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PROTON NMR STUDY OF IRON(II)-BLEOMYCIN: ASSIGNMENT OF RESONANCES BY SATURATION TRANSFER EXPERIMENTS

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Summary

The assignment of the paramagnetically shifted resonances of the Fe(II)-bleomycin complex in D_2O has been accomplished using the transfer of saturation method. A number of additional resonances arising from labile NH protons which are shifted by the metal ion are observed in the ^1H spectrum of the complex in H_2O. The temperature dependence of the chemical shifts is consistent with the formation of an isolated 1:1 complex, but does not obey either the Curie Law or the Curie-Weiss Law. The magnitude of the shifts suggests that the valeric acid hydroxyl (or carbonyl) group, the α-amino group, the imidazole N^{π}, the carbamoyl oxygen, the pyrimidine N_1 and/or the secondary amino group may be coordinated to the iron(II).

The bleomycins (Fig. 1) are antitumor antibiotics which form complexes with DNA, their putative pharmacological target, and with various polyvalent metal ions (1,2). In the presence of molecular oxygen and iron(II), the bleomycins cause single strand scission of DNA with the concommitant release of free nucleic acid bases (3-6). Studies of the binary complexes of the drug with nucleic acids (7-8) and with various polyvalent metal ions (9-14) suggest that the C-terminal end of the molecule binds to nucleic acids, while the N-terminus serves as a chelation site for metals (15,16).

Oppenheimer et al. (12) have recently reported a ^1H NMR study of a paramagnetic Bleo-A_2-Fe(II)[1] complex and a diamagnetic Bleo-A_2-Fe(II)-CO complex in D_2O. The Fe(II) complex exhibited paramagnetically shifted resonances both to low field and to high field of the spectrum of the metal-free antibiotic. In the present study, we report the use of saturation transfer experiments (17) in making the assignments of the resonances of the paramagnetic Fe(II)-

[1]Abbreviations: Bleo-A_2: Bleomycin-A_2; TSP: Trimethylsilyl propionate; ALA: β-amino alanine; PYR: [2]Pyrimidinyl propionamide; HIS: β-hydroxyhistidine; THR: threonine; G: Gulose; M: Mannose; VAL: valerate.

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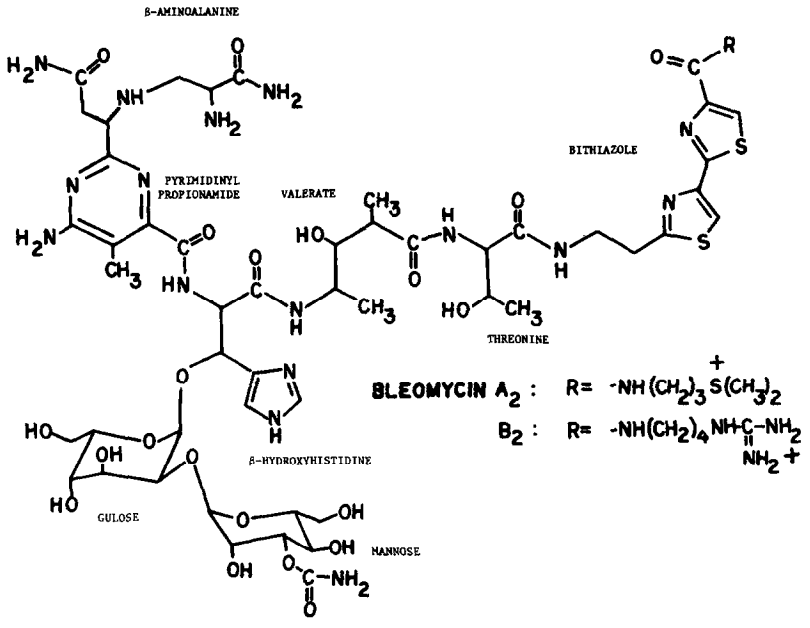

Figure 1 Primary structure of bleomycin congeners.

Bleo-A_2 complex. On the basis of these assignments we are able to identify hydrogens in close proximity to the metal and to suggest a set of probable ligation sites.

We have previously employed saturation transfer experiments in the determination of the kinetics of dissociation of the Zn(II) and Ga(III) complexes of the bleomycin antibiotics (18,19) as well as in the analysis of the kinetics of exchange of the peptide NH's of peptide hormones (20). The present study demonstrates the feasibility of a similar analysis of the kinetics of formation and dissociation of the Fe(II)-Bleo-A_2 complex. Detailed treatments of this method are available in the literature (17,20-22).

