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Demonstration of Nitrogen Coordination in Metal–Bleomycin Complexes by Electron Spin–Echo Envelope Spectroscopy†

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ABSTRACT: We have studied the Cu(II), Co(II), and Fe(III) complexes of the antineoplastic drug bleomycin by using electron spin–echo envelope spectroscopy. For all three complexes, nitrogen coordination of the metal ion is demonstrated. For the Cu(II)- and Co(II)–drug complexes, we have been able to identify imidazole as a metal ligand.

The bleomycins (Figure 1) are members of a family of antibiotic glycopeptides that have been isolated as Cu(II) complexes from bacterial cultures of *Streptomyces verticillus* (Umezawa et al., 1966a,b). The various bleomycins differ from each other only in their terminal functional group (Umezawa, 1979). Bleomycin breaks DNA in vitro (Suzuki et al., 1969) in a reaction requiring the O_2-dependent oxidation of drug-bound Fe(II) (Ishida & Takahashi, 1975; Sausville et al., 1976; Horwitz et al., 1979). It is believed that the in vitro antitumor activity is caused by the same reaction (Umezawa, 1979).

The structures of metal complexes of bleomycin (Muraoka et al., 1976; Sugiura, 1978) are of great interest since the in vitro activity of the drug requires the formation of an Fe(II) complex (Sausville et al., 1976, 1978b; Burger et al., 1979). Other metal ions such as Cu(II), Co(II), and Zn(II) are inactive although they inhibit the DNA–breakage reaction with Fe(II) by replacing iron in the metal–drug complex (Sausville et al., 1978a).

Structural determinations of metal–bleomycin complexes by X-ray crystallographic procedures have not met with any great success, save in a single instance (Iitaka et al., 1978), because of the difficulty of preparing samples suitable for analysis. Other physical techniques employed for structural determinations include polarography and optical, NMR, and EPR spectroscopy (Dabrowiak et al., 1978a,b; Oppenheimer et al., 1979a,b; Gupta et al., 1979; Antholine & Petering, 1979; Burger et al., 1979; Sugiura, 1979a,b; Sugiura & Ishizu, 1979; Sugiura & Mino, 1979; Sugiura et al., 1979; Solaiman et al., 1980; Lenkinski et al., 1980). Although conclusions drawn from these studies are, in many instances, inferential, they suggest that metal ions are bound to the drug via nitrogenous ligand atoms, including those from imidazole and pyrimidine.

Another physical probe, especially useful for the study of paramagnetic metalloproteins, is electron spin–echo envelope spectroscopy (Mims & Peisach, 1979a). This technique has proven to be a useful means of identifying ligands of paramagnetic metal ions and of determining the coupling between nuclei belonging to these ligands and the unpaired electron spin. In this paper, we present electron spin–echo data demonstrating unequivocably that Cu(II), Co(II), and Fe(III) are ligated to bleomycin via nitrogenous ligands. For Cu(II) and Co(II), we are able to identify imidazole as a ligand to the metal ion, based on the ^{14}N coupling frequencies. For the Fe(III) complex, the analysis is more difficult, and coupling to more than a single ^{14}N is suggested.

Materials and Methods

Bleomycin sulfate (Blenoxane) was a gift of Bristol Laboratories and contained approximately 60% bleomycin A_2, 30% bleomycin B_2, and 10% various other bleomycins. Cu(II)–BLM[1] was prepared by mixing equal volumes of 40 mM cupric acetate with 60 mM bleomycin and then diluting with an equal volume of glycerol. The pH was raised to 7.2 with concentrated NaOH. Fe(III)–BLM was prepared by using ferrous

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[1] Abbreviations used: BLM, bleomycin; TPP, tetraphenylporphyrin.

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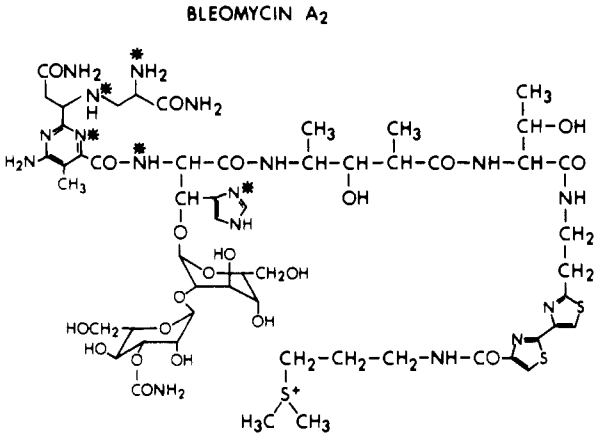

FIGURE 1: Chemical structure of bleomycin A_2. The asterisks in the figure designate the points of attachments of Cu(II) as indicated by an X-ray crystallographic study of the drug from which the terminal tripeptide fragment, the sugar moieties and their terminal amide, had been removed (Iitaka et al., 1978).

ammonium sulfate. Here, the same concentrations of ferrous salt and bleomycin were mixed as for the Cu(II)–BLM solution, but the pH was raised in this case to 7.6. Oxygen in the reagents readily effected the autoxidation of the iron (Burger et al., 1979).

