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LOCATION OF IRON IN THE Fe^{2+}-BLEOMYCIN COMPLEX AS OBSERVED BY ^{13}C NMR SPECTROSCOPY

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Summary: The antineoplastic action of bleomycin is currently thought to arise from the degradation of cellular DNA by the iron-bleomycin complex. Bleomycin A_2 has one iron binding site as revealed by the iron-titrations of bleomycin monitored optically. To probe the structure of the Fe^{2+}-bleomycin complex, we studied the paramagnetic effects of its high spin ferrous iron on the nuclear relaxation rates ($1/T_1$) of the natural abundance carbon-13 atoms in the molecule. The presence of Fe^{2+} in bleomycin predominantly enhances the $1/T_1$ of only four protonated carbon atoms in the molecule (C2, C3, C5, and C6). No other protonated carbon atoms are affected significantly. From the magnitudes of the paramagnetic effects of Fe^{2+} on the ^{13}C relaxation rates, we obtain distances of 3.6, 4.1, 4.0, and 3.6 Å from the metal to the C2, C3, C5, and C6 carbon atoms, respectively. These results are consistent with the metal ion-chelation of the α-amino group of the terminal diaminopropionic acid residue and the pyrimidine ring but do not implicate any other parts of the bleomycin molecule in binding to iron.

INTRODUCTION

Bleomycins are glycopeptide antibiotics (1) currently in use in chemotherapy of various malignancies. Bleomycin A_2 is the principal component of the clinically used drug (2). The antineoplastic properties of bleomycin are thought to arise from its interaction with cellular DNA (3-17). The complex of iron with bleomycin has been implicated in this molecule's ability to cause strand scission in DNA (4, 5, 12, 15, 16). Other metal ions, such as Cu^{2+} and Zn^{2+}, inhibit this reaction (17). We find that in the presence of oxygen, bleomycin acts as a ferrous oxidase and catalytically oxidizes Fe^{2+} to Fe^{3+}. This oxygen-dependent reaction of bleomycin with iron follows classical Michaelis-Menton kinetics, characteristic of enzymatic

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reactions, with one mole of bleomycin A_2 turning over ∿5,000 moles Fe^{2+} per min. The ability of bleomycin to bind DNA, and to cause continuous oxygen reduction and metal ion oxidation at this most important center in the cell, suggests the possibility that potentially reactive reduced oxygen species may be involved in DNA degradation. Our EPR studies reveal the formation of a superoxide radical intermediate in the metal-ion oxidation by bleomycin. In view of its importance in the bleomycin reaction, we have probed the anaerobic structure of the iron-bleomycin complex using paramagnetic effects of ferrous iron on ^{13}C nuclear relaxation times. In contrast to the non-specific and delocalized effects of Zn^{2+} and Cu^{2+} on the line widths and chemical shifts of the ^{1}H and ^{13}C NMR reported previously (18, 19), we report in this paper the observation of selective and localized effects of ferrous iron on the spin lattice relaxation times T_1 of the ^{13}C nuclei of bleomycin which permit direct identification of the chemical groups involved in the complexation of Fe^{2+} by bleomycin.

