2.472		
BLM-Fe(II)-^{14}NO	2.041	1.976	2.008	23.6	3
BLM-Fe(II)-^{15}NO	2.040	1.976	2.008	31.6	2
dep BLM-Fe(II)-^{14}NO	2.052	2.016	1.999	17.5	3
dep BLM-Fe(II)-^{15}NO	2.052	2.016	1.998	24.3	2
iso-BLM-Fe(II)-^{14}NO	2.040	1.976	2.008	23.6	3
deamido BLM-Fe(II)-^{14}NO (pH 6.2)	2.050	2.013	1.998	17.6	3
deamido BLM-Fe(II)-^{14}NO (pH 9.6)	2.039	1.969	2.008	23.8	3

The substantial difference for metal coordination between BLM and dep BLM is the fifth axial donor, because dep BLM clearly lacks the fifth axial α-amino nitrogen coordination. In the deamido BLM-Fe^{III} complex, therefore, change in the pH-dependent fifth axial donor is proposed on the basis of these results (see Figure 8). At pH 9.6, the α-amino nitrogen of deamido BLM coordinates to Fe(III) ion as the fifth axial donor as well as BLM, and then the rigid coordination environment with strong ligand field gives a low-spin Fe(III) state. At pH 6.2, the α-amino nitrogen does not bind to Fe(III) ion in either deamido or dep BLM. This weak ligand field leads to a high-spin Fe(III) state for dep BLM and deamido BLM at pH 6.2. The pK_a value of the α-amino group in deamido BLM is considerably higher than that in BLM. Probably, the pH-dependent axial change of deamido BLM ligand is correlated with the increasing basicity of the α-amino group. The presence of the NH_3^+–COO^- zwitterion such as amino acids contributes to the higher basicity of the α-amino group in the deamido BLM ligand. The smaller pK_a value of the α-amino group in BLM ligand reflects an electron-withdrawing effect of the $CONH_2$ group.

Fe^{II}·NO Adduct Complexes of BLM, Dep BLM, and Deamido BLM. We have already reported that the 1:1 BLM-Fe^{II} complex forms a stable nitrosyl adduct complex and that its ESR parameters are similar to those of the ferrous nitrosyl complexes of hemoproteins such as cytochrome P-450, peroxidase, and hemoglobin.[21] In the BLM-Fe^{II} complex which probably has square-pyramidal geometry and one axial nitrogen donor, the NO molecule is incorporated into the vacant sixth axial coordination site of the iron. In fact, the ESR spectrum of the BLM-$Fe^{II,14}$NO complex exhibits rhombic symmetry with a triplet hyperfine interaction in the central g_z signal, and its ESR feature is typical of six-coordinated type. Figure 9 shows the ESR spectrum of the dep BLM-$Fe^{II,14}$NO (or ^{15}NO) complex in comparison with that of the corresponding BLM complex. The ESR parameters (g_x = 2.052, g_y = 2.016, g_z = 1.999, and A^N = 17.5 G) of the dep BLM-$Fe^{II,14}$NO complex are clearly different from those (g_x = 2.041, g_y = 1.976, g_z = 2.008, and A^N = 23.6 G) of the BLM-$Fe^{II,14}$NO complex. The former is characteristic of the five-coordinate type (N splitting on g_{min}) and the latter of the six-coordinate type (N splitting on g_{mid}).[34] Dep BLM lacks the α-amino group of the β-aminoalanine portion in the BLM molecule and hence the fifth axial amino ligand is not expected. The substitution of ^{14}NO by ^{15}NO gave the transition from a triplet to a doublet in the g_z absorption with a concomitant change in the nitrogen hyperfine constant from 17.5 to 24.3 G (see Table II). These changes are consistent with the nuclear spin (I) and magnetogyric ratio (γ_N) of ^{14}N (I = 1 and γ_N = 1.934) and ^{15}N (I = $^1/_2$ and γ_N = −2.712) nuclei.[35] The three-line g_z signal for the BLM-$Fe^{II,14}$NO complex has been interpreted in terms of a weakened fifth axial ligand (α-amino group)-to-iron bonding with a concomitantly stronger NO-to-iron bonding.[21] The nitrosyl adduct of the 1:1 *i*-BLM-Fe^{II} complex exhibited an ESR spectrum the same as that of the BLM-Fe^{II}·NO complex, suggesting no participation of the carbamoyl group in the sugar portion toward NO coordination. The weak participation of the carbamoyl group in the BLM ligand has been presumed for Cu(II),[8] Ni(III),[36] and Zn(II)[37] coordinations.

