Interaction of Co·Bleomycin A2 (Green) with d(CCAGGCCTGG)$_2$: Evidence for Intercalation Using 2D NMR

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Bleomycin (BLM) is an antitumor antibiotic, the cytotoxicity of which is thought to be related to its ability to bind to and degrade DNA (Figure 1a).[1] A metal ion and O_2 are cofactors required for this process.[2] Since its discovery, much has been learned about the activation by metal ion and O_2 and the mechanism by which the resulting activated BLM initiates DNA degradation specifically by 4′-hydrogen atom abstraction from a pyrimidine in a d(GpPy) sequence. The structural basis for this specificity and the observed chemistry have remained elusive. This communication reports our initial efforts to define the basis for molecular recognition between a decameric oligonucleotide (d(CCAGGCCTGG)$_2$, **1**) (Figure 1b) and cobalt(III) BLM A2 (green) (CoBLM)[3] using 2D NMR.

The hydroperoxide form of CoBLM was the BLM cogener of choice for investigation by NMR since (1) it cleaves DNA only in the presence of light,[3] (2) its specificity and structure are similar to those of activated BLM,[4] (3) its ligands are exchange inert, and (4) it is diamagnetic. The first step in defining the structure of a DNA−drug complex is to solve the structure of CoBLM in solution. Recently Xu et al.[5] reported such a structure of a mixture of CoBLMs (A2 green and brown forms). We have also solved the structure of the pure A2 green form by NMR methods.[5] Another key to solving the structure of the complex is the choice of oligonucleotide. As outlined below, **1** possesses a single, light-mediated CoBLM cleavage site,[6] suggesting a single mode of binding to this oligomer.

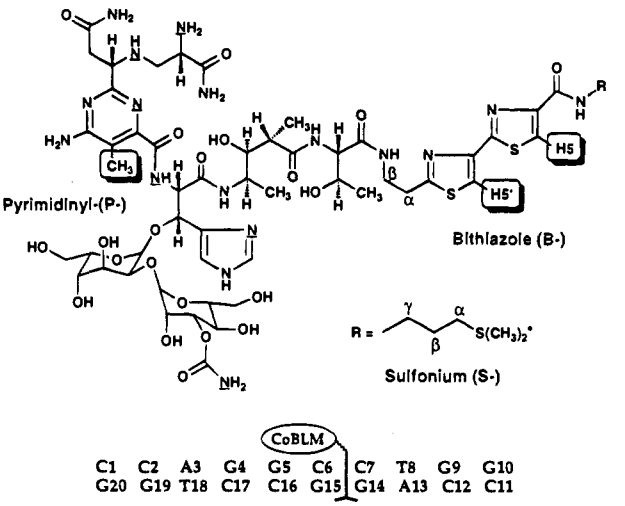

Figure 1. (a, top) Structure of BLM A2. The Ns are the proposed ligands coordinated to the metal. It is presently controversial as to whether the NH_2 group of the carbamoyl moiety of mannose[14] or the primary amino group of the β-aminoalanine is the fifth ligand in CoBLM A2 (green). (b, bottom) Model for interaction between CoBLM and **1**. The oval represents the CoBLM metal binding domain; the wavy line represents the peptide linker, and the thick lines represent the bithiazole tail.

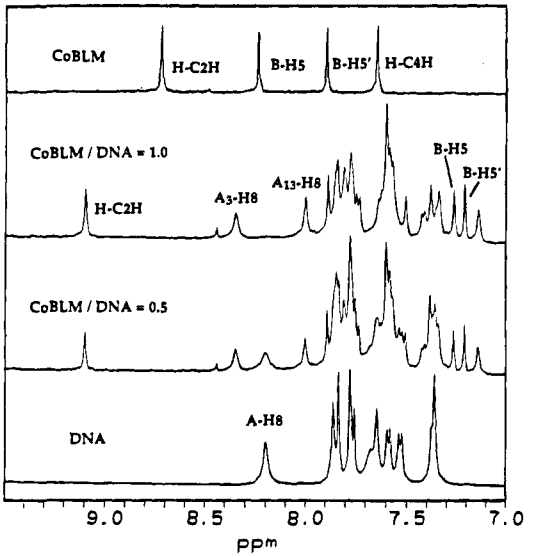

Figure 2. Titration of d(CCAGGCCTGG)$_2$ with CoBLM at 20 °C. Downfield region of the ^{1}H NMR: CoBLM in 50 mM sodium phosphate (pH 6.8) and decameric duplex DNA (2.0 mM) in 50 mM sodium phosphate (pH 6.8) with 0, 0.5, and 1 equiv of CoBLM added.

