Metal Coordination Environment and Dynamics in ^{113}Cadmium Bleomycin: Relationship to Zinc Bleomycin†

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ABSTRACT: The ^{13}C chemical shifts of Cd- and ZnBlm A_2 are almost identical throughout the entire molecule, suggesting that these structures adopt similar conformations. Nuclear magnetic resonance experiments with ^{113}Cd−bleomycin have defined part of the metal−ligand environment of the molecule. Nitrogen atoms from the primary amine, pyrimidine, and imidazole are bound to ^{113}Cd according to ^{13}C spectra showing ^{113}Cd−^{13}C spin−spin couplings. Bound and free forms of the secondary amine nitrogen may be in equilibrium, as suggested by temperature-dependent ^{13}C studies with Cd−bleomycin. In addition, a number of other carbon resonances are in chemical exchange over the temperature range 5−54 °C. The temperature dependence of the line widths of carbon atoms of Zn−bleomycin strongly resembles that of Cd−bleomycin. Examination of the ^{113}Cd resonance as a function of temperature also supports the presence of at least two differently coordinated forms of cadmium in the molecule. According to the position of the ^{113}Cd chemical shift, at most four nitrogen atoms are bound to Cd at low temperature. Titrations of ^{113}Cd−bleomycin with chloride or acetate demonstrate that these anions can bind to major and minor forms of the structure and that a minor species exists which does not associate with chloride.

Bleomycin is an antitumor antibiotic used in clinical drug combinations to treat human cancers. The prevailing view is that the mechanism of action of this glycopeptide involves DNA strand breakage by iron bleomycin (Petering *et al.*, 1990). The compound contains two functional domains, one to bind metal ions and the other to associate with DNA, that are bound together by a peptide linker region (Figure 1). The clinical mixture of bleomycins is composed mainly of bleomycin A_2 and B_2, which differ in the nature of the positively charged R group of the molecule that participates in DNA binding.[1]

Despite the intense interest in this structure and its mode of interaction with metal ions, the only complex for which the ligand environment is unequivocally known is a fragment of Cu(II)-Blm, which was examined by X-ray crystallography (Iitaka *et al.*, 1978). In this structure, which is missing the disaccharide moiety and the DNA-binding domain, copper is coordinated to the five nitrogen atoms noted with dots in Figure 1. A similar coordination geometry has been defined for crystals of a synthetic model of the metal-binding domain of Cu(II)-Blm (Brown & Mascharak, 1988).

NMR investigations have been conducted on various metallobleomycins, including Cu-, Co-, Fe-, and ZnBlm, to investigate both the structure of their metal-binding domains and to determine their overall conformations (Akkerman *et al.*, 1988a,b, 1990; Dabrowiak *et al.*, 1978; Dabrowiak & Tsukayama, 1981; Oppenheimer *et al.*, 1979a,b; Pillai *et al.*, 1980; Vos *et al.*, 1980; Xu *et al.*, 1994). For ZnBlm the most recent NMR analysis indicated that ligand donor atoms to the metal include nitrogens from primary and secondary amine, pyrimidine, imidazole, β-hydroxyhistidine amide, and mannose carbamoyl groups, which generate a 6-coordinate metal−ligand site (Akkerman, *et al.*, 1988a). It should be noted, however, that this coordination site was derived indirectly by *J*-coupling analysis, NOESY data, and chemical shift changes. A solution structure based on these coordination constraints plus NOE data depicted the molecule as adopting an extended conformation without significant secondary structure outside of the metal-binding domain (Akkerman *et al.*, 1988a, 1990).

The structures of Co^{III}Blm and HO_2^-−Co^{III}Blm, models for Fe^{III}Blm and the activated form of FeBlm, HO_2^-−Fe^{III}-Blm, appear to be distinct from that of ZnBlm (Xu *et al.*, 1994). There is extensive folding in the peptide linker region of both molecules, and the DNA domain is folded over the metal domain in the latter. These differences prompt questions about the use of ZnBlm as a model for relevant FeBlm structures.

The difference in behavior of ZnBlm and HO_2^-−Co^{III}-Blm is further emphasized by the recent communications on the interaction of these structures with DNA oligomers. ZnBlm was found to exhibit fast-exchange interaction with DNA in a manner consistent with minor groove binding and partial intercalation (Manderville *et al.*, 1994, 1995). In contrast, the cobalt species exhibited slow-exchange binding with clear evidence of classical intercalation of the bithiazole

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[1] Abbreviations: Blm, blenoxane, the clinical mixture of bleomycin congeners; Blm A_2 and Blm B_2, individual bleomycin congeners.

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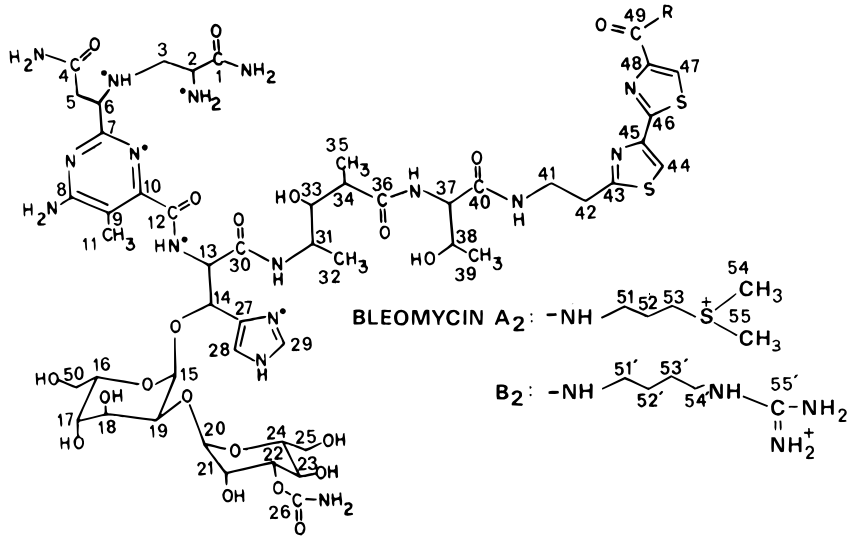