Materials and Methods

Sample Preparation. Bleomycin-A_2 chloride was purified from Blenoxane (Bristol Laboratories, Syracuse, NY),[2] the commercial mixture of bleomycin congeners, by chromatography on carboxymethyl Sephadex C-25 (23). Due to the extreme sensitivity of Fe(II)-bleomycin to oxidation, all samples were prepared under strict oxygen-free conditions. Bleomycin-A_2 (20.5 μmoles) lyophilized from D_2O was dissolved in 0.500 ml of ultrapure D_2O (99.96% D, Merck, Sharp and Dohme, Montreal, Canada). One half equivalent of a solution

[2] Iron(III) complexes of bleomycin have been found to be in fast exchange on the ^1H NMR chemical shift time scale. In contrast with the Fe(II) system, the Fe(III) complex exhibits relatively small shifts, but substantial broadening of the bithiazole resonances.

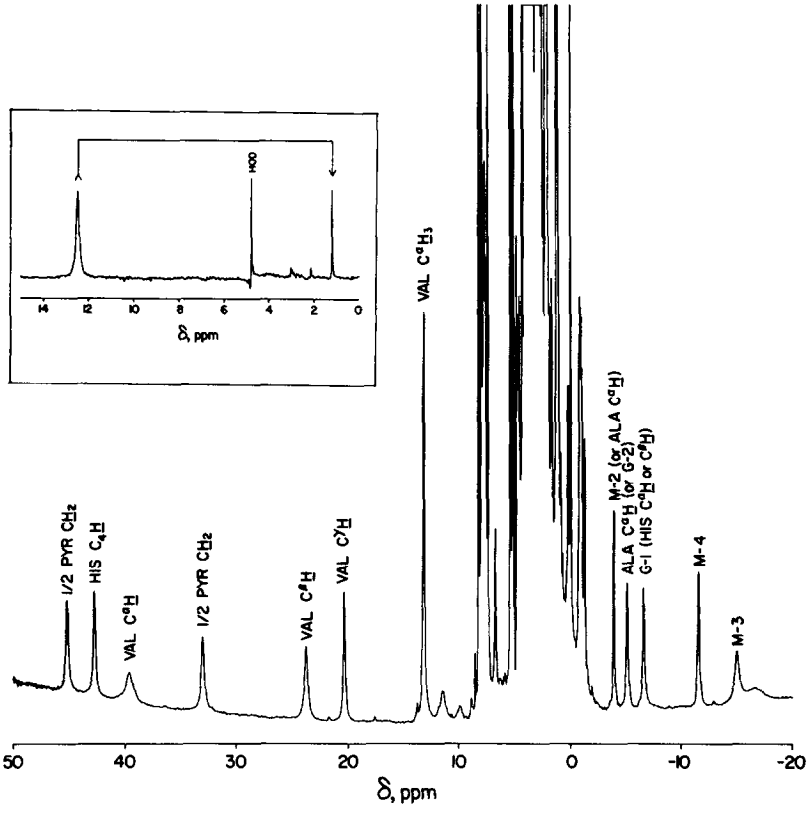

Figure 2 The 400 MHz ^{1}H NMR spectrum of Fe(II)-Bleo-A_2 in D_2O at 303°K; Bleo-A_2 = 38.5 mM; Fe(II) = 19.3 mM; pH = 6.30. The assignments of the shifted peaks are also shown. Inset: Representative difference spectrum from the transfer of saturation experiment at 303°K. The tail of the arrow represents the peak that is saturated and the head indicates the resonance to which magnetization is transferred. The HOD signal at 4.8 ppm is caused by signal "breakthrough."

of $FeSO_4 \cdot 7H_2O$ in D_2O was added to this solution and the pH (the meter reading uncorrected for the deuterium isotope effect) was adjusted to 6.30 with NaOD. The sample was transferred to a purged 5 mm NMR tube, which was immediately sealed. The absence of significant oxidation of Fe(II) to Fe(III) was ascertained from the characteristics of the NMR spectrum of the sample.[2] The Fe(II)-bleomycin-A_2 sample in H_2O was prepared by an analogous procedure.

NMR Spectra. Proton NMR spectra were obtained at 400 MHz on a Bruker WH-400 NMR spectrometer. All chemical shifts were calculated with respect to TSP as the internal standard. For the transfer of saturation experiments, the shifted resonances were selectively saturated using gated homonuclear decoupling with a decoupling time of 2-3 sec and ca. 15 db below .1 watt attenuation. The spectrum of Fe(II)-bleomycin-A_2 in H_2O was obtained using a Redfield 2-1-4-1-2 sequence (24).