Co(II)–BLM was prepared from solutions of components in 1:1 glycerol–H_2O that were made anaerobic with purified argon. Here, 18 mM cobalt(II) acetate was anaerobically added to an equal volume of 20 mM bleomycin in 60 mM phosphate buffer, pH 7.4.

In addition to metal–bleomycin complexes, various model compounds were also used in our studies. The copper(II) diethylenetriamine–imidazole complex (10 mM) was prepared as described previously (Mondovì et al., 1977). The copper(II) diethylenetriamine–pyrimidine complex (10 mM) was prepared by optical titration of copper(II) diethylenetriamine (10 mM) in glycerol–water with pyrimidine (16 mM) by using a Cary 14 spectrophotometer.

Co(II)–[^{14}N]TPP was purchased from Porphyrin Products. [^{15}N]TPP was prepared and then purified by reaction of benzaldehyde and [^{15}N]pyrrole (purchased from Merck, Sharpe & Dohme) by using established methods (Adler et al., 1967). Co(II)–[^{15}N]TPP was then prepared by ligation with cobalt(II) acetate in *N,N'*-dimethylformamide and then purified by previously reported procedures (Adler et al., 1970). the [^{14}N]- and [^{15}N]imidazole complexes of ^{14}N- and ^{15}N-labeled Co(II)–TPP were prepared anaerobically in tetrahydrofuran or in 35:65 dimethyl sulfoxide–$CHCl_3$. For spin–echo studies, the concentration of metal porphyrin was ~10 mM, and the imidazole concentration was about a 20-fold molar excess.

Two- and three-pulse echo decay envelopes for metal complexes of bleomycin and for model compounds were obtained at the X band as described previously (Mims & Peisach, 1976; Peisach et al., 1979). Those samples containing Co(II) were transferred to the microwave cavity (Mims & Peisach, 1976) and frozen under an argon atmosphere. For three-pulse studies, the data were manually extrapolated to $T + \tau = 0$ in order to facilitate Fourier transformation (Shimizu et al., 1979).

Results and Discussion

The single successful X-ray crystallographic study of a metal–bleomycin complex was performed on the Cu(II) complex of a drug fragment lacking the terminal tripeptide, the sugars and their terminal amide (Iitaka et al., 1978), and not

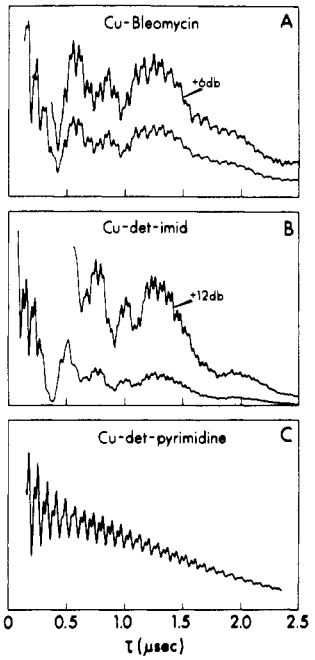

FIGURE 2: Two-pulse echo decay envelope of Cu(II) complexes of (A) bleomycin, (B) diethylenetriamine and imidazole, and (C) diethylenetriamine and pyrimidine. In traces A and B, one observes a modulation pattern arising from the interaction of Cu(II) with the remote ^{14}N of ligated imidazole. The respective magnetic fields and spectrometer frequencies are the following: (A) 3080 G, 9247 MHz; (B) 3195 G, 9251 MHz; (C) 2970 G, 9225 Hz.

of the complete drug molecule. The ligands to copper, indicated by asterisks in Figure 1, include nitrogen atoms from pyrimidine, imidazole, a side-chain amino group, and two peptidic nitrogen atoms.