FIGURE 1: Structure of bleomycin A_2 and B_2. Numbering refers to carbon atoms. Nitrogen atoms labeled with dots are ligands to Cu in a Cu−bleomycin metal domain fragment (Iitaka *et al.*, 1978).

moiety between base pairs (Q. Mao *et al.*, 1996; Wu *et al.*, 1994).

To examine in more detail a metal domain like that of ZnBlm, ^{113}CdBlm has been studied. In this structure the opportunity exists to use ^{113}Cd−^{13}C and ^{113}Cd−^{1}H spin−spin couplings to assign clearly the coordination structure of the drug (Summers, 1988). Because of the large chemical shift range of ^{113}Cd and the sensitivity of the ^{113}Cd resonance to ligand environment, it has also been possible to examine how the coordination properties of ^{113}CdBlm vary with temperature and anion concentration.

MATERIALS AND METHODS

Materials. Blenoxane, the clinical mixture of approximately 70% bleomycin A_2 and 30% bleomycin B_2 was a gift of Bristol Myers Co. To obtain pure bleomycin A_2 and B_2, the congeners were separated by CM-Sephadex chromatography using a linear gradient of 0.05−0.5 M ammonium formate as previously described (Fujii *et al.*, 1973). Desalting was achieved by lyophilization, redissolving in water, passage through a Sephadex G-10 column equilibrated with 0.02M ammonium carbonate, pH 7.5, and relyophilization. ^{113}CdO (91% atom %) was obtained from Oak Ridge National Laboratory and converted to the corresponding chloride and acetate salts by dissolving in concentrated hydrochloric and acetic acid, respectively, followed by evaporation and redissolution in water to a concentration of 0.5 M.

NMR Spectroscopy. NMR samples of CdBlm and CdBlm A_2 and B_2 were prepared by direct addition of aliquots of concentrated stock solutions (0.5 M) of enriched $^{113}CdCl_2$ or $^{113}Cd(OAc)_2$ or natural abundance $CdCl_2$ (12.2 atom % ^{113}Cd) to 1.5−2.0 mL solutions of bleomycin (40−70 mM) in 90% H_2O/10% D_2O, followed by adjustment to pH 7.6 with NaOH. ZnBlm samples were similarly prepared by addition of aliquots of 0.5 M $ZnSO_4$. All NMR spectra were acquired on a Bruker WM-250 spectrometer using 10 mm NMR tubes. ^{13}C spectra were obtained at 62.9 MHz with continuous broad band proton decoupling. Assignments of CdBlm signals were made on the basis of their very close chemical shift correspondence to the previously assigned resonances of ZnBlm (Akkerman *et al.*, 1988b; Williamson *et al.*, 1990). Assignments were checked by confirming the presence of expected one-bond and long-range ^{13}C−^{1}H spin−spin couplings either in proton-coupled ^{13}C spectra obtained with inverse gated decoupling to maintain the favorable NOE or in two-dimensional ^{13}C−^{1}H HMQC spectra (Bax & Subramanian, 1986). The latter experiments used a 10 mm inverse detection probe (Cryomagnet Systems) and were conducted on samples of ZnBlm and CdBlm dissolved in 99.8% D_2O and placed in 10 mm NMR tubes. ^{113}Cd spectra were obtained at 55.5 MHz with broad-band proton decoupling gated on during the acquisition period only to eliminate unfavorable NOE effects. ^{13}C chemical shifts were referenced to external tetramethylsilane (TMS). ^{113}Cd chemical shifts are reported in parts per million downfield from the ^{113}Cd resonance of 0.1 M $Cd(ClO_4)_2$.

RESULTS

^{13}C NMR Spectrum of ^{113}CdBlx. The structure of the zinc complex with bleomycin has been well characterized by two-dimensional proton NMR (Akkerman *et al.*, 1988a) and complete assignments of the ^{1}H and ^{13}C spectra of both free Blm and ZnBlm have been made using a combination of homo- and heteronuclear NMR methods (Haasnoot *et al.*, 1984; Akkerman *et al.*, 1988b; Williamson *et al.*, 1990). When Blm is titrated with ^{113}Cd(II), spectral changes are observed in the ^{13}C NMR spectrum that are very similar to those induced by Zn(II). Addition of 0.5 equiv of Cd(II) generates a spectrum containing resonances of equal intensity from both free Blm and CdBlm, indicative of tight metal binding and slow exchange between bound and free forms of the drug. The resonances most affected by Cd(II) and Zn(II) binding (Table 1) are those from carbon atoms in the left-hand portion of the structure depicted in Figure 1. Large upfield shifts are observed for carbons 3, 5, 6, 7, 10, and 14, and large downfield shifts are observed for carbons 8, 13, and 30. Because of the close correspondence of ^{13}C chemical shifts from ZnBlm and CdBlm (Table 1), as well as those of the spin-coupled protons observed in ^{1}H−^{13}C HMQC spectra (data not shown), assignments of the CdBlm signals can be made with confidence.

