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# Metal Binding to Modified Bleomycins. Zinc and Ferrous Complexes with an Acetylated Bleomycin†

Norman J. Oppenheimer,* Luis O. Rodriguez,‡ and Sidney M. Hecht§

ABSTRACT: We have studied the DNA- and metal-binding properties of a bleomycin A2 derivative in which the $\alpha$-amino group of the $\beta$-aminoalanine moiety has been N-acetylated. The modified antibiotic has been shown to be without activity in mediating the in vitro release of [$^3$H]thymine from PM-2 DNA. Fluorescence experiments indicate that the degree of quenching by DNA of the bithiazole fluorescence is unaffected by N-acetylation of bleomycin. Furthermore, $^1$H NMR experiments demonstrate that N-acetylation does not alter the stoichiometry of metal binding. The Fe(II)–Ac-bleomycin A2 complex, however, has been found to be stable in the presence of both $O_2$ and CO, and thus inactivation appears to be accounted for by the loss of the ability to bind and/or reduce $O_2$. Comparison of the $^1$H NMR spectra of the Fe(II)–bleomycin and Fe(II)–Ac-bleomycin A2 complexes indicates that either a drastic reorganization of the ligands with respect to the central iron atom has occurred or that an altered spin state is stabilized. These experiments establish that the ability of bleomycin to cause DNA damage is sensitive to even minor structural alterations within the antibiotic.

The bleomycins are a family of structurally related antibiotics isolated from *Streptomyces verticillus* by Umezawa et al. (1966). In addition to their antibacterial activity, the bleomycins inhibit the growth of transformed mammalian cells both in culture and in experimental animals (Remers, 1979). Significantly, a mixture consisting primarily of bleomycins A2 and B2 has been used successfully in the clinical treatment of certain types of cancers, such as squamous cell carcinomas and malignant lymphomas (Umezawa, 1979). Bleomycin is believed to function by strand scission of DNA in the affected cells, a process which seems to be mediated via a metal chelate of bleomycin that is capable of generating a reduced form of oxygen in proximity to a susceptible site(s) on the DNA (Hecht, 1979).

Although the structure shown in Figure 1 has been proposed for bleomycin (Takita et al., 1978a) and many of the structural components have been verified by unambiguous synthesis [see, e.g., Hecht et al. (1979)], the detailed structures of the chelates that bleomycin forms with several metals are less clear. For example, P-3A (a putative biosynthetic intermediate structurally related to bleomycin) has been crystallized as its Cu(II) chelate and the structure has been determined by X-ray crystallographic analysis (Iitaka et al., 1978). While simple

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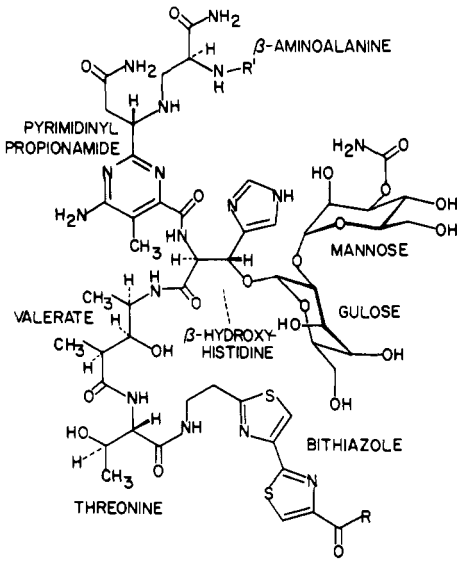


FIGURE 1: Structure of bleomycin. R = $NH(CH_2)_3S^+(CH_3)_2$; R′ = H, bleomycin A2; R′ = $-COCH_3$, acetylbleomycin A2.

extrapolation of the Cu(II)–P-3A structure to that of Cu(II)–bleomycin and Fe(II)–bleomycin has been suggested (Takita et al., 1978b), it has also been noted that P-3A lacks many of the potential ligating groups present in bleomycin (Oppenheimer et al., 1979a,b). In fact, recent $^1$H NMR experiments with a diamagnetic Fe(II)–bleomycin–CO complex (Oppenheimer et al., 1979b), that seems to be analogous spectroscopically to the "active" complex of bleomycin (Burger et al., 1979a), have suggested strongly that the "active" complex of bleomycin involves different groups as metal ligands than does P-3A.