Results

NMR Spectrum in D_2O. The ^{1}H NMR spectrum of the Fe(II)-Bleo-A_2 complex in D_2O is shown in Figure 2. The spectrum is a superposition of the res-

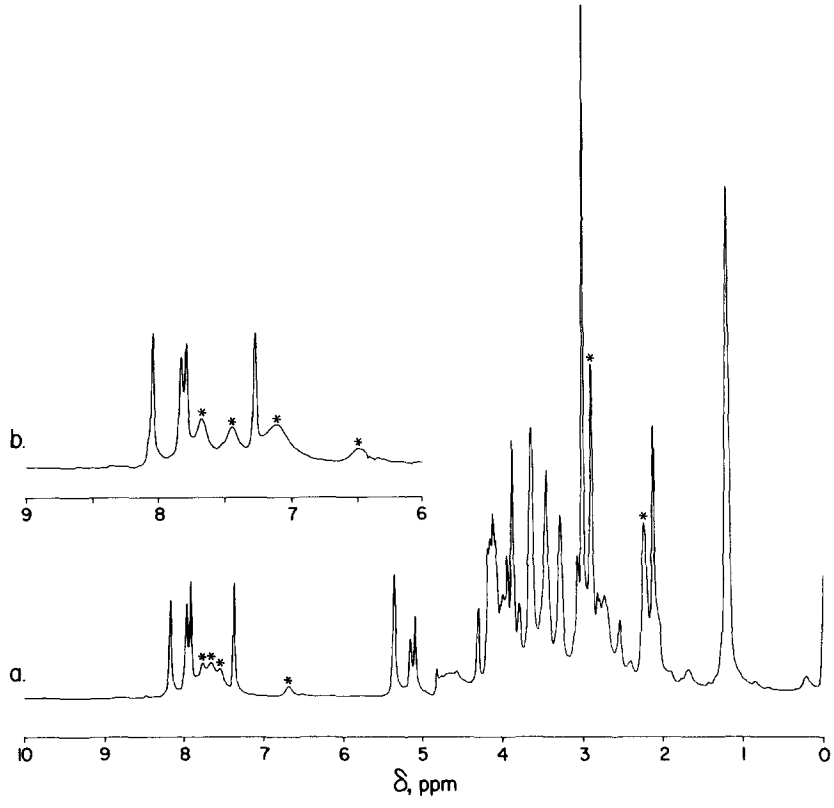

Figure 3 The diamagnetic region of the 400 MHz ^{1}H NMR spectrum of Fe(II)-Bleo-A_2 in D_2O. (a) pH = 6.30, T = 303°K; (b) pH = 8.0, T = 313°K. Asterisks (*) indicate resonances that belong to the complex.

onances of free Bleo-A_2, which are limited to the normal diamagnetic spectral range (9-0 ppm) and those of the paramagnetic iron(II) complex, which extend between 50 and -20 ppm. The diamagnetic region also contains a few resonances of the metal complex as shown in Figure 3. All the resonances outside the diamagnetic region have the intensity of a single proton, except the resonance at 13.1 ppm which corresponds to 3 protons and the cluster of resonances at ∿ -1 ppm which consists of 5 or 6 protons. The assignment of the shifted resonances was accomplished using saturation transfer experiments. A typical difference spectrum is shown in the inset of Figure 2. Saturation of the 3-proton resonance of the complex at 13.1 ppm results in transfer of saturation (∿ 10%) to 1.13 ppm, which corresponds to the α-methyl resonance of valeric acid of free bleomycin. The assignments of the other resonances of the complex were made in an analogous manner using the known assignments of the resonances of the free drug (11,12,25) and our recent assignments of some of the previously unassigned sugar resonances which occur between 3.5 and 4.1 ppm

(e.g.: M-4 at 3.88 ppm, G-4 at 3.91 ppm, G-2 at 4.04 ppm, M-2 at 4.06 ppm and G-3 at 4.12 ppm at 303°K, pH = 6.3). The results of the transfer of saturation experiment are summarized in Table I.

Temperature Dependence of Shifts. The temperature dependence of the chemical shifts is shown in Table I. Plots of the chemical shifts vs temperature were linear between 298°K and 333°K, and appear to extrapolate to the diamagnetic region (9 to 0 ppm) at infinite temperature. This behavior is consistent with the formation of an isolated 1:1 complex as reported by Sausville et al. (15). However, the diamagnetic shifts obtained by extrapolation of the plot to infinite temperature do not agree with those obtained from the saturation transfer experiments. The changes in the chemical shifts ($\Delta\delta$) also do not obey the simple Curie Law ($\Delta\delta$ proportional to $1/T$) or the Curie Weiss Law ($\Delta\delta$ linear in $1/(T + \text{constant})$).