As indicated in Figure 2, titration of DNA with drug suggests the formation of a 1:1 complex that is in slow exchange on the NMR time scale. The free oligomer, **1**, is palindromic and hence possesses a single resonance at 8.20 ppm for A-H8.[7] Upon titration, this resonance disappears, and two new resonances appear at 8.35 (A_3-H8) and 8.00 ppm (A_{13}-H8) (Figure 2).[8] Using ^{13}C−^{1}H correlation spectroscopy (Figure 3, supplemen-

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(3) Chang, C. H.; Meares, C. F. *Biochemistry* **1982**, *21*, 6332. CoBLM has been purified to homogeneity and its stability characterized by C18 RP-HPLC and ^{1}H NMR spectroscopy. In our hands, and in contrast to a report of Xu et al.,[5] this compound is stable for at least 4 months at pH 6.8. Details of the characterization of CoBLM by electrospray mass spectroscopy and 2D NMR methods are in preparation.

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(5) Xu, R. X.; Nettesheim, D.; Otvos, J. D.; Petering, D. H. *Biochemistry* **1994**, *33*, 907. While our structure is in reasonable agreement with that reported by Xu et al., our ability to purify A2 (green) to homogeneity has allowed the assignments of all the sugar protons and all the carbon chemical shifts by HMQC and HMBC methods (Bax, A.; Subramanian, S. *J. Magn. Reson.* **1986**, *67*, 565. Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093). These studies have allowed the detection of several previously unreported NOEs which define the screw sense of this compound and the ligation state (Wu et al., in preparation).

(6) Incubation of [5′-^{32}P]**1** with 1, 2, and 4 equiv of CoBLM indicates a single cleavage at C_6 using a 20% polyacrylamide sequencing gel (data not shown).

(7) Assignments of ^{1}H chemical shifts for **1** were made by standard 2D COSY, NOESY, and TOCSY methods. Wüthrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley & Sons, Inc.: New York, 1986.

(8) Assignments of ^{1}H chemical shifts for the complex of **1** with CoBLM were made by standard methods[7] and are given for the base protons and H1′ protons in Table 1, supplementary material.

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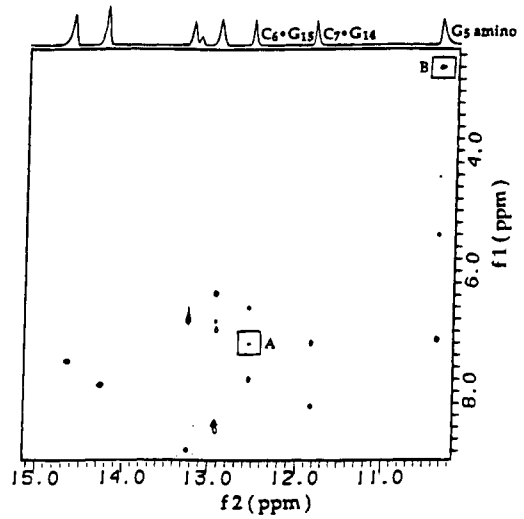

Figure 4. (a) Expanded NOESY spectrum (200 ms mixing time) of a 1:1 complex of CoBLM and **1** in H_2O. CoBLM and **1** (5.0 mM) in 90% H_2O/10% D_2O in 50 mM sodium phosphate (pH 6.8) at 20 °C. The boxed cross peaks are (A) the C_6•G_{15} imino to B-H5′ and (B) the G_5 amino to pyrimidinylmethyl (P-CH_3). Figure 4b (supplementary material) shows the imino region of the spectrum.

tary material), the assignments of the bithiazole protons B-H5 and B-H5′ (Figure 1a) have been made in both the CoBLM and the complex.[9] The B-H5 shifts from 8.17 ppm in CoBLM to 7.27 ppm in the complex, while the B-H5′ shifts from 7.82 to 7.21 ppm (Figure 2). These upfield shifts suggest an intercalative mode of binding of CoBLM to **1**.[10]