Attention has focused on the primary amine of the β-aminoalanine moiety as a determinant of biological activity. The biological inactivation of bleomycin caused by enzymatic deamidation of the β-aminoalaninecarboxamide (Umezawa et al., 1974) has been attributed to competition between the newly formed carboxylate and the primary amine for coordination to the metal. Sugiura et al. (1979) have shown that the aerobic Fe(II) complex with the deamidobleomycin is much less efficient than bleomycin in the production of hydroxyl radicals. This result parallels the low activity of the deamido form of the bleomycin congener, pepleomycin, in causing [$^3$H]thymine release from DNA (Takahashi et al., 1979).

The need to define the factors that determine the nature of the metal binding and especially those that permit the resulting metal complexes to reduce $O_2$ has prompted us to study other bleomycin analogues dysfunctional in DNA degradation. Presently, we describe DNA- and metal-binding properties of N-acetylated bleomycin,[1] an analogue of bleomycin that does not degrade DNA.

## Experimental Section

*Materials.* The clinical mixture of bleomycin A2 and B2 (blenoxane) was obtained from the National Cancer Institute and ferrous ammonium sulfate was obtained from MCB. Calf thymus DNA was purchased from Calbiochem and glass fiber disks were from Schleicher & Schuell.

Bleomycin A2 was prepared by fractionating blenoxane (160 mg), according to the procedure of Chien et al. (1977), with a recovery of 94 mg of bleomycin A2. The $^1$H NMR and fluorescence spectra of this material, as well as its ability to release [$^3$H]thymine from radiolabeled PM-2 DNA, were unaffected by the separation procedure. The preparation of acetylated bleomycin A2 has been improved as follows. Bleomycin A2 (8 mg, ~6 μmol) was acetylated with acetic anhydride in aqueous solution containing DEAE-cellulose ($HCO_3^-$ form) as described previously (Oppenheimer et al., 1979a). After removal of the DEAE-cellulose by centrifugation, the reaction mixture was fractionated on Sephadex C-25 ($HCOO^-$ form) as described. Fractions 20–27 (70 $A_{290}$ units) were pooled, diluted to a salt concentration of <0.05 M, and applied to a Sephadex C-25 column (1.1 × 38 cm; $HCO_3^-$ form). The column was washed with 50 mL of 0.05 M ammonium bicarbonate and then with a linear gradient of ammonium bicarbonate (900-mL total volume; 0.05–0.5 M; 5-mL fractions) at a flow rate of 50 mL/h. Fractions 15–25 were pooled and desalted by repeated evaporations of portions of water (bath temperature 25 °C), affording 70 $A_{290}$ units (88%) of N-acetylated bleomycin A2 as a clear glass after drying under desiccation (0.05 mmHg/5 °C) for 20 h.

*Fluorescence Measurements.* Fluorescence measurements were conducted on a Farrand Mark 1 spectrofluorometer with emission and excitation correction. An excitation wavelength of 295 nm (10-nm slit width) was used for the experiments. All measurements were made at 25 °C in a septum-stoppered cell where $N_2$ was bubbled through each solution for at least 1–2 min prior to spectral determination. Solutions were made < 24 h in advance of the experiments and stored at <–20 °C. The pH of each solution was adjusted to 6.8 with Tris-HCl, to a final concentration of 5 mM. Glutathione was added to a final concentration of 34 μM to prevent formation of Fe(III); its addition was without effect on the fluorescence spectra. Bleomycin A2 (or Ac-bleomycin A2), Fe(II), and DNA were added to final concentrations of 17, 34, and 710 μM, respectively. Spectra of Fe(II)–bleomycin under $N_2$ with and without DNA were obtained on degassed, metal-free samples to which deaerated solutions of $Fe(NH_4)_2(SO_4)_2$ were added anaerobically via syringe.

