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PROTON NMR STUDIES OF TERNARY COMPLEXES OF BLEOMYCIN WITH METAL IONS AND NUCLEIC ACID

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Summary

The formation of a ternary complex of bleomycin of the type metal ion-Bleo-A_2-poly(dA-dT) is indicated by the observation of spin diffusion in the ^1H NMR spectra when nuclear Overhauser experiments are performed on samples containing poly(dA-dT) and Zn(II)-Bleo-A_2 or CO-Fe(II)-Bleo-A_2. The enhancement of the transverse relaxation rates of the resonances of Fe(II)-Bleo-A_2 by poly(dA-dT) demonstrates the formation of the putative active complex Fe(II)-Bleo-A_2-poly(dA-dT). The absence of substantial changes in the chemical shifts suggests that the geometry of metal ion co-ordination is minimally perturbed by polynucleotide binding, which is consistent with the bifunctional nature of bleomycin.

Introduction

The antibacterial and antitumor activity of the bleomycins (Figure 1) has been attributed to the ability of these glycopeptides to degrade DNA in the presence of molecular oxygen and a metal ion cofactor such as Fe(II) (1-4). A number of quasi-enzymatic and free radical mechanisms have been proposed for this reaction (5-7). Characterization of the putative active ternary complex, Fe(II)-Bleo-DNA which can bind molecular oxygen is essential to the determination of the mechanism of action of these antibiotics.

Binary complexes of bleomycin with metal ions have been studied extensively (8-11), but there is still some controversy regarding their structures. Investigations of binary complexes of the drug with nucleic acids have also been reported (12-14). From the study of binary complexes it is clear that the C-terminal end of the antibiotic binds to DNA while the N-terminus chelates the metal ion.

There is as yet little evidence about the structure of the ternary complexes. That such complexes are formed in solution is suggested by the quenching of the fluorescence of the bithiazole group of bleomycin by DNA in the presence of Cu(II) or Fe(II) (15,16). Similar results were reported by Oppenheimer *et al.* using CO-Fe(II)-Bleo-A_2, an inactive analogue of O_2-

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Abreviations: Bleo-A_2: Bleomycin-A_2; TSP: Trimethylsilylpropionate; NOE: Nuclear Overhauser Effect.

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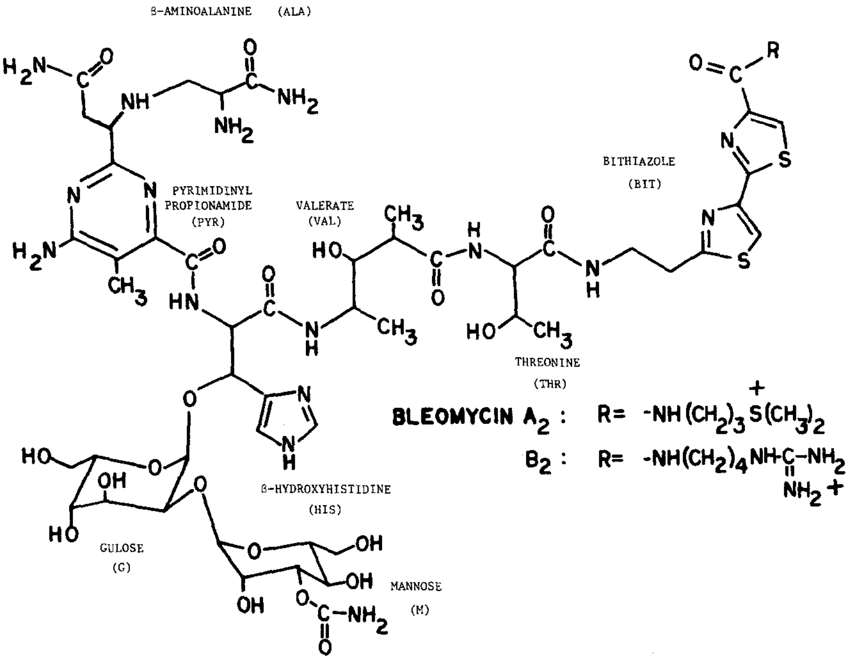

Figure 1 Primary structure of bleomycin congeners.