An experiment examining the imino hydrogens of **1** and the complex with CoBLM in H_2O[11] also provides strong support for a 1:1 complex. Free **1** has four detectable imino hydrogens at 20 °C: 14.10 ppm for the A•T base pair and 12.89−13.07 ppm for the three G•C base pairs (Table 2, supplementary material). Upon addition of drug, eight imino hydrogens are now observed (Figure 4a). There are now two A•T imino hydrogens at 14.62 and 14.24 ppm, and there are four G•C imino hydrogens. As anticipated for an intercalator,[10] the C_6•G_{15} and C_7•G_{14} imino hydrogens have shifted upfield from 12.94 to 12.54 ppm and from 12.89 to 11.84 ppm, respectively. An intermolecular NOE between the B-H5′ and C_6•G_{15} imino proton is detected, again consistent with the intercalative mode of binding by the bithiazoles (A in Figure 4a).

Additional evidence supporting the intercalation mode of binding for the bithiazole tail comes from detection of intermolecular NOEs between B-H5 and B-H5′ and **1** (Table 3, supplementary material). Of particular note is that the H5 of the terminal thiazolium ring has strong NOEs to both the G_{14} and G_{15} H8 and H1′ protons,[8] while the H5′ of the penultimate thiazolium ring shows weak cross peaks to the H5 protons of both C_6 and C_7. These observations and the inability to detect sequential NOEs between G_{15}-H8 and G_{14}-H1′[12] and between the two imino hydrogens of C_6•G_{15} and C_7•G_{14} (Figure 4b, supplementary material) indicate that the bithiazole tail is intercalated between base pairs C_6•G_{15} and C_7•G_{14}. C_6, as indicated by these studies and our cleavage studies,[6] is thus the target of light-mediated degradation of CoBLM, and the bithiazole tail enters the minor groove, 3′ to the cleavage site, between C_6 and C_7 (Figure 1b).

Finally, an exchangeable proton with an unusual chemical shift of 10.36 ppm is also observed. This resonance has tentatively been assigned to one of the two hydrogens of the 2-amino group of G_5.[13] It has intramolecular NOEs to the other 2-amino hydrogen, to the H1′ of G_5, and to imino hydrogens of G_5•C_{16} and G_4•C_{17} (Figure 4b, supplementary material). It also exhibits an intermolecular NOE to the methyl group of the pyrimidinyl moiety of the CoBLM (B in Figure 4a), positioning the drug's metal binding domain between G_5 and C_6 (Figure 1). The above studies present the first structural insight into the basis of a metallo-BLM binding to a single site of DNA (Figure 1b) and hold promise for detailed structural elucidation.

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Supplementary Material Available: Figure 3, expanded HMQC spectrum; Figure 4b, imino region of the NOESY spectrum; Tables I, II, and III, listing the chemical shifts and the intermolecular NOEs (5 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(9) Assignments of the B-H5 and B-H5′ in the complex were made using HMQC methods. The assignments were based on the assumption that the ^{13}C chemical shifts are not altered greatly between bound and free CoBLM. CoBLM, 127.5 (C5) and 121.3 ppm (C5′); in complex, 126.7 (C5) and 117.4 ppm (C5′).

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(12) Spectral crowding of the resonances is noted in the regions of 7.6 and 5.9 ppm (Table I, supplementary material). However, examination of the NOEs in the base to H1′, base to H2′ and H2″, and base to base regions of the spectrum have allowed the assignments of all cross peaks. We thus feel confident that the connection between G_{15}-H8 and G_{14}-H1′ is missing.

(13) We are unable to determine from NMR methods whether the resonance at 10.36 ppm is due to a G_4 or G_5 amino proton. Previous studies of Kuwahara and Sugiura using deoxyinosine substitutions in DNA fragments have shown the importance of the 2-amino group of dG in d(GpPy) sequences to BLM-mediated cleavage (Kuwahara, J.; Sugiura, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 2459). This information and our observed cleavage at C_6 allow us to favor the assignment of the 10.36 resonance to a G_5 amino proton.

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