Comparing a ^{13}C spectrum of Blm A_2 complexed with isotopically enriched ^{113}Cd(II) to that of a complex with

Table 1: ^{13}C Chemical Shifts for Free Bleomycin and Its Complexes with Zn^{2+} and Cd^{2+} at pH 7.6, 44 °C

fragment	carbon	Blm	Zn−Blm	Cd−blm	$J_{^{113}Cd-^{13}C}$ (Hz)
A	1	175.7	174.4	176.7	4
	2	53.9	53.0	52.0	
	3	49.5	45.5	46.0	
P	4	176.8	176.9	177.4	
	5	41.0	34.4	36.2	
	6	60.6	56.6	57.0	
	7	166.2	162.2	162.3	18
	8	165.3	168.5	167.4	
	9	112.9	114.7	114.4	8
	10	153.0	149.1	151.1	13
	11	11.5	11.6	11.0	
	12	168.4	169.8	170.2	25
H	13	57.8	62.0	61.8	
	14	74.0	71.0	71.2	7
	27	135.6	136.1	136.9	3
	28	118.5	118.7	117.7	10
	29	137.6	138.4	138.7	18
	30	169.8	172.4	173.4	
G	15	98.3	96.1	97.1	
	16	67.8	68.0	67.4	
	17	69.8	70.6	70.5	
	18	68.7	67.1	68.2	
	19	71.0	68.8	70.2	
	50	61.0	61.8	61.5	
M	20	98.9	97.8	99.0	
	21	69.1	68.8	68.6	
	22	75.1	75.9	75.8	
	23	65.5	65.0	65.0	
	24	74.2	75.2	74.5	
	25	61.6	61.6	61.6	
	26	158.6	158.1	158.0	
V	31	48.2	47.8	47.0	
	32	15.5	15.4	16.5	
	33	75.1	75.2	75.1	
	34	43.3	43.5	42.7	
	35	12.5	12.5	12.7	
	36	178.2	177.9	178.0	
T	37	59.8	59.8	59.8	
	38	67.8	67.9	67.8	
	39	19.6	19.7	19.8	
	40	172.6	172.6	172.6	
B	41	39.7	39.9	39.7	
	42	32.7	32.8	32.7	
	43	171.7	171.5/171.4[a]	171.4/171.3	
	44	119.7	119.9	119.8	
	45	147.6	147.8	147.6	
	46	163.7	163.6/163.4	163.5/163.3	
	47	126.1	125.9/125.5	125.8/125.4	
	48	149.5/149.8	149.7/150.0	149.5/149.7	
	49	164.0/163.6	164.2/163.7	164.0/163.6	
DMS	51	38.3	38.5	39.3	
	52	24.3	24.4	24.3	
	53	41.6	41.8	41.6	
	54/55	25.5	25.6	25.5	
AGM	51′	39.7	39.9	39.7	
	52′	26.5	26.7	26.6	
	53′	26.2	26.3	26.2	
	54′	41.6	41.8	41.6	
	55′	157.5	157.7	157.5	

[a] The two chemical shift values are for the A_2 and B_2 congeners, respectively.

$^{113}Cd^{2+}$ at natural abundance (12.2%) allows resonances that exhibit resolved $^{113}Cd-^{13}C$ spin−spin couplings to be identified (Figure 2). As indicated in Figure 3 and Table 1, nine resonances arising from carbon atoms 1, 7, 9, 10, 12, 14, 27, 28, and 29 are split into doublets by *J* couplings ranging from 3−25 Hz. These two- and three-bond scalar coupling interactions prove unambiguously that nitrogen atoms from the primary amine, pyrimidine, and imidazole groups are ligands to the $^{113}Cd(II)$ ion. Interestingly, no splittings of the resonances from carbons 2, 3, 5, and 6 are observed, as would be expected for coordination by the secondary amine nitrogen of the β-aminoalanine group. Although one explanation of this result is that the secondary amine nitrogen is not a ligand, it should be pointed out that resonances 2, 3, 5, and 6 are significantly broader at this temperature (39 °C) than most other resonances (see below) and the splittings may, therefore, be obscured. The failure to observe spin coupling to carbons 13 and 30 as well as to the proton attached to carbon 13 suggests that the amide nitrogen between carbons 12 and 13 is not a ligand as previously assumed for ZnBlm (Akkerman *et al.*, 1988a). Similarly, the lack of splitting of carbon 26 argues against ligation by the carbamoyl group of the mannose moiety, although the possibility exists that the coupling constant is simply too small to be detected.

Temperature Dependence of ^{13}C Spectra. A large number of resonances in the ^{13}C spectra of CdBlm and ZnBlm undergo substantial changes in line width as a function of temperature that are believed to result from modulations of the ligand coordination structure. This behavior is illustrated in Figure 4 for $^{113}CdBlm$, where the upfield region of the spectrum is shown at 5, 24, 39, and 54 °C. A number of resonances, such as those from carbons 2, 6, 15, 19, and 20, are relatively narrow at high temperature, become significantly broader at intermediate temperature, and then sharpen again at low temperature. These spectral changes suggest a transition between moderately fast exchange at high temperature involving two or more coordination states and moderately slow exchange at low temperature. Other resonances, such as those of carbons 3 and 5, remain quite broad even at 54 °C. A summary of the carbon atoms in ZnBlm and CdBlm whose resonances are broadened by more than 10 Hz by chemical exchange between 5 and 54 °C is given in Figure 5. It can be seen that the temperature effects are not localized to a single region of the molecule but extend from the β-aminoalanine and pyridinylpropionamide region to the disaccharide and methyl valerate region. Except for the fact that the environments of the imidazole carbons are affected more by temperature in ZnBlm than CdBlm, it appears that both the zinc and cadmium complexes are undergoing similar modulations of their structures. Since it was not possible at 5 °C to "freeze out" the equilibrating species, it is not known how many conformational states are present in solution or what their relative concentrations are. It is clear, however, that the coordination structure of CdBlm and, by inference, ZnBlm are undergoing dynamic conformational change and cannot be considered to be a static 6-coordinate structure as previously believed.