MATERIALS AND METHODS

Purified bleomycin A_2 was a generous gift from the National Cancer Institute, Bethesda, Md. The spin state of iron in Fe^{2+}-bleomycin was established by magnetic susceptibility measurements, which were carried out at 360 MHz on a Bruker WH-360 MHz spectrometer, using the NMR method (20). To obtain the electron spin relaxation time τ_s of Fe^{2+}, water proton relaxation measurements were carried out at 8, 15 and 24.3 MHz on a spin echo spectrometer, at 100 MHz on a Varian XL-100 and at 360 MHz on a Bruker WH-360 instrument. For natural abundance ^{13}C NMR, bleomycin A_2 was dissolved in double distilled deionized water, containing 10% D_2O for field-frequency lock, to a final concentration of ∿0.05M (pH = 5.5 ± 0.2). Field-dependent ^{13}C T_1 measurements on metal free bleomycin were made at 67.9 MHz on a Bruker WH-270 at NIH and at 45.3 MHz on a Bruker WH-180 at the University of Pennsylvania. The paramagnetic effects of iron on ^{13}C relaxation rates were measured at 67.9 MHz only. All measurements were made under anaerobic conditions in an atmosphere of nitrogen at 24 ± 1°C. ^{13}C T_1 determinations were made in the presence of proton-noise decoupling using the fast inversion recovery method. The transverse relaxation rates were estimated from ^{13}C line-widths. All of the ^{13}C resonances in the NMR spectrum of bleomycin have previously been assigned to 55 individual carbon atoms in the molecule (21, 22). The ^{1}H-^{13}C spin multiplet patterns observed by us, in the absence of proton decoupling, were consistent with these assignments. The paramagnetic effects of ferrous iron on the longitudinal ($1/T_{1p}$) and transverse ($1/T_{2p}$) relaxation rates of a ^{13}C nucleus were evaluated by subtracting the relaxation rates of metal-free bleomycin from those obtained in the presence of iron. For a ^{13}C nucleus that exchanges between a paramagnetic and a diamagnetic environment rapidly with respect to the difference between the resonance frequencies of the two environments, such that an exchange-averaged resonance is observed, the effect of the paramagnetic environment on the relaxation rates can be described by the following equations (23):

$$\frac{1}{fT_{1p}} = \frac{1}{T_{1M}} \qquad [1]$$

$$\frac{1}{fT_{2p}} = \frac{1}{T_{2M}} + \frac{\tau_M(1-f)^2(\Delta\omega_M)^2}{\pi} \qquad [2]$$

T_{1M}^{-1} and T_{2M}^{-1} are the longitudinal and transverse relaxation rates in the paramagnetic environment and τ_M is the residence time in the paramagnetic environment. $\Delta\omega_M$ is the frequency shift experienced by the ^{13}C nucleus under consideration in the iron-bleomycin complex and may be paramagnetic or diamagnetic in origin. f is the ratio of the concentration of Fe^{2+}-bleomycin complex to the total bleomycin concentration. When dipolar interactions predominate, the distance dependencies of T_{1M} and T_{2M} are described by the following equations (24):

$$\frac{1}{T_{1M}} = \frac{2\mu_S^2\gamma_I^2}{15r^6}\left\{\frac{3\tau_S}{1+\omega_I^2\tau_S^2} + \frac{7\tau_S}{1+\omega_S^2\tau_S^2}\right\} \qquad [3]$$

$$\frac{1}{T_{2M}} = \frac{2\mu_S^2\gamma_I^2}{15r^6}\left\{2\tau_S + \frac{1.5\tau_S}{1+\omega_I^2\tau_S^2} + \frac{6.5\tau_S}{1+\omega_S^2\tau_S^2}\right\} \qquad [4]$$

where symbols characterizing the electron and nuclear moments have their usual meaning. For ferrous iron the electron spin relaxation time τ_S is the correlation time for the metal-nuclear dipolar interaction (25). Although not included in equation 4, a sizable contribution from the dipolar-interaction of nuclei with the thermal average of the electron spin ("Curie spin") may also be present in $1/T_{2p}$ (26). The same mechanism, however, gives rise to a vanishingly small contribution to $1/T_{1p}$ (26). The distance dependence of $1/T_{1M}$ described by equation 3 may be used to calculate distances from iron to various carbon atoms of the bleomycin molecule. However, implicit in the derivation of equations 1 and 3 are the assumptions that T_{1p} is not exchange-limited and that the outersphere and contact hyperfine contributions to paramagnetic effects on $1/T_{1p}$ are negligible (23). We consider the validity of these assumptions later in this paper.