*Bleomycin-Mediated DNA Degradation.* [$^3$H]PM-2 DNA (21 × $10^6$ cpm/mol; containing radiolabeled thymine) was prepared as described (Espejo & Canelo, 1968) and used as a substrate for degradation by bleomycin A2 and Ac-bleomycin A2. DNA degradation was measured as a function of the release of [$^3$H]thymine from acid-insoluble products. The reaction was carried out in 50 mM sodium cacodylate, pH 6.9 (450-μL total volume), containing 68 μM glutathione, 1.7 μM PM-2 DNA (1.5 × $10^4$ cpm of [$^3$H]thymine), 34 μM $Fe(NH_4)_2(SO_4)_2$, and 34 μM bleomycin A2 (or Ac-bleomycin A2) with Fe(II) and bleomycin being added last, in that order. The reaction mixtures were incubated at 38, 48, or 59 °C, and 50-μL aliquots were removed at predetermined time intervals and applied to glass fiber disks that had been presoaked with 30 mM thymine and 10% aqueous trichloroacetic acid solutions. The dried disks were washed thoroughly with 5% aqueous trichloroacetic acid, dried, and used for determination of radioactivity.

*NMR Parameters.* Spectra were obtained at 360 MHz on a Bruker HXS-360 NMR spectrometer equipped with a Nicolet Technologies 1180 computer/Fourier transform system and a computer-controlled homonuclear decoupling accessory. Quadrature detection was employed and both 16K and 32K Fourier transforms were obtained with a spectral width of 3610 Hz. Spectra of the Fe(II) complexes were obtained with a spectral width of 36 000 Hz and a 32K Fourier transform was

[1] Abbreviation used: Ac-bleomycin, bleomycin in which the α-amino group of the β-aminoalanine moiety has been N-acetylated.

Table I: Fluorescence Quenching of Bleomycin $A_2$ and Acetylated Bleomycin $A_2$ by Calf Thymus DNA[a]

| compd tested ($\mu$M) | conditions | $I_{DNA}$[b] + | $I_{DNA}$[b] − | % fluorescence quenching[b] |
|---|---|---|---|---|
| bleomycin $A_2$ | | | | |
| 17 | $O_2$ | 41.0 | 76.0 | 46 |
| 17 | $N_2$ | 37.0 | 69.5 | 47 |
| 17 | $N_2$ + Fe(II) | 26.0 | 52.0 | 50 |
| *N*-acetylbleomycin $A_2$ | | | | |
| 17 | $O_2$ | 39.5 | 77.5 | 49 |
| 17 | $N_2$ | 36.5 | 72.0 | 49 |
| 17 | $N_2$ + Fe(II) | 32.5 | 60.0 | 46 |

[a] Carried out in the presence of 34 $\mu$M Fe(II), as described under Experimental Section. Fluorescence emission was recorded at 353 nm. [b] In the presence vs. absence of 710 $\mu$M calf thymus DNA.

employed. Typically 128 transients were accumulated with 2.7 s between pulses and using a 60° pulse angle. Equilibrium intensities were therefore observed for all the resonances under these conditions with the possible exception of those of the bithiazole moiety. Spectra obtained in $H_2O$ used a "214" long pulse sequence (Redfield et al., 1975) to suppress the solvent resonance.

The unbuffered binary Fe(II) complexes were prepared as previously outlined (Oppenheimer et al., 1979b) and the Zn(II)–Ac-bleomycin A2 complex was prepared according to Oppenheimer et al. (1979a). All concentrations were determined spectrophotometrically and the final concentration of 5 mM in antibiotic. After obtaining the spectrum of Ac-bleomycin A2 and Zn(II)–Ac-bleomycin A2 in $D_2O$, the samples were lyophilized and 90% $H_2O$–10% $D_2O$ (v:v) was added to obtain the spectra of the exchangeable (–NH) resonances. Dithionite (0.2 $\mu$mol) was added routinely to the samples containing Fe(II) to protect against traces of $O_2$. The pD or pH of the solutions was measured in the NMR tube subsequent to spectral acquisition by using an Ingold 4-mm diameter glass electrode. For samples containing Fe(II) a stream of nitrogen was directed at the NMR tube to prevent any oxidation until after the measurement had been made. The standard electrode correction has been employed: pD = meter reading + 0.4 (Glasoe & Long, 1960). The internal standard, sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate (TSP), was used, and the chemical shifts were corrected (where needed) for the protonation of the carboxylate of TSP ($pK_a \sim 5$).