^{113}Cd NMR Spectra. Further insight into the structural dynamics of the interaction of zinc and cadmium with bleomycin is provided by ^{113}Cd spectra of $^{113}CdBlm$ at different temperatures. As shown in Figure 6, separate resonances are observed at 5 °C for the A_2 and B_2 congeners at 239.2 and 241.2 ppm, respectively. These are confirmed in spectra of the separate A_2 and B_2 structures. The only difference between these forms of bleomycin is the identity of the positively charged R group attached to the bithiazole portion of the molecule. Since ZnBlm has been described as an extended structure in which there is no interaction

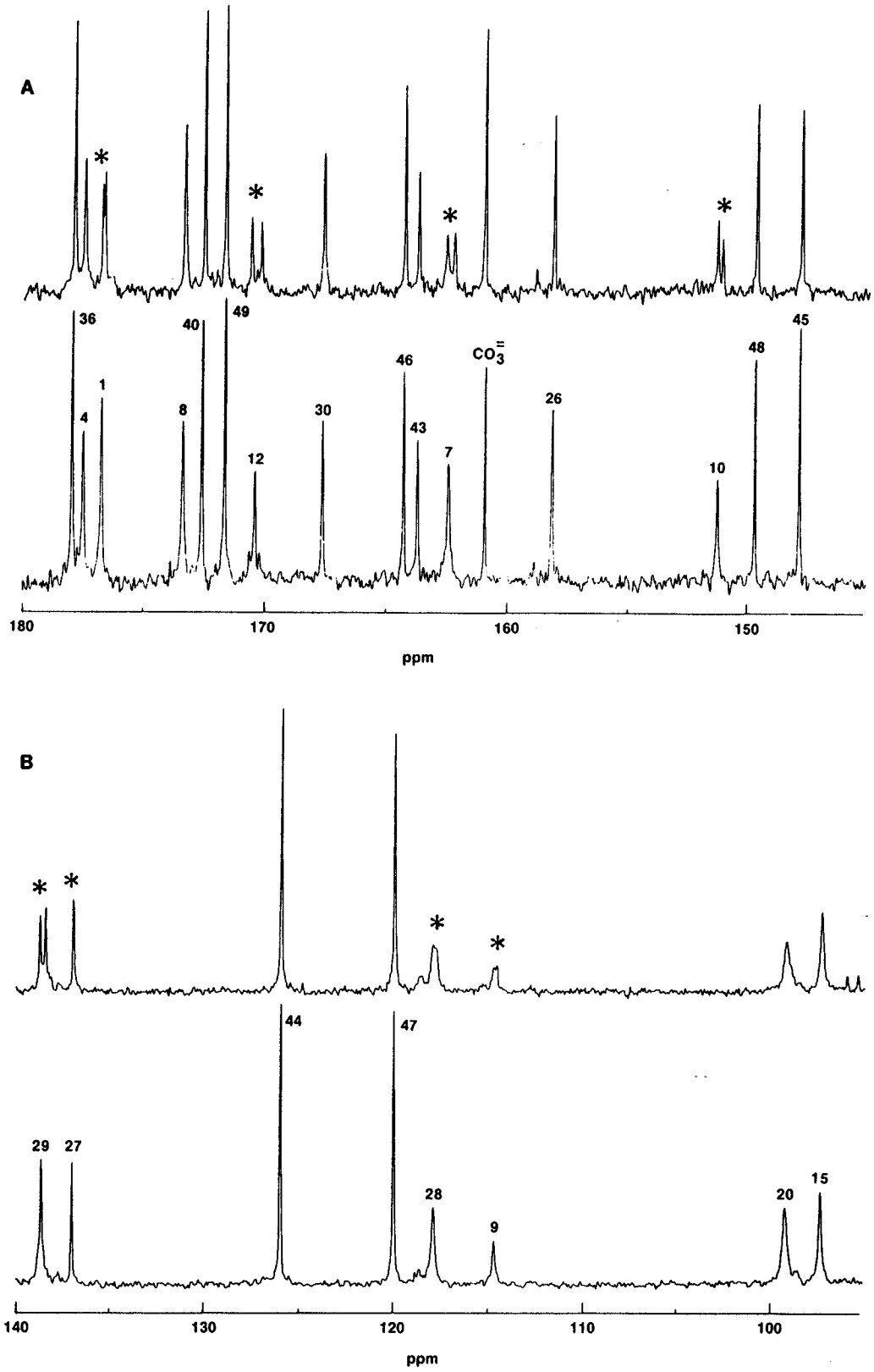

FIGURE 2: ^{13}C NMR spectra recorded at 39 °C of CdBlm A_2 prepared with enriched ^{113}Cd (upper spectrum in A and B) and natural abundance Cd (12% ^{113}Cd) (lower spectrum in A and B). A and B display adjacent parts of the ^{13}C spectrum. Asterisks indicate those resonances that exhibit resolved $^{113}Cd-^{13}C$ coupling.