RESULTS AND DISCUSSION

Addition of bleomycin to iron causes significant changes in the optical spectrum of the metal which are observable in the visible region. In the presence of a saturating level of bleomycin, a sizable change in the visible absorption occurs at a wavelength of 460 nm ($\varepsilon_{mM}^{460} = 0.225$) in titrations of bleomycin with Fe^{2+} and at 430 nm ($\varepsilon_{mM}^{430} = 2.0$) in titrations with Fe^{3+}. In both cases, iron-titrations of bleomycin show sharp breaks at equimolar concentrations of bleomycin and iron and reveal the existence of only a single iron binding site per molecule of the drug in either oxidation state of the metal. Complexation of bleomycin with Mn^{2+} was also studied measuring free Mn^{2+} directly by EPR spectroscopy. A scatchard plot of the EPR data revealed the existence of only one tight site for Mn^{2+} in the bleomycin molecule. Addition of Fe^{2+}, Fe^{3+}, Zn^{2+}

or Cu^{2+} to a solution containing Mn^{2+} and bleomycin at non-saturating levels increases the amplitude of the free Mn^{2+} signal due to displacement of Mn^{2+} by other metal ions, suggesting that the binding of various metal ions to bleomycin is mutually exclusive. In contrast to other metal ions investigated, Mg^{2+}, however, shows little or no affinity for bleomycin. This finding may offer an explanation for the observation that Mg^{2+} has no significant inhibitory effect on the activity of bleomycin (12). It would appear that the inhibition of bleomycin by various metal ions results from the displacement of iron from the iron-bleomycin complex by inactive metals.

Since Fe^{2+} is required for bleomycin action, we probed its magnetic state in the iron-bleomycin complex by susceptibility measurements which revealed the presence of a high spin ferrous iron (S=2) under anaerobic conditions. To further probe the structure of the active iron-bleomycin complex, the paramagnetic effects of the ferrous iron on the longitudinal nuclear relaxation rates ($1/T_1$) of ^{13}C nuclei in the bleomycin molecule were studied. However, since signal-to-noise considerations require the use of high levels of bleomycin in solution for the study of the paramagnetic effects of iron on the ^{13}C nuclei of bleomycin in natural abundance, it became necessary to show that no significant aggregation of bleomycin occurred at these levels. For this purpose, the overall rotational correlation time of the bleomycin molecule was determined from field-dependent studies of T_1 values of protonated carbon atoms at 67.9 and 45.3 MHz (Table I). A small but significant dependence of T_1 on the strength of the magnetic field is observed. An analysis of the field-dependent relaxation data yielded a rotational correlation time of 6×10^{-10} s which is close to the value of 4×10^{-10}s obtained from Stoke's law for a spherical monomeric molecule of M.W. $\sim$1420, indicating the absence of any significant aggregation of bleomycin under our conditions.

The presence of ferrous iron in a solution of bleomycin greatly enhances the $1/T_1$ of several carbon atoms in the molecule, presumably due to the paramagnetic effects of the iron. Such effects are not observed with diamagnetic metals. The

TABLE I

Paramagnetic effects of Fe^{2+} on nuclear spin relaxation times of protonated natural abundance ^{13}C atoms in bleomycin A_2 (54 mM) at 67.9 MHz (T = 24°C) under anaerobic conditions.