## Results

*DNA Binding Studies.* The affinity of the unmodified and modified bleomycins for calf thymus DNA was measured by comparison of the extent to which the fluorescence of the bithiazole moieties in the individual bleomycins was quenched upon addition of DNA. As shown in Table I, the intensity of fluorescence emission of an aerated solution of bleomycin was diminished to the extent of ~46% in the presence of 0.71 mM calf thymus DNA. Similar results were obtained both from measurements on solutions that had been deaerated by bubbling $N_2$ through them prior to spectral determination and also in deaerated solutions that additionally contained 34 $\mu$M Fe(II). When the identical experiments were repeated using Ac-bleomycin A2, essentially the same results were obtained. Thus, the extent of fluorescence quenching of bleomycin A2 by DNA, which is presumably a measure of the affinity of the antibiotic for DNA, was unaffected by acetylation of the primary amino group in the $\beta$-aminoalaninecarboxamide substituent or by the presence of $O_2$ or Fe(II).

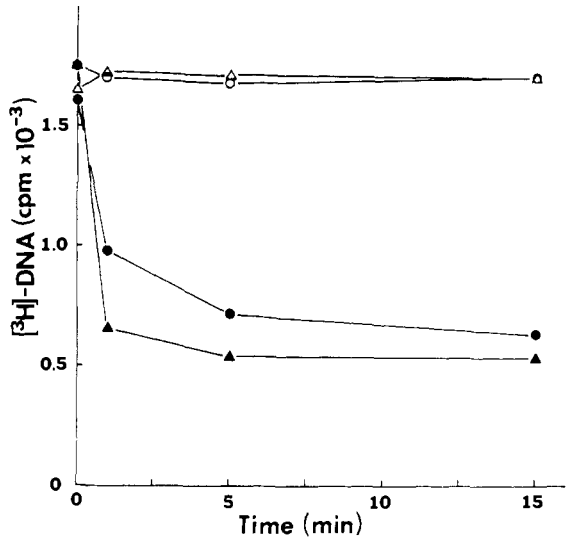


FIGURE 2: Release of [$^3$H]thymine from radiolabeled PM-2 DNA in the presence of bleomycin A2 (solid symbols) or Ac-bleomycin A2 (open symbols) at 38 (triangles) and 48 °C (circles).

The affinity of Ac-bleomycin A2 for DNA suggested that it might also be able to degrade the macromolecule, as has been noted for bleomycin itself (Oppenheimer et al., 1979b; Takeshita et al., 1976; Sausville et al., 1976; Lown & Sim, 1977). As illustrated in Figure 2, the course of DNA degradation by bleomycin can be monitored conveniently by the release of [$^3$H]thymine from radiolabeled PM-2 DNA. The time course of thymine release is evident in the figure and can be seen to proceed slightly less quickly at 48 °C than at 38 °C. Also evident from the figure is the absence of thymine release at either temperature upon administration of Ac-bleomycin A2 to a solution of [$^3$H]PM-2 DNA. Repetition of the experiment at 59 °C (data not shown) revealed a somewhat slower thymine release by bleomycin A2 but no degradation by the modified bleomycin.

*$^1H$ NMR Studies of Ac-bleomycin A2. (1) Titration Studies.* The pD dependence of the chemical shifts for the previously assigned propionamide and $\beta$-aminoalanine resonances (Oppenheimer et al., 1979a) is shown in Figure 3. These results establish that acetylation of the primary amine of the $\beta$-aminoalanine moiety causes the $pK_a$ of the secondary amine to increase from 2.7 to 5.7.

The increase in $pK_a$ of the secondary amine may be attributed to the loss of the electrostatic interaction with the $\alpha$-nitrogen and possibly also to a disruption of hydrogen-bonding interactions. The $pK_a$ of the imidazole has been determined to be 5.1, based on the pD dependence of the chemical shifts of the ring protons (see Figure 4). We further note in Figure 4 that the $\alpha$-CH resonance of the valerate moiety in Ac-bleomycin A2 is sensitive to a group with a $pK_a$ of 5.1 (the imidazole) and not 5.7 (the secondary amine, the only other group that titrates in this pD range). A similar sensitivity to the protonation of the imidazole is observed in bleomycin (Oppenheimer et al., 1979a). This result suggests that the environment or spatial relationship that appears to exist between the imidazole and the valerate moieties is unaltered by acetylation of the primary amine.