between the metal and the DNA domain, the observed ^{113}Cd chemical shift difference is surprising. Above 5 °C, the resonances from both $^{113}CdBlm$ A_2 and B_2 are observed to broaden considerably and move downfield, indicative of the onset of chemical exchange between multiple species. Although there is not an exact correspondence between ^{113}Cd chemical shift and the nature and number of ligands in the first coordination sphere of the metal (Armitage & Otvos, 1982; Summers, 1988), the observed chemical shift of about 240 ppm is similar to that observed for carbonic anhydrase (with three imidazole nitrogen ligands) and falls within the chemical shift range 210−310 ppm that is observed for

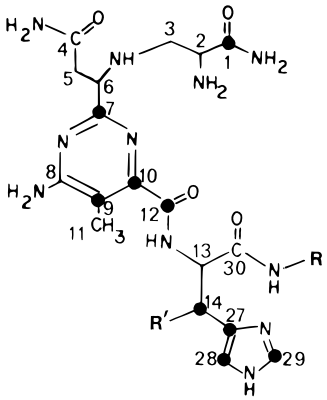

FIGURE 3: Structure of Blm showing carbons that exhibit resolved $^{113}Cd-^{13}C$ splittings.

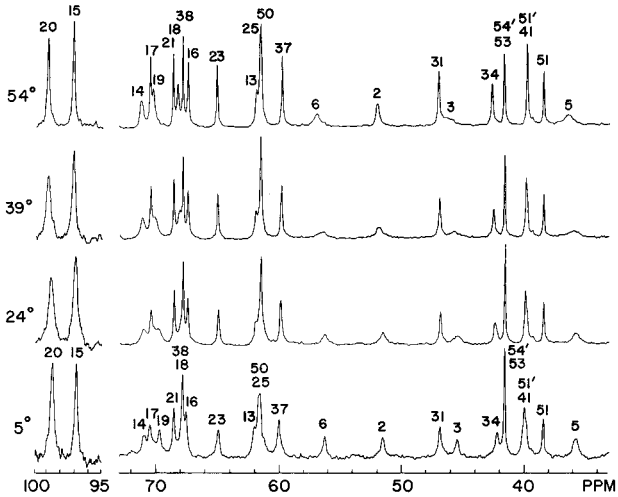

FIGURE 4: ^{13}C NMR spectra of $^{113}CdBlm$ at pH 7.6 obtained at the four different temperatures indicated.

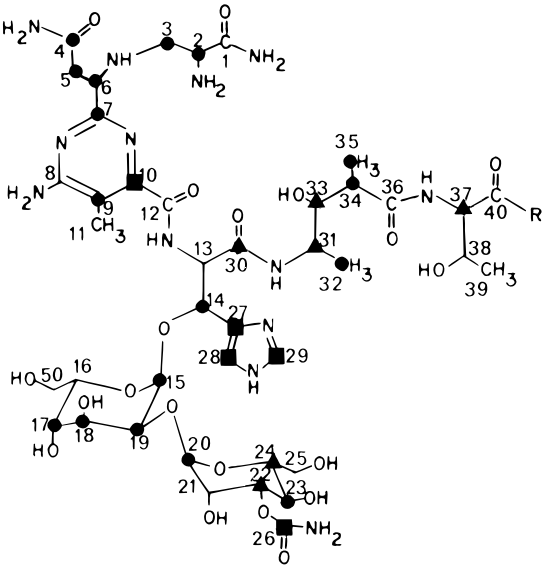

FIGURE 5: Structure of Blm showing the carbons whose ^{13}C resonances are broadened by greater than 10 Hz by chemical exchange between 5 and 54 °C in CdBlm and ZnBlm. (●) Carbons broadened in both structures, (■) carbons broadened only in ZnBlm, and (▲) carbons broadened only in CdBlm.

tetrahedral coordination by three nitrogen donor atoms (Summers, 1988).

In order to probe further the nature of the coordination sphere in the cadmium−bleomycin complex, ^{113}Cd NMR

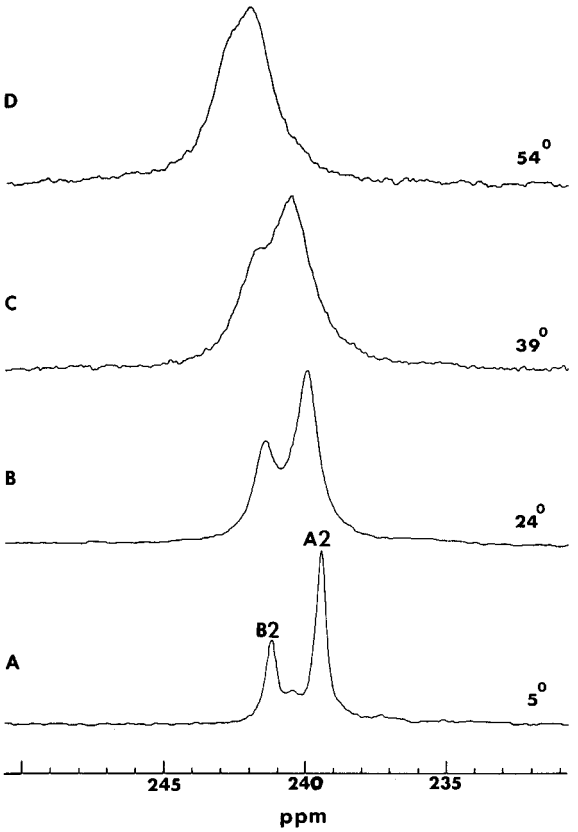

FIGURE 6: ^{113}Cd NMR spectra of $^{113}CdBlm$ obtained at the four different temperatures indicated (in °C).