Carbon	T_1[a]	T_1[b]	T_1[c]	T_{1p}^{-1}[b]	T_{1p}^{-1}[c]	T_{1M}^{-1}
#	sec			sec^{-1}		
22,33	0.16(0.15)	0.17	0.18	-0.4	-0.7	
19	0.17(0.15)	0.17	0.19	0.0	-0.6	
24	0.15(0.14)	0.15	0.15	0.0	0.0	
18	0.15(0.15)	0.15	0.16	0.0	-0.4	
17	0.16(0.16)	0.16	0.16	0.0	0.0	
21	0.18(0.16)	0.17	0.15	0.3	1.1	
14	0.15(0.13)	0.15	0.14	0.0	0.5	
16,38	0.19(0.14)	0.16	0.18	1.0	0.3	
23	0.17(0.14)	0.19	0.19	-0.6	-0.6	
25	0.11(0.10)	0.11	0.11	0.0	0.0	
50	0.14(0.15)	0.14	0.12	0.0	1.2	
02	0.19(0.16)	0.07	0.04	9.0	20.	35 ± 2
37	0.18(0.15)	0.18	0.20	0.0	-0.6	
13	0.15(0.13)	0.14	0.14	0.5	0.5	
06	0.28(0.25)	0.08	0.04	8.9	21.	36 ± 2
31	0.19(0.13)	0.15	0.15	1.4	1.4	
03	0.14(0.13)	0.09	0.06	4.0	9.5	16 ± 2
34	0.16(0.13)	0.19	0.17	-1.0	-0.4	
53	0.45(0.36)	0.42	0.41	0.2	0.2	
05	0.13(0.13)	0.08	0.05	4.8	12.	20 ± 2
41	0.11(0.12)	0.13	0.11	-1.4	0.0	
51	0.19(0.12)	0.19	0.18	0.0	0.3	
42	0.12(0.09)	0.12	0.13	0.0	-0.6	
52	0.28(0.25)	0.27	0.27	0.1	0.1	
29	0.14	0.14	0.14	0.0	0.0	
28	0.14	0.14	0.13	0.0	0.5	
44	0.16	0.14	0.14	0.9	0.9	
47	0.15	0.14	0.13	0.5	1.0	
15	0.15	0.14	0.15	0.5	0.0	
20	0.15	0.14	0.14	0.5	0.5	

[a]In the absence of Fe^{2+}; [b]With 14 mM Fe^{2+}; [c]With 31 mM Fe^{2+}.
Numbers in parentheses were obtained at 45.3 MHz.

magnitudes of the paramagnetic effects of ferrous iron on $1/T_1$ of protonated carbon atoms in the bleomycin molecule measured at 67.9 MHz are quite selective and are shown in Table I. The presence of ferrous iron predominantly enhances the $1/T_1$ of only four carbon atoms in the bleomycin molecule. All other carbon atoms are affected to a much smaller extent. The selective nature of the paramagnetic effects observed here by itself justifies our assumption that they arise from the complexation of bleomycin with iron and are not outersphere in origin. Assuming the observed effects to arise from the iron-bleomycin complex, the larger magnitude of effect on $1/T_2$ may be explained by $\Delta\omega_M$ contribution in equation 2 and/or by the

TABLE II

Calculation of Fe^{2+}-^{13}C distances for iron-bleomycin A_2 complex.

Carbon[a] #	$(fT_{1p})^{-1}$	$(fT_{2p})^{-1}$	τ_S	$r(^{13}C\text{-}Fe^{2+})$
	sec^{-1}		$x10^{12}$ sec	Å
02	35 ± 2	330 ± 50	1.0 ± 0.5	3.6 ± 0.3
03	16 ± 2	200 ± 50	1.0 ± 0.5	4.1 ± 0.3
05	20 ± 2	200 ± 50	1.0 ± 0.5	4.0 ± 0.3
06	36 ± 2	330 ± 50	1.0 ± 0.5	3.6 ± 0.3

[a]These are the only carbon atoms upon which significant paramagnetic effects were observed.

effects of Curie magnetization on $1/T_{2M}$ (26). Further, the observed largest paramagnetic effect on $1/T_2$ sets a lower limit on $1/\tau_M$, the rate of dissociation of the iron-bleomycin complex. Since the largest $1/T_{2p}$ exceeds the largest $1/T_{1p}$ on a ^{13}C nucleus by an order of magnitude (Table II), $\tau_M << T_{1M}$. Exchange-limitation on the longitudinal relaxation is therefore unlikely. The observed variation in the magnitude of paramagnetic effects on various carbons also argues against τ_M-limited relaxation which would predict essentially similar effects on all affected carbons, τ_M being the same for all carbon atoms within a molecule. Thus, by the above criteria, the observed paramgnetic effects on $1/T_1$ may be used for calculating Fe^{2+}-^{13}C distances.