Fe(II)-Bleo-A_2 (10). The observation by Sugiura and Ishizu (17) that the ESR spectrum of NO-Fe(II)-Bleo-A_2 is perturbed by calf thymus DNA also provides evidence for the formation of a ternary complex to which the inactive oxygen analog NO is bound.

In this paper we report ^{1}H NMR studies using nuclear Overhauser effects which demonstrate the formation of the diamagnetic complexes Zn(II)-Bleo-A_2-poly(dA-dT) and CO-Fe(II)-Bleo-A_2-poly(dA-dT). We also report enhanced relaxation rates of the resonances of Fe(II)-Bleo-A_2 in the presence of poly(dA-dT) as direct evidence for the formation of the putative active ternary complex. These experiments demonstrate the feasibility of performing NMR studies of the molecular structure of the active ternary complex and its various inactive analogues. They also provide a basis for correlating the structures of the binary and ternary complexes of the antibiotic.

Materials and Methods

The purification of poly(dA-dT) (P-L Biochemicals, Milwaukee, Wis) and Bleo-A_2 (isolated from Blenoxane, Bristol Laboratories, Syracuse, N.Y) as well as the preparation of [A(D-8)]poly(dA-dT) has been previously described (13).

All samples were prepared with 9 μmoles of poly(dA-dT), 50 or 500 μmoles NaCl and 1 μmole of TSP. Sufficient Bleo-A_2 was added to yield the required nucleic acid to drug ratio (P/D) and the samples were lyophilized vs D_2O, dissolved in 500 μl D_2O and purged free of oxygen. $FeSO_4$ in D_2O, equivalent to the concentration of Bleo-A_2 was added to each sample under strict oxygen-free conditions and the pH (meter reading, uncorrected for isotope effects) was adjusted to 7.0. Complexes with CO were prepared by bubbling ultrapure CO through the solution for a few minutes and readjusting the pH to 7.0. The samples were transferred to a purged 5 mm NMR tube and sealed. The Zn(II) complexes were prepared with similar concentrations of reagents, but without taking precautions to exclude oxygen. Appropriate controls for the above complexes were also prepared, such as Fe(II)-Bleo-A_2-CO and Zn(II)-Bleo-A_2, under oxygen-free conditions when necessary.

NMR Spectra. Proton NMR spectra were recorded at 400 MHz on a Bruker WH-400 spectrometer operating in the FT mode using quadrature detection. The NOE experiment was performed by saturating the dimethylsulfonium resonance using the gated homodecoupling mode (24 db below .1 watt attenuation).

Results and Discussion

NOE Experiments. The application of NOE's to obtain qualitative structural information, and in favorable cases, internuclear distances, is well documented (18-20). Direct dipole-dipole interaction between observed and irradiated protons is the most probable cause of NOE's in the NMR spectra of macromolecules. When the dimethylsulfonium group of Zn(II)-Bleo-A_2 or CO-Fe(II)-Bleo-A_2 was irradiated in the presence of poly(dA-dT), a negative NOE was observed for all Bleo-A_2 as well as poly(dA-dT) resonances (Figures 2 and 3). This observation of spin diffusion demonstrates that the two spin systems-- metal-bound Bleo-A_2 and poly(dA-dT) are in thermal contact and hence a ternary complex is formed. Other features of the spectra also support this conclusion. The characteristic shift of the pyrimidine methyl resonance from 2.0 to 2.4 ppm which accompanies metal ion binding as reported by Oppenheimer et al. in Zn(II)-Bleo-A_2 (9) and in CO-Fe(II)-Bleo-A_2 (10) is clearly evident in the spectra. The broadening of the bithiazole resonances which was observed as a result of complexation with nucleic acids (15,16) is also evident. Since metal ion binding is slow while nucleic acid binding is fast on the NMR chemical shift time scale, the observation of simultaneous perturbations of Bleo-A_2 resonances by the metal ion and the polynucleotide clearly indicates the formation of a ternary complex.