A variety of physical probes have been applied to metal–bleomycin complexes in solution. An analysis of the EPR spectrum (Peisach & Blumberg, 1974) of the Cu(II) complex of the drug (Dabrowiak et al., 1978a) has led to the suggestion that the metal ion is predominantly nitrogen coordinated. This interpretation is not conclusive as no superhyperfine structure attributable to ^{14}N can be observed in the X-band spectrum. Other physical studies have led to the suggestion that Cu(II) and Zn(II) are linked to similar ligands in the drug (Dabrowiak et al., 1978a,b) although here, too, no direct evidence for metal–nitrogen coordination is presented. Recent NMR studies of the Zn(II)– and Fe(II)–CO complexes (Dabrowiak et al., 1978b; Oppenheimer et al., 1979a,b) conclude that both pyrimidine and imidazole nitrogen atoms, among others, are bound to the metal atom. In these, as well as in previous NMR studies (Dabrowiak et al., 1978a), large changes in the spectrum of the drug upon metal ligation afford evidence for complex formation but give no *direct* evidence as to the nature of the ligands.

In Figure 2A, we show the two-pulse electron spin–echo decay envelope for the Cu(II) complex of bleomycin in a frozen solution prepared with excess drug at near neutral pH. In addition to the short periods of 38 and 76 ns attributable to drug and solvent proton interactions with the Cu(II) (Mims et al., 1977), one observes a complex pattern containing periods of ~260 ns (three cycles can be seen) and also a slower period of ~700 ns which is observed at longer values of τ. A similar periodicity can be seen for the model compound copper(II) diethylenetriamine–imidazole (Figure 2B).

Previous studies had shown that there is no modulation of the echo envelope associated with the coupling between Cu(II) and directly coordinated ^{14}N (Mims & Peisach, 1976; Mondovì

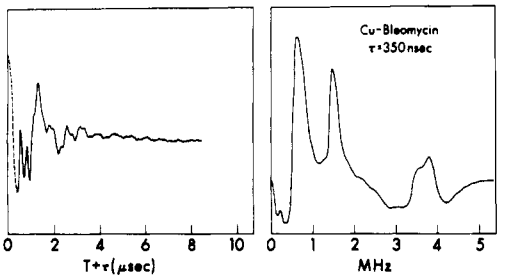

FIGURE 3: Three-pulse electron spin–echo decay envelope (left) and its cosine Fourier transform (right) for copper(II)–bleomycin. The dotted line in the experimental curve is a manual extension of the data to $T + \tau = 0$. The spectrometer frequency was 9247 MHz, the magnetic field was 3280 G, and τ, the time between the second and third pulses of the three-pulse sequence, was 350 ns.

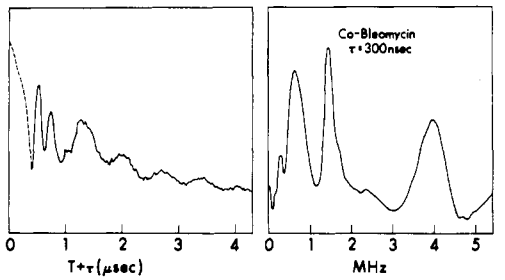

FIGURE 4: Three-pulse electron spin–echo decay envelope (left) and cosine Fourier transform (right) for cobalt(II)–bleomycin. The spectrometer frequency was 9250 Hz, the magnetic field was 3175 G, and τ was 300 ns.

et al., 1977). [The reason for this is presented elsewhere (Mims & Peisach, 1978).] The modulation pattern observed with the Cu(II)–drug complex, as well as with the model compound, is due to the interaction of the Cu(II) spin with the remote ^{14}N of ligated imidazole (Mims & Peisach, 1978) and establishes the ligation of imidazole to the Cu(II) in both cases.

Pyrimidine ligation to Cu(II) cannot be verified by our experimental approach. In Figure 2C, we see the two-pulse echo decay envelope for the copper(II) diethylenetriamine–pyrimidine complex. Only the pattern attributable to proton interaction is observed (Mims et al., 1977). Continuous-wave EPR as well as optical titrations of copper(II) diethylenetriamine with pyrimidine suggests that pyrimidine is bound to the Cu(II). The lack of an effect in the electron spin–echo study of the copper(II)–bleomycin complex indicates that either the Cu(II) is bound to the amino ^{14}N of the drug's 4-aminopyrimidine moiety, in which case no nuclear modulation pattern from ^{14}N is expected, or the ligation of the ring ^{14}N is so different from that in imidazole that interaction with the remote ^{14}N in the heterocycle is not observed in X-band spin–echo experiments.