On the other hand, deamido BLM formed two iron nitrosyl complex species as well as the previous Fe(III) complexes at the variation of reaction pH. At pH 9.6, the ESR feature of the deamido BLM-$Fe^{II,14}$NO complex is remarkably close to that of the BLM-$Fe^{II,14}$NO complex, and its central resonance at g = 2.008 has a well-resolved triplet hyperfine structure with 23.8-G splitting. At pH 6.2, on the contrary, the deamido BLM-Fe^{II}·^{14}NO complex exhibited an ESR spectrum similar to the dep BLM-$Fe^{II,14}$NO complex. The ESR parameters of these iron nitrosyl complex species are also shown in Table II. The fingings strongly indicate the pH-dependent axial donor change in the deamido BLM-Fe^{II}·NO complex, as seen in the deamido BLM-Fe^{III} complexes. A similar phenomenon was also observed in the 1:1 Co(II) complex of deamido BLM.[38] At a physiological pH region, it is interesting that deamido BLM has a weaker axial donor such as a carboxyl group in contrast with the stronger axial α-amino nitrogen coordination of BLM.

Implication for Action Mechanism of DNA Cleavage by BLM and for Inactivation Mechanism of BLM by BLM Hydrolase. As discussed above, studies with BLM and its related compounds strongly indicated that the axial donor ligand is crucial for the

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(36) Sugiura, Y.; Mino, Y. *Inorg. Chem.* **1979**, *18*, 1336–1339.

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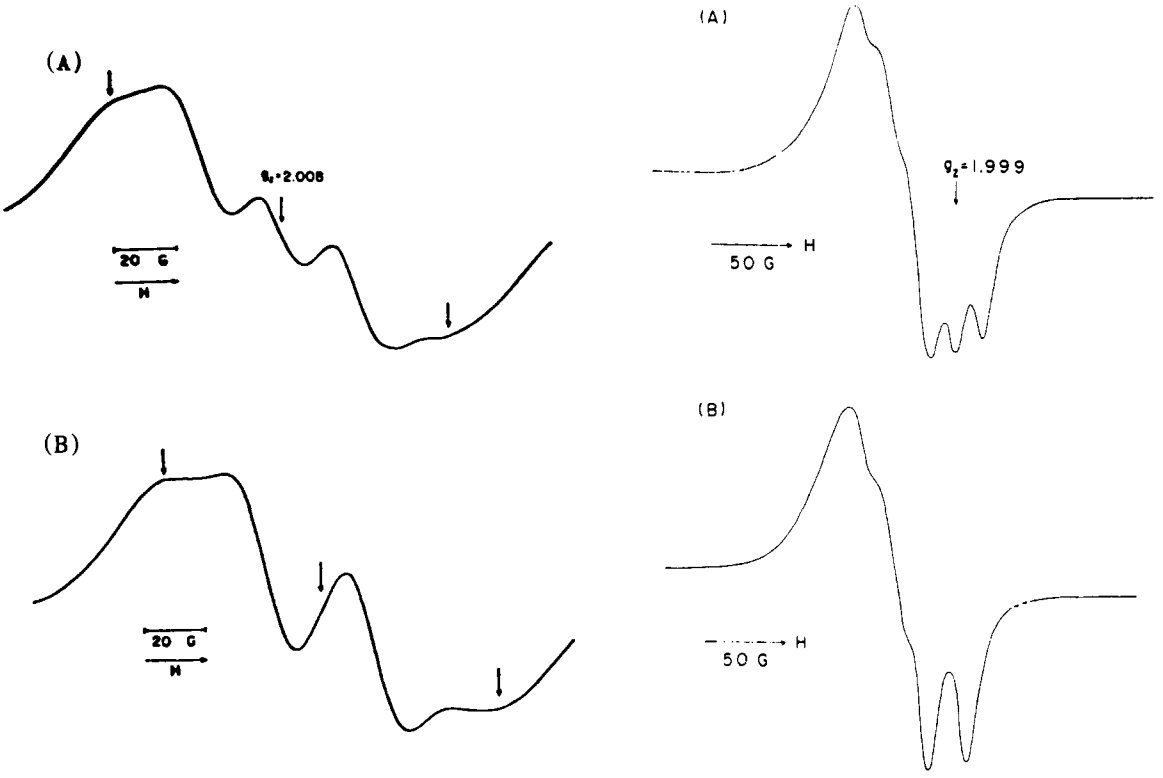