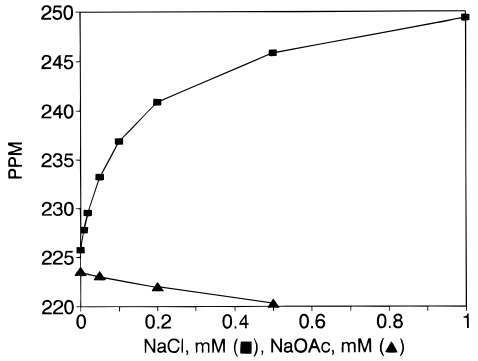

FIGURE 7: ^{113}Cd chemical shift dependence of $^{113}CdBlm$ as a function of added sodium chloride and sodium acetate concentration at 25 °C. CdBlm recovered from Blm and cadmium acetate.

spectra of $^{113}CdBlm$ A_2 were obtained in the presence of varying concentrations of chloride or acetate ion. As summarized in Figure 7, addition of increasing amounts of sodium chloride induced a progressive downfield shift of the ^{113}Cd resonance while addition of sodium acetate caused a slight upfield shift. Such behavior is diagnostic of a coordination complex containing one or more "open" coordination sites occupied by a water molecule(s) which can undergo fast exchange interaction with a competing anion (Summers, 1988).

Low-temperature (5 °C) spectra of $^{113}CdBlm$ at three different concentrations of chloride ion are shown in Figure 8. Under these slow exchange conditions, expansion of the vertical scaling showed that minor forms of $^{113}CdBlm$ coexist in solution with the major species (labeled number 4). As seen in Figure 8A, three additional broad resonances are observed downfield of 240 ppm. The peaks labeled 2 and

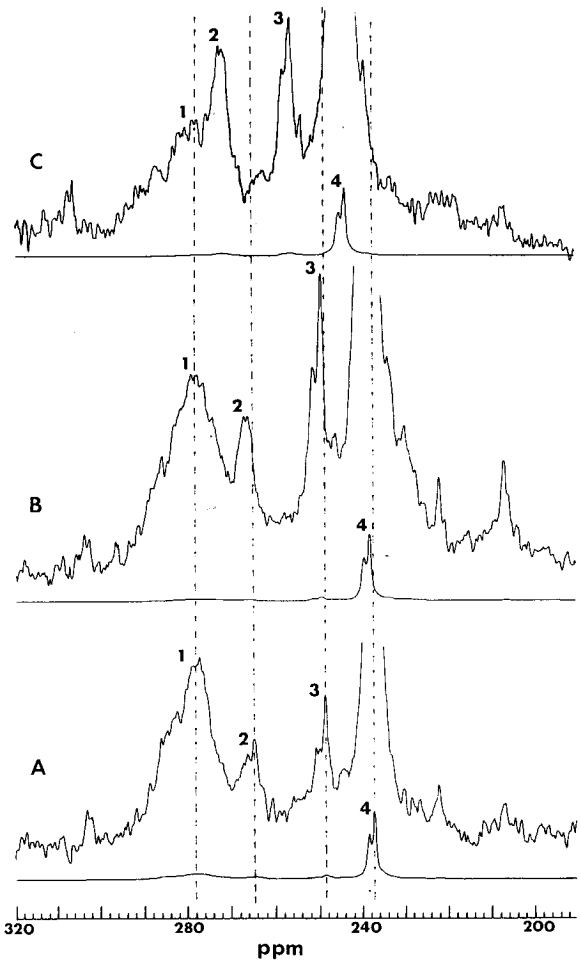

FIGURE 8: ^{113}Cd NMR spectra of ^{113}CdBlm at 5 °C as a function of Cl^- concentration: (A) 0.1 M Cl^-; (B) 0.15 M Cl^-; and (C) 0.4 M Cl^-.

3, which together account for about 10% of the total ^{113}Cd signal intensity, also move downfield with increasing chloride ion concentration as do the major peaks from ^{113}CdBlm A_2 and ^{113}CdBlm B_2. Thus, they represent species that also have at least one open coordination site that can interact with Cl^-. In contrast, the most downfield peak at about 280 ppm, labeled number 1, which represents about 20% of the total integrated intensity, is not affected by chloride ion concentration. It is, therefore, likely that this resonance represents a complex in which an additional donor atom(s) from the drug completes the inner coordination sphere and closes off access of the metal ion to solvent or anion molecules. At temperatures higher than 5 °C, interconversion between these multiple-coordination complexes becomes more rapid and, as shown in Figure 6, an exchange-averaged ^{113}Cd signal is observed somewhat downfield of 240 ppm.