*(2) Zn–Ac-Bleomycin Complex.* Addition of less than stoichiometric amounts of Zn(II) to a solution of Ac-bleomycin A2, along with sufficient NaOD to maintain the pD at 6.5, results in a decrease of the intensity of the resonances for free Ac-bleomycin A2 with a proportional appearance of a new set

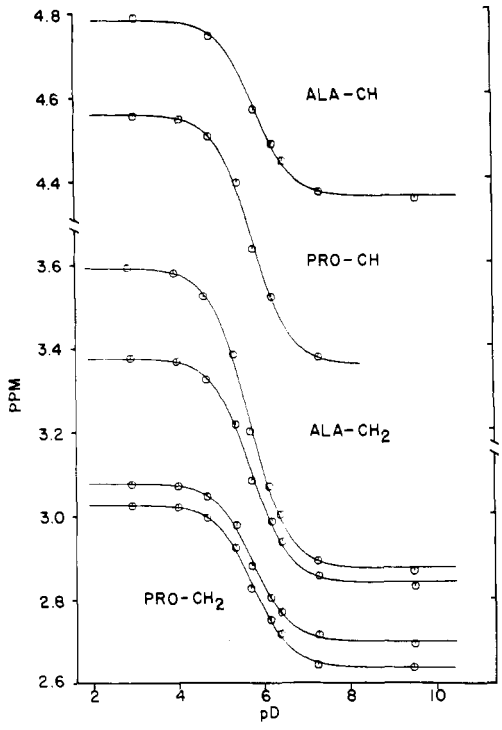


FIGURE 3: pD dependence of the chemical shifts for the proton resonances of the $N^{\alpha}$-acetyl-$\beta$-aminoalanine (ALA) and propionamide (PRO) moieties of Ac-bleomycin A2. All protons show only the influence of the titration of the secondary amine, $pK_a = 5.7$.

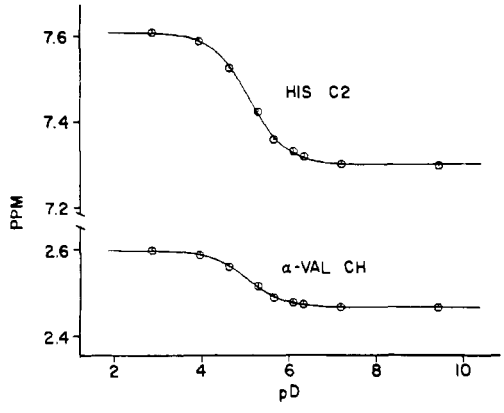


FIGURE 4: pD dependence of the chemical shifts for the proton reasonances of the imidazole C-2 proton and the $\alpha$-CH proton of the valerate moiety (VAL) in Ac-bleomycin A2. Computer analysis of the data for the imidazole C-2 and C-4 protons establishes the $pK_a$ of this group to be 5.1 and that the $\alpha$-CH of the valerate moiety is sensitive to a group with the same $pK_a$.

of resonances. At a Zn–Ac-bleomycin A2 ratio of 1:1, the $^1$H NMR spectrum does not indicate any resonances due to metal-free Ac-bleomycin A2 as shown in Figure 5. Further increase in the concentration of Zn(II) to a 2:1 ratio causes no additional changes in either the pD of the solution or the $^1$H NMR spectrum, consistent with the absence of other binding sites. When metal-free and metal-complexed forms are both present, there is no indication of any line broadening, even for resonances that differ by only a few hertz. Consequently the exchange rate of the metal between these species is estimated to be slower than 1 $s^{-1}$, similar to that of Zn(II)–bleomycin (Cass et al., 1978; Lenkinski & Dallas, 1979) and Fe(II)–bleomycin–CO (Oppenheimer et al., 1979b). The resonances of the metal complex have been assigned by homonuclear decoupling and analogy to the spectral parameters observed and previously discussed for Ac-bleomycin A2 and the Zn(II)–bleomycin complex (Oppenheimer et al., 1979a), and the chemical shifts are reported in Table II.