Although the observation of a steady state NOE demonstrates the formation of a ternary complex, no structural information can be obtained since the effects are distributed over the entire molecule (spin diffusion). However, transient NOE's (20) should help identify the protons on the antibiotic and the polynucleotide which are adjacent to each other and thus relax each other through dipolar interactions. Transient NOE experiments may also yield internuclear distances and thus help formulate a model for the structure of the ternary complex. Such experiments are currently in progress in this laboratory.

Relaxation Rate Enhancement. ^1H NMR spectra which demonstrate the effects of adding poly(dA-dT) to Fe(II)-Bleo-A_2 in the presence of 100 mM NaCl and 1.0 M NaCl are shown in Figure 4. As previously reported for the Fe(II)-Bleo-A_2 complex (21), the binary complex satisfies the slow exchange limit on

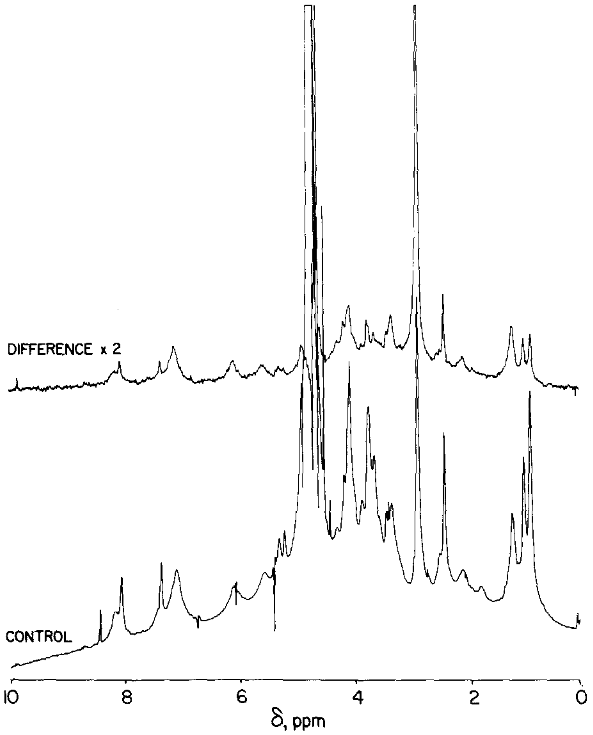

Figure 2 400 MHz ^1H NMR spectra of poly(dA-dT) + Zn(II)-Bleo-A_2 in D_2O at pH 6.7 and 25°C; Zn(II) = Bleo-A_2 = 3 mM, poly(dA-dT) = 18 mM, NaCl = 0.1 M. (bottom): control spectrum, (top): NOE difference spectrum obtained by irradiating the dimethyl sulfonium resonance at ∿3 ppm.

the chemical shift time scale. In contrast, nucleic acid binding to the drug satisfies the fast exchange criterion for both binary (13) and ternary complexes. The hyperfine shifted resonances of the metal complex which lie outside the normal diamagnetic region of the spectrum provide convenient probes for monitoring the interactions of the antibiotic with both the metal ion and the nucleic acid. The linewidths and chemical shifts of these resonances are summarized in Tables I and II. It can be seen that the hyperfine shifted resonances are broader in the presence of 100 mM NaCl compared to when 1 M NaCl is present. Since the linewidth of the TSP resonance remains approximately the same, non-specific broadening due to viscocity or other effects may be ruled out. Bleomycin is released from DNA by high salt concentrations (D.M. Chen and J.D. Glickson, unpublished data); thus the linebroadening is most likely to be due to the formation of a ternary complex of the type Fe(II)-Bleo-poly(dA-dT). It must be pointed out that the resonances in the diamagnetic region are also broadened by poly(dA-dT) in the presence of 100 mM NaCl, but remain sharp when 1 M NaCl is present.