The correlation time for the metal-nuclear dipolar interaction required for distance calculations via equation 3 was determined from the frequency dependence of paramagnetic effects of Fe^{2+}-bleomycin complex on the relaxation rate of water protons in the same complex. This approach is valid since the correlation time for both the Fe^{2+}-^{13}C and the Fe^{2+}-^{1}H (H_2O) dipolar interactions is dominated by τ_S, the electron spin relaxation time of ferrous iron. The magnitude of the longitudinal component of molar paramagnetic relaxivity of the Fe^{2+}-bleomycin complex for water protons remained unchanged from 8-100 MHz at $\sim$400 $s^{-1}M^{-1}$ but decreased by $\sim$2-fold at 360 MHz. The observed paramagnetic effect of Fe^{2+}-bleomycin on $1/T_1$ of water protons was in the fast exchange-region on the NMR time scale, since the magnitude of the effect decreased by $\sim$10% with a 20°C increase in the sample

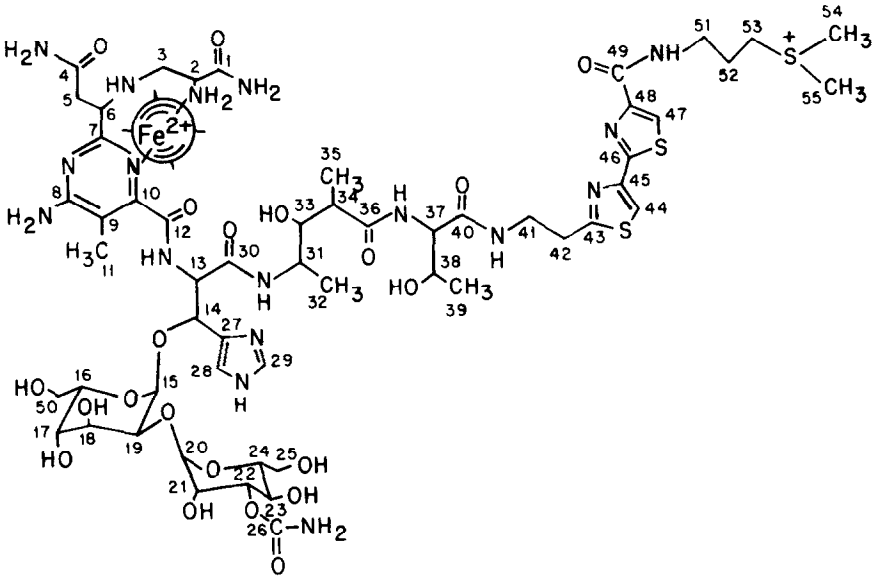

Fig. 1: Location of ferrous iron in the structure of bleomycin A_2.

temperature. Exchange-limited paramagnetic relaxation would be expected to show a more pronounced temperature dependence in the opposite direction (23), contrary to our observations. An analysis of the frequency-dependence of water proton relaxation via equation 3 yielded a value of $\sim 1 \times 10^{-12}$s for the τ_S and a value of 4 ± 1 for the number of exchanging water molecules in the innersphere of iron in the Fe^{2+}-bleomycin complex.

The paramagnetic effects of ferrous iron on bleomycin were measured at two different levels of Fe^{2+} (14 and 31 mM) under anaerobic conditions (Table I). At either level only four protonated carbon atoms are significantly affected by the presence of the ferrous iron. The protonated aromatic carbons, notably the carbon atoms of the imidazole ring, are essentially unaffected by iron-complexation. The variation in the magnitude of the paramagnetic effect of Fe^{2+} for different carbon atoms arises from the distance-dependence of the effect. From the observed paramagnetic effects of Fe^{2+}, using the correlation time τ_S of $\sim 1 \times 10^{-12}$s, derived from the water proton relaxation data for the Fe^{2+}-bleomycin complex, we obtain Fe^{2+}-^{13}C distances of 3.6, 4.1, 4.0 and 3.6 Å from the metal to the C2, C3, C5, and C6 carbon atoms, respectively. These

results are consistent with the metal ion- chelation of the α-amino group of the terminal diaminopropionic acid residue and the pyrimidine ring (Fig. 1) but do not implicate participation of the disaccaride moeity or the imidazole ring in binding of iron by bleomycin.

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