Table II: $^1$H Chemical Shifts[a]

| | shifts (ppm) | |
|---|---|---|
| | Zn-Ac-bleomycin (pD 6.5; pH 5.1) | Ac-bleomycin (pH; pD 2.9) |
| $\beta$-aminoalanine | | |
| $\alpha$-CH | 4.405 | 4.791 |
| $\beta$-$CH_2$ | 3.588, 3.300 | 3.593, 3.377 |
| -NH-COC$H_3$ | 1.919 | 2.075 |
| -N*H*-$COCH_3$ | 8.569 | 8.719 |
| propionamide | | |
| $\alpha$-CH | 4.225 | 4.558 |
| $\beta$-$CH_2$ | 3.08, 2.92 | 3.078, 3.026 |
| pyrimidine | | |
| -$CH_3$ | 2.483 | 2.086 |
| -$NH_2$[b] | 7.116 | 6.774 |
| $\beta$-hydroxyhistidine | | |
| C-2 | 7.435 | 7.608 |
| C-4 | 8.159 | 8.810 |
| $\alpha$-CH | 5.057 | 5.128 |
| $\beta$-CH | 5.315 | 5.568 |
| $\alpha$-NH | | 9.062 |
| mannose | | |
| C-1 | 4.918 | 5.043 |
| C-3 | 4.05 | 4.709 |
| -$OCONH_2$ | [c] | 6.310[d] |
| valerate | | |
| $\alpha$-CH | 1.834 | 2.598 |
| $\beta$-CH | 3.373 | 3.754 |
| $\gamma$-CH | 3.690 | 3.970 |
| $\alpha$-$CH_3$ | 0.955 | 1.165 |
| $\delta$-$CH_3$ | 0.974 | 1.165 |
| $\gamma$-NH | 7.748 | 8.30 |
| threonine | | |
| $\alpha$-CH | 4.225 | 4.226 |
| $\beta$-CH | 4.091 | 4.168 |
| $\beta$-$CH_3$ | 1.099 | 1.095 |
| $\alpha$-NH | 7.723 | 8.025 |
| bithiazole | | |
| C-2 | 8.254, 8.107 | 8.245, 8.069 |
| NH | 8.300 | 8.30 |
| primary amides | 7.685, 7.656 | 7.815, 7.743 |
| | 7.116, 7.038 | 7.294, 7.080 |

[a] Values are reported in parts per million relative to internal TSP. Spectra were recorded at 28 °C and with a concentration of 5 mM. Zn(II)–Ac-bleomycin in $H_2O$ was pH 5.1 to suppress exchange broadening. [b] Model compound: $\beta$-(4-amino-6-carboxamido-5-methylpyrimidin-2-yl)propionamide, 6.78 ppm. [c] Exchange broadening of the $NH_2$ resonances of the carbamoyl moiety at 28 °C precluded an accurate measurement of their chemical shift. [d] Model compound: 3′-carbamoyl-$\alpha$-methyl-D-mannose, 6.43 ppm.

(*3*) *Zn(II)-Binding Site.* Coordination of ligands to diamagnetic Zn(II) causes alterations in the chemical shift of resonances both by electron withdrawal (similar to the shifts resulting from protonation at the binding site) and by changes in conformation that alter the juxtaposition of protons with respect to shielding or deshielding environments. Both effects have a relatively short range and thus the general area of metal coordination will be reflected by changes in the chemical shifts of adjacent resonances.

The changes in chemical shifts for the Zn(II)–Ac-bleomycin A2 complex relative to metal-free Ac-bleomycin A2 at pD 2.9 are shown schematically in Figure 6. The diprotonated form of Ac-bleomycin A2 was chosen as a reference for comparison since it should be most closely analogous to the complex with the divalent Zn(II). The largest changes in chemical shifts occur for the proton resonances of the following substituents: pyrimidinylpropionamide, $N^{\alpha}$-acetyl-$\beta$-aminoalanine, pyrimidine methyl and amine, $\beta$-hydroxyhistidine, methyl valerate,