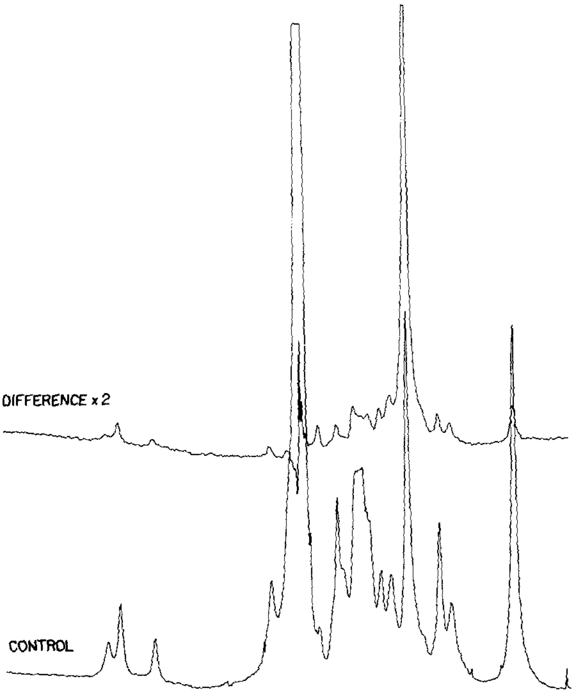

Figure 3 400 MHz ^1H NMR spectra of poly(dA-dT) + CO-Fe(II)-Bleo-A_2 in D_2O at pH 7.0 and 25°C; Fe(II) = Bleo-A_2 = poly(dA-dT) = 18 mM, NaCl = 0.1 M. (bottom): control spectrum, (top): NOE difference spectrum obtained as in Figure 2.

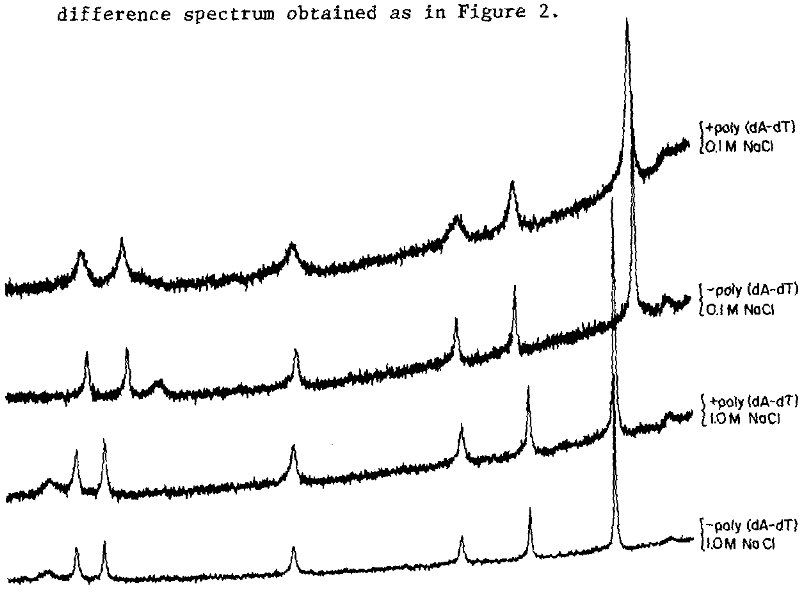

Figure 4 Effect of poly(dA-dT) and NaCl on the chemical shifts and linewidths of the hyperfine shifted resonances in the proton NMR spectra of Fe(II)-Bleo-A_2 samples: Fe(II) = Bleo-A_2 = 6 mM, poly(dA-dT) = 0 or 18 mM, NaCl = 0.1 or 1.0 M. Only the low field resonances are shown.