DISCUSSION

Structural characteristics of ZnBlm have been studied for years using NMR spectroscopy, because it has been thought that this metallobleomycin represents an accessible, diamagnetic model for iron bleomycin (Dabrowiak *et al.*, 1978; Haasnoot *et al.*, 1984; Akkerman *et al.*, 1988a,b; Williamson *et al.*, 1990). Recently, CoBlm was shown to be an excellent surrogate model for FeBlm in its reaction with O_2 (Fulmer & Petering, 1994; Xu *et al.*, 1992b, 1994). The solution structures of both of its Co(III) species, HO_2^-−Co^{III}Blm and Co^{III}Blm, are highly folded whereas that of ZnBlm is extended in conformation (Akkerman *et al.*, 1988). Structural differences are also seen in the interactions of ZnBlm A_2 and HO_2^-−Co^{III}Blm A_2 with DNA (Manderville *et al.*, 1994, 1995; Q. Mao *et al.*, 1996; Wu *et al.*, 1994). In these studies, it was assumed that both complexes are 6-coordinate as described in previous studies, bearing the same net charge and only differing in an axial ligand−carbamoyl group for ZnBlm and peroxide for the cobalt structure (Akkerman *et al.*, 1988a; Xu *et al.*, 1994). Thus, it is not apparent why their interactions with DNA differ so much.

In order to learn more about the nature of the metal−ligand environment in ZnBlm, temperature-dependent ^{13}C NMR studies of both ZnBlm and CdBlm were carried out. Zn(II) and Cd(II) have very similar coordination preferences, and it was, therefore, assumed that the two metal ions would form nearly identical complexes with bleomycin. In keeping with this expectation, the ^{13}C chemical shifts of ZnBlm and CdBlm are closely similar, as are the temperature-induced changes in line width of many of the resonances (Table 1, Figures 4 and 5). These results strongly support the view that inferences made about the structural and dynamic properties of CdBlm on the basis of both ^{13}C and ^{113}Cd NMR evidence can be extended directly to ZnBlm.

By employing ^{113}Cd as a spin $^1/_2$, NMR-active analog of Zn, it was initially anticipated that the number and nature of metal-coordinating groups in the drug could be identified by the carbon resonances that are split into doublets by two-bond or three-bond spin−spin coupling interactions with the metal ion. Although numerous ^{113}Cd−^{13}C spin couplings were, indeed, observed (Figure 2), a simple structural interpretation is not possible owing to the apparent existence in solution of multiple, interconverting configurational states of the metal−drug complex. Evidence that more than one form of CdBlm is present under our experimental conditions is provided by both ^{13}C and ^{113}Cd NMR experiments. Over the temperature range 5−54 °C, the ^{13}C resonances of a large number of carbon atoms in the metal-binding region of Blm exhibit substantial changes in line width due to chemical exchange (Figures 4 and 5). At high temperatures, there is relatively rapid equilibration between the interconverting states, and most resonances are in the fast-exchange regime and have narrow line widths. However, a few signals, notably from carbons 2, 3, 5, and 6, remain quite broad at 54 °C (Figure 4), indicating that the propionamide and β-aminoalanine segments of the molecule experience the largest structural perturbation in going from one configurational state to the other(s). At 5 °C it was hoped that the rate of interconversion between states would be slow enough to allow separate signals from each state to be observed, which would have enabled the number of states and their relative concentrations to be determined. However, as seen in Figure 4, this did not occur. Although the resonances from carbons 2, 3, 5, and 6 are narrower at 5 °C than at 24 °C, indicative of entry into the slow-exchange regime, no other signals from less abundant configurational states are detectable.

^{113}Cd NMR spectra at 5 °C proved to be more informative. Because of the sensitivity of ^{113}Cd chemical shifts to small differences in ligand coordination structure, it was possible to detect several broad signals from minor configurational states that appear downfield from the major resonance at

about 240 ppm (Figure 8A). The largest and broadest of these signals at about 280 ppm accounts for about 20% of the total ^{113}Cd signal intensity, while the others represent less than 10%. Therefore, CdBlm does not exist as a single species, but rather as a mixture of one major and several minor configurational forms that are in equilibrium with each other.

Some insight into the possible structural identities of these species is provided by the magnitudes of the observed ^{113}Cd chemical shifts and the reponsiveness of the different resonances to the concentration of added chloride and acetate ion. When Blm A_2 is complexed with $^{113}Cd(OAc)_2$ at pH 7.6, the chemical shift of the signal from the major species appears at about 225 ppm. Addition of increasing concentrations of chloride ion causes a progressive downfield shift of the signal, which reaches a value of about 250 ppm in the presence of 1 M NaCl. Such behavior is characteristic of a Cd complex that has one or more sites in the inner coordination sphere occupied by water, which can undergo exchange with competing anions (Armitage & Otvos, 1982; Summers, 1988). In contrast, the signal at 280 ppm from the most abundant minor configurational state appears to be unaffected by competing anions, which implies that it originates from a coordination complex in which there are no readily exchangeable ligands such as H_2O. As such, the species may be the same as the 6-coordinate structure assumed by Akkerman *et al.* (1988a) in their two-dimensional ^{1}H NMR study of ZnBlm. Although no ^{113}Cd chemical shift information exists for complexes with a similar array of ligands, the observed 280 ppm chemical shift of the minor CdBlm form is at least consistent with available data on octahedral complexes having six nitrogen ligands (Summers, 1988). For example, Cd(imidazole)$_6$ in the solid state is reported to have a chemical shift of 238 ppm (Mennitt *et al.*, 1981).

Inferences about the structures of the major CdBlm species can be made on the basis of the ^{113}Cd−^{13}C spin−spin coupling information obtained from ^{13}C NMR spectra as well as ^{113}Cd chemical shift considerations. As summarized in Figure 3, there are nine carbon atoms whose resonances exhibit spin coupling to the ^{113}Cd ion. These two-bond and three-bond interactions establish unambiguously that three of the ligands to the metal are nitrogen atoms from the primary amine, pyrimidine, and imidazole groups. With no direct evidence that any other donor atoms from the drug are involved as ligands, one might reasonably propose the existence of a tetrahedral complex consistent with the ^{113}Cd chemical shift of 225 ppm observed in the absence of chloride ion. By comparison, the Cd NMR signal from the tetrahedral metal-binding site of bovine carbonic anhydrase A, which consists of three imidazole nitrogens and a water molecule, appears at 220 ppm (Jonsson *et al.*, 1980).