Figure 9. ESR spectra of ^{14}NO (A) and ^{15}NO (B) adducts of the BLM-Fe^{II} (left) and BLM-Fe^{II} (right) complexes at 77 K.

Table III. Biological Activity of DNA Cleavage Reaction and Spin Concentration of Oxygen Free Radical by the Iron(II) Complex Systems of Bleomycin and Its Related Compounds

	Biological Activity	Spin Concentration
Bleomycin	100 %	1
Iso-Bleomycin	32	1/1.4
Deamido Bleomycin	1	1/90
Depyruvamide Bleomycin	0.6	1/100

binding and spin state of iron. The importance of the fifth axial ligand for the activation of molecular oxygen at the sixth coordination site has been inferred from a vast number of works on porphyrins. Therefore, BLM and its modified ligands were investigated and compared from the viewpoint of oxygen activation. The ESR spectrum which was generated by the oxygen bubbling to the 1:1 BLM-Fe^{II} complex (1.0 mM), in the presence of *N*-*tert*-butyl-α-phenyl nitrone (BPN), can be analyzed in terms of the parameters $g = 2.0057$ and $a^N = 15.3$ G which are identical with those found for the ·OH spin adduct of BPN.[15a] At 0.02 mM concentration of the BLM-Fe^{II} complex, the ESR spectrum with $g = 2.0057$, $a^N = 14.9$ G, and $a_\beta^H = 2.8$ G is typical of that of $O_2^{-}\cdot$ or $HO_2\cdot$ adduct of BPN.[15a] The pK_a for the acid-base equilibrium ($HO_2\cdot \rightleftharpoons O_2^{-}\cdot + H^+$) of $HO_2\cdot$ radical species is 4.4 ± 0.4,[39] and it is uncertain as to whether BPN traps $O_2^{-}\cdot$ directly or $HO_2\cdot$ followed by protonation. The result of the present BPN spin trapping provides evidence that the hydroxyl and superoxide radicals are effectively produced from the BLM-Fe^{II}·O_2 complex system. The hydroxyl radical was also detected by the DMPO spin adduct which has a 1:2:2:1 quartet pattern, $a^N = a_\beta^H = 15.2$ G, and $g = 2.0058$.[15b] It has been reported that the $O_2^{-}\cdot$ spin adduct of DMPO is considerably unstable ($T_{1/2}$ = 35 s at pH 8 and 80 s at pH 6).[40] In addition, these reactions were stongly inhibited by the addition of catalase (5 mg) or superoxide dismutase (5 mg) which is a scavenger of H_2O_2 or $O_2^{-}\cdot$. On the basis of the present spin trapping and the above-mentioned ESR study of the BLM-Fe complexes, it is proposed that the superoxide and hydroxyl radicals are generated during the reversible redox reaction of the 1:1 BLM-Fe complex.[41] Figure 10 shows the results of the spin-trapping experiment by these Fe(II) complex systems at pH 6.9. In contrast with the BLM-Fe^{II}·O_2 system, the production of the hydroxyl radical by the corresponding deamido and dep BLM complex systems was remarkably low. The radical spin concentrations of the deamido BLM and dep BLM complex systems were estimated to be approximately one-nineth and one-hundredth of that of the BLM complex system, respectively. It is presumed that the large difference in the oxygen activation is attributed to the difference of the fifth axial coordination donor among BLM, deamido BLM, and dep BLM. Here, it is of particular importance to note that the fifth axial nitrogen donor and rigid square-pyramidal coordination environment are essential for effective oxygen binding and efficient oxygen reduction by the iron complexes of the BLM antibiotics. In addition, the biological activity of deamido BLM and dep BLM for DNA cleavage reaction has been estimated to be approximately 1% to that of BLM.[42] Table III shows the roughly parallel relationship between the spin concentration of hydroxyl radical BPN adduct