In Figure 3, we show the three-pulse echo decay envelope and the cosine Fourier transform obtained for the Cu(II) complex of the drug. The advantage of using a three-pulse electron spin–echo sequence rather than a two-pulse sequence is that the modulation pattern can be observed over a longer period of time, and, with the appropriate choice of τ, the time between the second and third microwave pulses, the contribution of protons to the echo decay envelope can be suppressed (Peisach et al., 1979; Mims & Peisach, 1981). The peaks obtained in the Fourier transform spectrum near 1.5 and 3.8 MHz correspond to the periods of 670 and 260 ns which can be seen in the echo decay envelope. These frequencies are characteristic of imidazole coordination to Cu(II) in a variety of copper(II)–proteins and imidazole-containing model compounds (Mims & Peisach, 1976, 1978, 1979b; Mondovì et al., 1977; Zweier et al., 1979; Kosman et al., 1980).

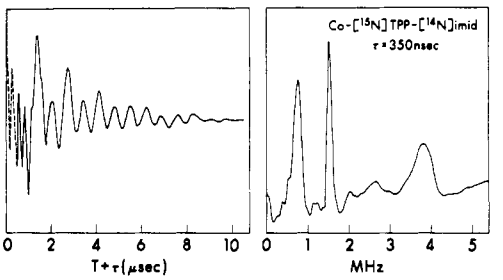

FIGURE 5: Three-pulse echo decay envelope (left) and cosine Fourier transform (right) for the cobalt(II)–[^{15}N]tetraphenylporphyrin–[^{14}N]imidazole complex in a dimethyl sulfoxide–chloroform solution. The spectrometer frequency was 8973 MHz, the magnetic field was 2760 G, and τ was 350 ns.

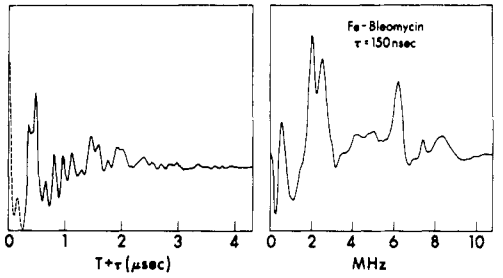

FIGURE 6: Three-pulse echo decay envelope (left) and cosine Fourier transform (right) for low-spin iron(III)–bleomycin. The spectrometer frequency was 9247 MHz, the magnetic field was 3040 G, and τ was 150 ns.

A similar spectrum (Figure 4) can be obtained for the low-spin Co(II) complex of the drug (Sugiura, 1978). Here too, the frequencies are assigned to the remote ^{14}N of ligated imidazole. This assignment is based on a model study of ^{15}N-labeled Co(II)–TPP complexes with [^{14}N]imidazole.[2] The three-pulse modulation pattern (Figure 5) arises from the interaction of the electron spin of Co(II) with ^{14}N in the axial ligand. This pattern is not seen in the echo decay envelope when [^{15}N]imidazole is substituted for [^{14}N]imidazole. When [^{14}N]imidazole is ligated to Co(II)–TPP, each of the Co(II) nuclear hyperfine features of the continuous-wave EPR spectrum is split into a triplet which arises from the coordination of a single imino ^{14}N of the imidazole (Walker, 1970). The imino ^{14}N does not contribute to the modulation pattern observed in the electron spin–echo decay envelope for the same reasons as in the case of copper(II)–imidazole complexes. It is concluded, then, that the frequencies observed must arise from the remote ^{14}N of the coordinated imidazole. Thus, low-spin Co(II) resembles Cu(II) in its magnetic coupling to imidazole and coordinates to imidazole in the metal–drug complex.

For the low-spin Fe(III) complex (Burger et al., 1979), the three-pulse echo decay envelope is more complicated, as is the Fourier transform spectrum of the data (Figure 6). Of the frequencies that can be seen, those at 0.4 and 2.0 MHz are in the same range as those observed for low-spin heme complexes ligated to imidazole (0.4, 1.7, and 2.1 MHz). The higher frequencies, ~6 and ~8 MHz, likely arise from the interaction of the spin of Fe(III) with a different ^{14}N ligand.

[2] These are reasonable models for Cu(II)–BLM since the continuous-wave EPR spectrum of the drug and the porphyrin complex is characteristic of pentacoordinate, low-spin Co(II) in an axial environment as is found in Co(II)-substituted hemoproteins [see, for example, Yonetani et al. (1974)].

The occurrence of a narrow peak at 6 MHz suggests that the coupling between the Fe(III) spin and the ^{14}N nucleus in this ligand is comparable to the coupling between the Fe(III) and the ^{14}N nuclei of the porphyrin in low-spin hemoproteins (Peisach et al., 1979).

In summary, we have shown by the echo envelope spectroscopic technique that Co(II), Cu(II), and Fe(III) are coordinated to bleomycin via nitrogenous ligands. For Co(II) and Cu(II), imidazole coordination is indicated.

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