It is not possible to rule out conclusively the presence of structures with higher coordination numbers on the basis of either ^{113}Cd chemical shift considerations or the observed absence of spin splitting of certain ^{13}C resonances. ^{113}Cd signals from octahedral complexes generally appear upfield from those of tetrahedral complexes with comparable ligand donor sets (Summers, 1988). For example, the chemical shift of Cd occupying the octahedral (three imidazole, three H_2O) Zn sites of hexameric insulin is reported to be 165 ppm (Sudmeier *et al.*, 1981), which is significantly upfield of the 220 ppm shift seen for the above mentioned tetrahedral (three imidazole, one H_2O) carbonic anhydrase complex. The observed ^{113}Cd chemical shift of the major peak of CdBlm is, thus, also consistent with a 5- or 6-coordinate complex containing more than three nitrogen ligands from the drug.

If there are more than three nitrogen donor atoms to ^{113}Cd, the question arises as to why more ^{13}C resonances do not exhibit resolved ^{113}Cd−^{13}C splittings. One possibility is that the coupling constants are too small to detect. Another is that the splittings are obscured by the large natural line widths of the several ^{13}C signals that are broadened by chemical exchange. The region of the molecule most affected by chemical exchange is that surrounding the secondary amine of the β-aminoalanine moiety, particularly, carbons 2, 3, 5, and 6. If the secondary amine nitrogen were coordinated to ^{113}Cd, one would expect the ^{13}C signals of all of these carbons to exhibit spin−spin splittings, yet none was observed at either high or low temperatures. However, it should be noted that the line widths of these signals are large enough to obscure any splittings arising from coupling constants smaller than about 10 Hz. This is the probable explanation for our inability to detect the spin−spin splitting of the signals of carbons 3 and 6 that would be anticipated from coordination by the primary amine and pyrimidine nitrogen, respectively. Because of the complications introduced by the temperature-dependent structural modulation in the β-aminoalanine portion of the drug, it is possible that the secondary amine is a fourth nitrogen ligand to the metal. A stronger case can be made, however, against the involvement of the hydroxyhistidine amide nitrogen and the mannose carbamoyl group as ligands, since the relevant signals which failed to exhibit ^{113}Cd−^{13}C couplings, those of carbons 30, 13, and 26, are quite narrow and largely unaffected by chemical exchange broadening.

The ^{113}Cd and ^{13}C NMR results demonstrate clearly that CdBlm and by inference ZnBlm do not exist in solution as a single 6-coordinate species with all of the ligands provided by the drug but rather that they exist as a mixture of one major species and two or more minor species in dynamic equilibrium. If our hypotheses are correct about the structures of the major and minor species of Cdblm and ZnBlm in solution, one might expect that a major structural reorganization would be involved in their interconversion. Evidence for the presence of global conformational equilibria is provided by the large number of ^{13}C resonances outside the metal domain that experience temperature-induced changes in line width including resonances from the disaccharide, methyl valerate, and threonine regions of the structure (Figures 4 and 5).

The nature of the temperature-dependent chemical exchange processes involving carbons from both the metal domain and linker is not known. Possibly, they reflect temperature sensitive binding of the secondary amine to ^{113}Cd. As the 4-coordinate metal−drug center forms, it may provide the necessary structural framework to support folding in the linker region.

Experiments that monitor the ^{113}Cd resonance support this possibility. According to Figure 6 the ^{113}Cd chemical shifts of ^{113}CdBlm A_2 and B_2 are readily distinguishable. Since the chemical shifts of all of the ^{13}C resonances in the common part of ^{113}CdBlm A_2 and B_2 are virtually identical, these results imply that the DNA and metal-binding domains are not spatially isolated from one another in solution. It is possible that the peptide linker folds as it does in Co^{III}Blm

species, bringing the bithiazole and variable R group into proximity with the metal chelation site (Xu *et al.*, 1994). This appears to be favored at lower temperature when the Cd-binding domain may be 4-coordinate.

The low-temperature NMR NOESY data for ZnBlm A_2 suggest that some folding occurs at low temperature even though that structure was described as an extended conformation (Akkerman *et al.*, 1988a). In particular, a long range NOE between H4 of the imidazole ring and the valerate α-methyl group was reported. Following this line of reasoning, if ZnBlm like CdBlm is composed of several interconverting species involving different metal domain configurations and, perhaps, different degrees of folding in the rest of the molecule, then it would present a much different structure to a DNA-binding site than do O_2-Co^{II}-Blm, $HO_2^- - Co^{III}$Blm, O_2-Fe^{II}Blm, and $ON-Fe^{II}$Blm. In these latter complexes, current evidence suggests that their metal domains interact with DNA (Antholine & Petering, 1979; Chikira *et al.*, 1991; Fulmer & Petering, 1994; Q. Mao *et al.*, 1996; Wu *et al.*, 1995). In contrast, a recent structure of a ZnBlm−DNA adduct shows no such interactions (Manderville *et al.*, 1995). Considering these apparent differences, it is suggested that different structures of these metal domains affect the conformations of the rest of the molecule and the modes of binding of these metallobleomycins to DNA.

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