(39) Sehested, K.; Rasmussen, O. L.; Fricke, H. *J. Phys. Chem.* **1968**, *72*, 626-631.
(40) Buettner, G. R.; Oberley, L. W. *Biochem. Biophys. Res. Commun.* **1978**, *83*, 69-74.
(41) Our cyclic voltammorgram showed a reversible redox reaction and E_p (pH 6.9) = +165 mV for the BLM-Fe complex.
(42) Takahashi, K.; Ekimoto, H.; Aoyagi, S.; Koyu, A.; Kuramochi, H.; Yoshioka, O.; Matsuda, A.; Fujii, A.; Umezawa, H. *J. Antibiot.* **1979**, *32*, 36-42.

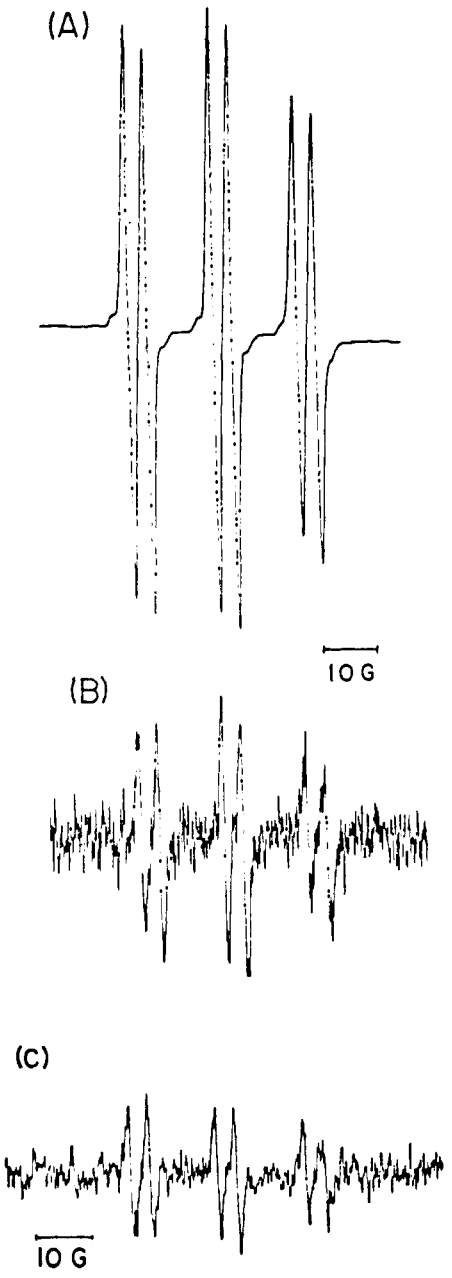

Figure 10. ESR spectra obtained by oxygen bubbling of the BLM-Fe^{II}, deamido BLM-Fe^{II}, and dep BLM-Fe^{II} complexes in the presence of BPN and pH 6.9: (A) 1.0 mM BLM-Fe^{II} complex and 0.08 M BPN; (B) 1.0 mM deamido BLM-Fe^{II} complex and 0.08 M BPN; (C) 1.0 mM dep BLM-Fe^{II} complex and 0.08 M BPN. Conditions of ESR spectroscopy: microwave powder, 10 mW; modulation amplitude, 0.5 G; time constant, 0.01 (A), 0.03 (B), and 0.1 (C) s.

and the biological activity of DNA breakage reaction in these BLM antibiotics. These observations clearly indicate that potential reactive oxygen radicals such as $O_2^{-}\cdot$ and ·OH generated from the BLM-$Fe^{II}\cdot O_2$ complex system are responsible for the oxidative cleavage of DNA by BLM. Caspary et al. have reported that the oxidation reaction of Fe(II) and the reduction reaction of oxygen by BLM follow classical Michaelis-Menten kinetics (V_{max} = 0.27 μ mol/min/mL and K_m = 1.8 mM), and that 1 mol of BLM turns over approximately 5000 mol of Fe(II)/min.[43] The antineoplastic properties of BLM have been thought to arise from its strand scission of cellular DNA.[12,44] Fluorescence and ^1H NMR

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(44) (a) Suzuki, H.; Nagai, K.; Akutsu, E.; Yamaki, H.; Tanaka, N.; Umezawa, H. *J. Antibiot.* **1970**, *23*, 473-480. (b) Haidle, C. W. *Mol. Pharamcol.* **1971**, *2*, 645-652.