NMR Spectrum in H_2O. The ^1H NMR spectrum of Fe(II)-Bleo-A_2 in H_2O is shown in Figure 4. In addition to the resonances observed in the spectrum measured in D_2O (Figures 2 and 3) and those of free bleomycin in H_2O (25), a number of resonances (indicated by asterisks) are observed, including one at 67 ppm (the lowest field Bleo-A_2 resonance yet observed). These resonances observed only in H_2O originate from NH protons which interact with the paramagnetic metal ion. Experiments are still in progress to identify these resonances, but are complicated by the shorter relaxation times of these protons.

Discussion

The quantitative interpretation of the paramagnetic shifts herein identified would require estimates of the relative contributions of the Fermi contact and dipolar mechanisms to each of the observed shifts. Although these estimates are not available, useful qualitative information can be obtained from the present data. It is likely that resonances exhibiting very large paramagnetic shifts originate from hydrogens in close proximity to the metal ion. Small shifts, however, do not necessarily imply remoteness from the metal binding site since numerous effects can diminish the magnitude of the shifts. Detailed reviews of the analysis of paramagnetic shifts have appeared in the literature (26-28).

It can be seen from Table I that the shifts exhibited by the dimethylsulfonium group and the bithiazole protons are all relatively small. This is in agreement with previous studies which indicate minimal involvement of the C-terminus of bleomycin in metal binding (9-11). The large shift exhibited by the imidazole C_4H is consistent with the metal coordination of the imidazole N^{π}. It is noteworthy that a shifted imidazole C_2H resonance has not been observed either by saturation transfer experiments or by comparing the spectrum

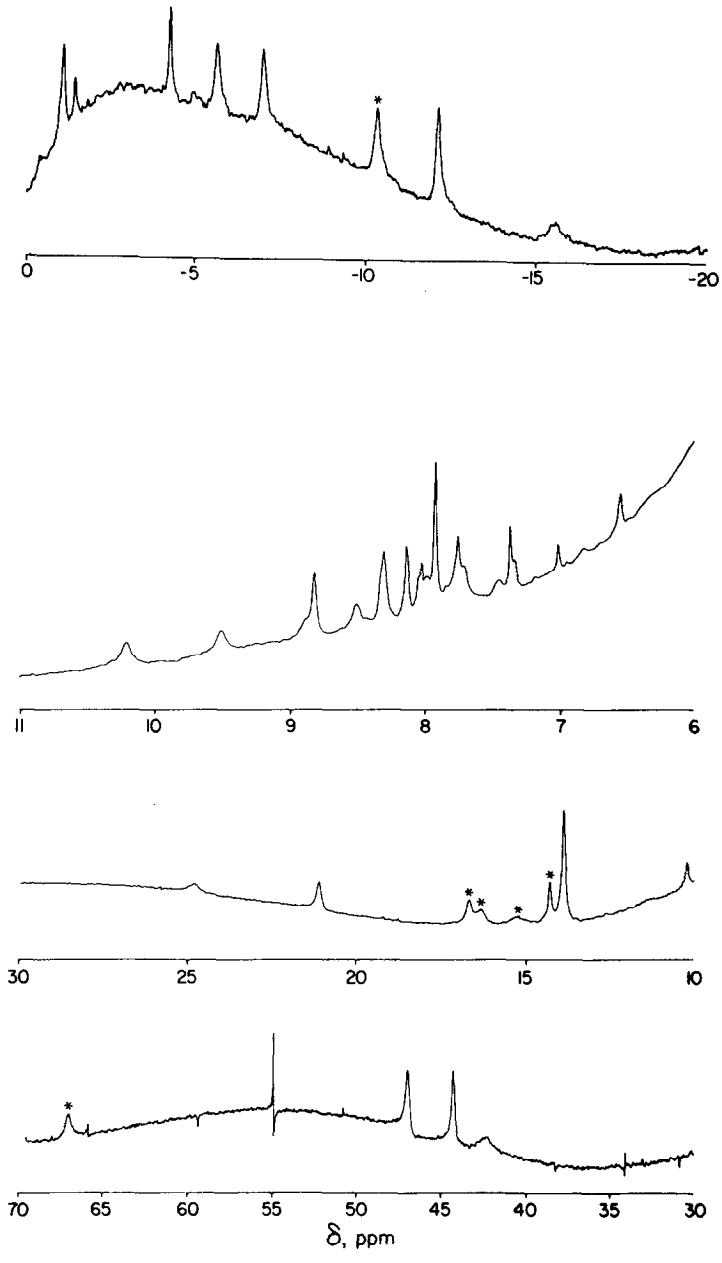

Figure 4 The 400 MHz ^{1}H NMR spectrum of Fe(II)-Bleo-A_2 in H_2O at 297°K, Bleo-A_2 = 46.3 mM, Fe(II) = 23.2 mM, pH = 6.28. Asterisks (*) indicate shifted NH resonances.

with that of a sample in which the imidazole $C_2\underline{H}$ is replaced by deuterium.[3] The close proximity of the imidazole $C_2\underline{H}$ to N^{π} could readily shift its resonance

[3] Bleo-A_2 in D_2O was heated for 12 hours at 85°C.