Table I

Comparison of Linewidths of Fe(II) Complexes of Bleomycin

Linewidth in Hertz

Resonance	Poly(dA-dT) + Fe(II)-Bleo-A_2, 0.1 M NaCl	Fe(II)-Bleo-A_2, 0.1 M NaCl	Poly(dA-dT) + Fe(II)-Bleo-A_2, 1.0 M NaCl	Fe(II)-Bleo-A_2, 1.0 M NaCl
1/2 PYR $C\underline{H}_2$	232	95	111	97
HIS $C_4\underline{H}$	209	86	85	70
VAL $C^{\alpha}\underline{H}$	>500	280	360	323
1/2 PYR $C\underline{H}_2$	330	123	145	104
VAL $C^{\beta}\underline{H}$	280	106	124	99
VAL $C^{\gamma}\underline{H}$	180	62	53	59
VAL $C^{\alpha}\underline{H}_3$	148	57	60	47
TSP	9	12	11	8
M-2 (ALA $C^{\alpha}\underline{H}$)	93	48	42	35
G-2 (ALA $C^{\alpha}\underline{H}$)	175	75	86	64
G-1 (HIS $C^{\alpha}\underline{H}$ or $C^{\beta}\underline{H}$)	170	80	96	63
M-4	141	65	80	61
M-3	∿440	151	225	155

Table II

Comparison of Chemical Shifts of Fe(II) Complexes of Bleomycin

Chemical Shifts (ppm)

Resonance	Poly(dA-dT) + Fe(II)-Bleo-A_2, 0.1 M NaCl	Fe(II)-Bleo-A_2, 0.1 M NaCl	Poly(dA-dT) + Fe(II)-Bleo-A_2, 1.0 M NaCl	Fe(II)-Bleo-A_2, 1.0 M NaCl
1/2 PYR $C\underline{H}_2$	45.629	45.354	46.005	46.036
HIS $C_4\underline{H}$	43.239	43.026	44.368	44.429
VAL $C^{\alpha}\underline{H}$	41.105	41.200	47.540	47.700
1/2 PYR $C\underline{H}_2$	33.304	33.161	33.365	33.406
VAL $C^{\beta}\underline{H}$	23.664	23.745	23.501	23.542
VAL $C^{\gamma}\underline{H}$	20.379	20.328	19.586	19.576
VAL $C^{\alpha}\underline{H}_3$	13.555	13.332	14.522	14.572
TSP	0.000	0.000	0.000	0.000
M-2 (ALA $C^{\alpha}\underline{H}$)	-3.905	-3.925	-2.898	-2.847
G-2 (ALA $C^{\alpha}\underline{H}$)	-5.237	-5.288	-5.664	-5.685
G-1 (HIS $C^{\alpha}\underline{H}$ or $C^{\beta}\underline{H}$)	-6.590	-6.640	-5.664	-5.685
M-4	-11.603	-11.603	-9.956	-9.905
M-3	-15.0	-14.8	-10.5	-10.342

It can be seen from Table II that the chemical shifts of the resonances are not significantly altered by the polynucleotide, indicating that the geometry of metal ion co-ordination is minimally perturbed. This is consistent with the bifunctional nature of the drug, which predicts binding of metal ions and nucleic acid at opposite ends of the drug. The perturbations caused by high concentrations of NaCl may be due to the binding of Cl^- ion and/or due to small changes in the structure of the complex.

The ^1H NMR spectra of samples containing varying concentrations of Fe(II)-Bleo-A_2, constant poly(dA-dT) and 100 mM NaCl are shown in Figure 5. It can be seen that the linewidths (summarized in Table III) increase with increasing P/D, whereas the linewidths of the TSP resonances exhibit the opposite trend. The narrowing of the TSP resonances is caused by the decrease in the concentration of the paramagnetic metal ion with increasing P/D. This observation excludes paramagetic impurities as the cause of the increased linewidth of the Bleo-A_2 resonances. Since the concentration of the polynucleotide is kept constant, and the viscocity is therefore also expected to be unchanged, these data provide further evidence that the linebroadening is not caused by viscocity effects.