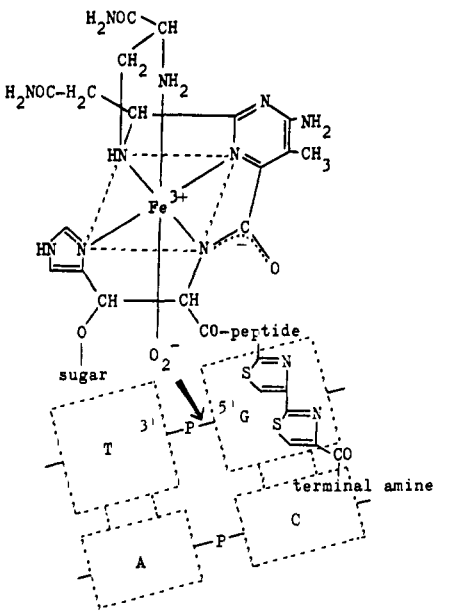

Figure 11. Active iron-oxygen species of bleomycin for DNA cleavage.

spectroscopic data have indicated that BLM binds preferentially to the guanine base of DNA with its bithiazole portion and that the equilibrium constant for the BLM-DNA complex is 1.2×10^{-5} M^{-1}.[6] In the presence of ferrous ion and molecular oxygen, in fact, BLM promotes cleavage at G-T and G-C sequences.[3,7] The inhibition of this DNA degradation reaction by Co(II), Cu(II), and Zn(II) ions[5] can be explained by block of the Fe(II) binding to BLM by these metals, consistent with the order of the formation constants for the BLM-metal complexes, Cu(II) > Zn(II) > Co(II) > Fe(II).[8] The inhibition by chelating agents such as EDTA and deferoxamine[5] is probably due to trace amounts of the Fe(II) ion trapped in the reaction system. In the stimulation of the DNA cleavage by thiol compounds,[44a] it is speculated that thiols play a role in recycling the redox reaction of the BLM-Fe complex.

BLM hydrolase, which is distributed in a variety of organs and is a kind of aminopeptidase,[19] inactivates BLM to produce deamido BLM. This enzyme activity in tumor cells is related to their sensitivity to BLM, that is, the higher the enzyme activity, the lower the BLM sensitivity. The lower biological activity of deamido BLM is probably due to the less effective oxygen activation of its Fe(II) complex, of which the fifth axial coordination donor is not occupied by the α-amino nitrogen, but predominantly by the carboxyl group or aquo molecule, at the physiological pH region. Thus, the inactivation mechanism of BLM hydrolase is interpreted in terms of decreasing oxygen activation, as based on axial donor change to iron.

In conclusion, two characteristics are necessary for antineoplastic action of BLM. First, the amine-pyrimidine-imidazole portion of the drug is capable of oxygen activation by the complexation with Fe(II) ion. Second, the bithiazole region of the antibiotic has an affinity for the guanine base of DNA. In the presence of BLM and Fe(II), cleavage at G-C and G-T sequences was promoted and pyrimidine bases located to the 3′ side of guanosine were released preferentially.[3] Therefore, the "site-specific oxygen radical", produced from an active BLM-Fe species as illustrated in Figure 11, would account for the action mechanism of DNA cleavage by BLM.

Acknowledgments. Gratitude is due to Professor Hamao Umezawa for encouragement, Dr. Tomohisa Takita, Proessor Kazuhiko Ishizu, and Professor Hisashi Tanaka for pertinent advice, and M. Ohara for comments on the manuscript. This study was supported in part by a grant from the Ministry of Education, Science, and Culture, Japan.