Table I

Summary of Saturation Transfer Experiments at 303°K and pH = 6.3

Saturated Peak	Saturation Transfer observed at	Assignment[§]	dδ/dT
45.2 ppm[†]	2.83	1/2 PYR $C\underline{H}_2$	-0.26
42.7	7.38	HIS $C_4\underline{H}$	-0.23
39.6	2.55	VAL $C^{\alpha}\underline{H}$	-0.41
32.9[†]	2.58	1/2 PYR $C\underline{H}_2$	-0.22
24.7	3.80	VAL $C^{\beta}\underline{H}$	-0.16
20.3	3.98	VAL $C^{\gamma}\underline{H}$	-0.12
13.1	1.21	VAL $C^{\alpha}\underline{H}_3$	-0.12
7.9[‡]	8.12	BITHIAZOLE $C_5\underline{H}$	
7.7[‡]	7.83	BITHIAZOLE $C_5'\underline{H}$	
7.1[‡]	1.13	VAL $C^{\gamma}\underline{H}_3$	
6.5[‡]	4.22	THR $C^{\alpha}\underline{H}$	
2.8	2.94	DIMETHYLSULFONIUM $C\underline{H}_3$	
0.6[‡]	5.01	M-1	
-1 ppm (cluster of peaks)	Peaks ranging from 3.8 - 4.1 ppm	Sugar $C\underline{H}$'s, ALA or PYR $C\underline{H}$	
-3.9	4.13	M-2 (or ALA $C^{\alpha}\underline{H}$)	0.05
-5.1	4.10	ALA $C^{\alpha}\underline{H}$ (or G-2)	0.07
-6.6	5.37	G-1 (HIS $C^{\alpha}\underline{H}$ or $C^{\beta}\underline{H}$)	0.07
-11.6	3.89	M-4	0.10
-15.1[†]	4.80	M-3	0.10

[†]Temperature = 323°K

[‡]pH = 8.0, Temperature = 313°K

[§]The uncertainty in the assignment of some of these resonances arises out of the complexity of the spectrum of the free drug between 3.8 and 4.1 ppm where several $C\underline{H}$ resonances overlap.

out of the observed spectral range or broaden it beyond detection. The shifts observed for several resonances of the mannose residue indicate that the carbamoyl group may be coordinated to the metal ion. The very large and non-equivalent shifts of the two distinct pyrimidine $C\underline{H}_2$ resonances suggest that this group may be adjacent to the metal ion binding site due to ligation of the pyrimidine N_1 and/or the secondary amine. The perturbation of the $C\underline{H}$ proton of the alanine moiety implicates the α-amino group as a ligand. These results are compatible with the results of other studies of metal-bleomycin complexes in the literature (9-13).

The shifts exhibited by the $C^{\alpha}\underline{H}$, $C^{\beta}\underline{H}$, $C^{\gamma}\underline{H}$, $C^{\alpha}\underline{H}_3$ and $C^{\gamma}\underline{H}_3$ resonances of the valeric acid residue suggest that this residue may also be ligated to the

metal ion. Oppenheimer et al. (11) have attributed the anomalous shifts of the valeric acid resonances of the Bleo-A_2 complex with Zn(II) (which might serve as a diamagnetic analogue of Fe(II)) to placement of this residue over the imidazole ligation site or to direct coordination of the metal ion. Our data tends to favor the latter explanation with the hydroxyl group or the carbonyl group serving as the ligand.

The above considerations suggest the following coordination sites for the Fe(II) complex: α-amino group, imidazole N^{π}, carbamoyl oxygen, valeric acid OH or CO, pyrimidine N_1 and/or the secondary amino group. With space filling models, we have been able to construct a number of plausible structures for such a complex. However, in view of the uncertainties associated with the interpretation of the present as well as other experimental data, it is premature to formulate any detailed model for the structure of this complex. Experiments are now in progress in this laboratory to further define the structure of iron(II)-bleomycin and to determine its magnetic and kinetic properties.

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