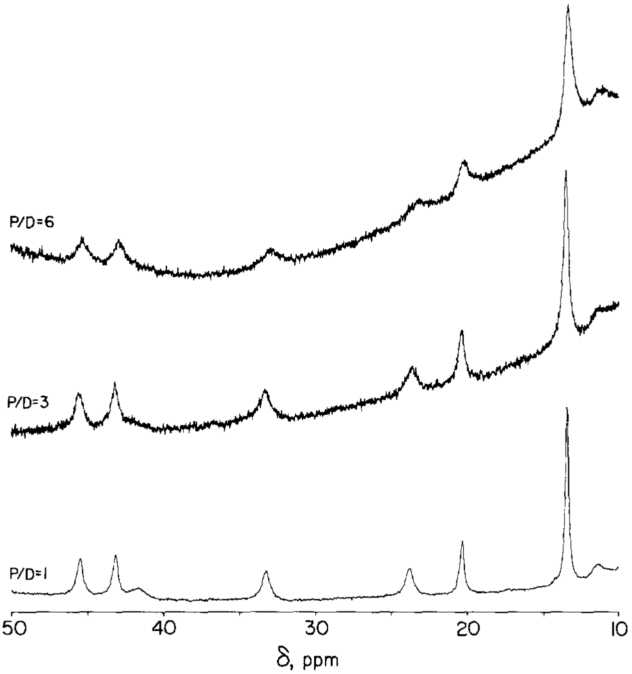

Figure 5 Proton NMR spectra of Fe(II)-Bleo-A_2-poly(dA-dT) at varying P/D at pH 7.0 and 25°C; poly(dA-dT) = 18 mM, Fe(II) = Bleo-A_2 = 3, 6 or 18 mM, NaCl = 0.1 M. Only the low field hyperfine shifted resonances are shown.

Table III

Dependence of Linewidths of Hyperfine Shifted Resonances on P/D ratio

Resonance	Linewidth in Hertz P/D = 1.0	P/D = 3.0	P/D = 6.0
1/2 PYR $C\underline{H}_2$	172	232	385
HIS $C_4\underline{H}$	126	209	350
VAL $C^{\alpha}\underline{H}$	466	>500	Too broad
1/2 PYR $C\underline{H}_2$	198	330	450
VAL $C^{\beta}\underline{H}$	204	280	>500
VAL $C^{\gamma}\underline{H}$	126	180	317
VAL $C^{\alpha}\underline{H}_3$	92	148	235
TSP	12	9	6
M-2 (ALA $C^{\alpha}\underline{H}$)	68	93	120
G-2 (ALA $C^{\alpha}\underline{H}$)	103	175	227
G-1 (HIS $C^{\alpha}\underline{H}$ or $C^{\beta}\underline{H}$)	111	170	300
M-4	105	141	350
M-3	265	0	Too broad

Since Fe(II)-Bleo-A_2 is in slow exchange with the free antibiotic and the metal ion on the chemical shift time scale, the linewidths of the shifted resonances in the absence of nucleic acid binding are given by (22)

$$1/T_2 = 1/T_{2M} + 1/\tau_M \qquad (1)$$

where $1/T_2$ is the observed transverse relaxation rate of the resonance, $1/T_{2M}$ its relaxation rate in the co-ordination sphere of the metal ion, and $1/\tau_M$ is the exchange rate between the free and bound environments. On the other hand, the dissociation of poly(dA-dT) from the drug or its metal complex is fast on the chemical shift time scale. Hence the enhancement of the relaxation rate of the shifted resonances ($1/T_{2p}$) as a consequence of the formation of the ternary complex, in the absence of significant chemical shift perturbations, is given by

$$1/T_{2p} = P_M/(T_{2M(t)} + \tau'_M) \qquad (2)$$

where P_M is the fraction of the binary metal ion complex that is complexed to the polynucleotide, $1/T_{2M(t)}$ is the relaxation rate in the ternary complex, and $1/\tau'_M$ is the exchange rate of the polynucleotide from the ternary complex. Thus the increase in the linewidths of these resonances with increasing P/D can be attributed to the increase in the relative concentration of the ternary complex with respect to the binary complex. The present data also indicates that the exchange contribution is not the dominant part in equation 2, since the magnitude of the linebroadening is not the same for all the resonances.

It is clear from the present investigation that the ternary complex retains the structural characteristics of the binary complexes of the drug with metal ions and with nucleic acids. Thus useful information about the structure of the active ternary complex can be obtained from the study of appropriate binary complexes. The study of diamagnetic analogs of the ternary complex may also be of importance, and are being undertaken in this laboratory.

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