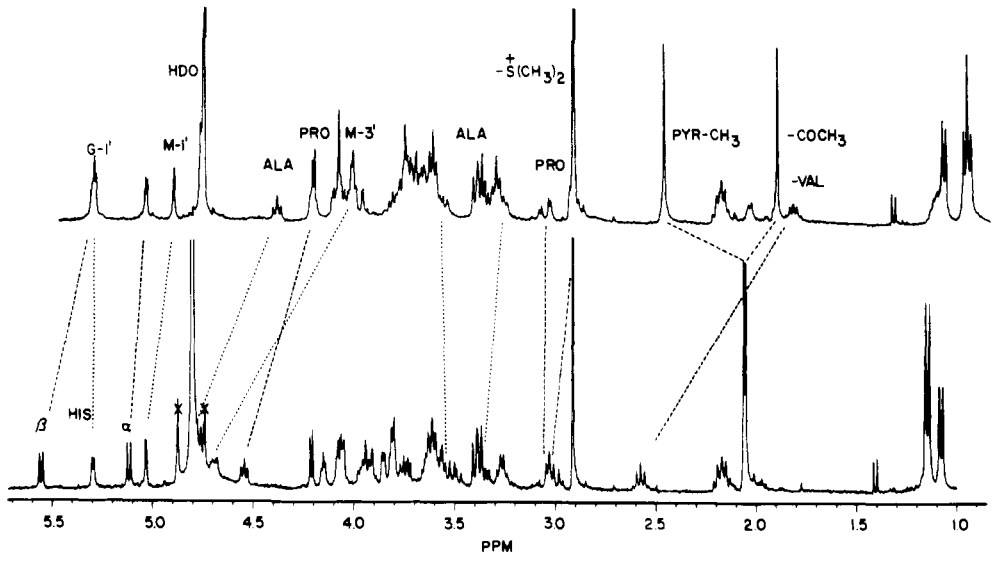


FIGURE 5: Comparison of a portion of the $^1H$ NMR spectra of Zn(II)–Ac-bleomycin A2, pD 6.5 (top), with Ac-bleomycin A2, pD 2.9 (bottom). Abbreviations used: G, gulose; M, mannose; ALA, β-aminoalanine; PRO, propionamide; PYR, pyrimidine; VAL, valerate.

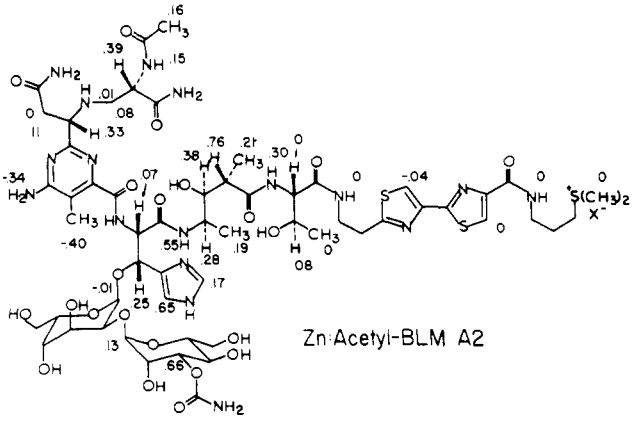


FIGURE 6: Schematic representation of the chemical shift changes, in parts per million, induced by formation of the Zn(II)–Ac-bleomycin A2 complex. Positive values indicate upfield shifts upon coordination to Zn(II) and negative values indicate downfield shifts relative to Ac-bleomycin A2 at pD 2.9. Data for the exchangeable NH resonances were obtained for a solution in 90% $H_2O$–10% $D_2O$ at the same pH and temperature. Values have been rounded off to the nearest 0.01 ppm.

Table III: Coupling Constants[a]

| | | Ac-bleomycin, pD 2.9 | | Zn-Ac-bleomycin, pD 6.5 | |
|---|---|---|---|---|---|
| | $J$ | Hz | rotamer population[b] | Hz | rotamer population |
| β-amino-alanine | $^3J$ | 5.9 | 12% *gg* | 6.8 | <10% *gg* |
| | $^3J$ | 6.6 | | 7.6 | |
| $CHCH_2$ | $^2J$ | −13.1 | | −13.4 | |
| propion-amide | $^3J$ | 5.2 | <10% *gg* | 3.9 | <10% *gg* |
| | $^3J$ | 7.5 | | 8.6 | |
| $CHCH_2$ | $^2J$ | −16.7 | | −16.7 | |
| β-hydroxy-histidine | $^3J_{\alpha-\beta}$ | 6.5 | 47% trans | 3.2 | <10% trans |
| valerate | $^3J_{\alpha-\beta}$ | 7.1 | 54% trans | 6.2 | 44% trans |
| | $^3J_{\beta-\gamma}$ | 4.9 | 28% trans | 5.8 | 39% trans |

[a] Coupling constants are accurate to within ±0.2 Hz. [b] Rotamer populations have been calculated by using the parameters $J_{trans} = 11$ Hz and $J_{gauche} = 2.5$ Hz; *gg* designates the gauche-gauche rotamer.

and the mannose 3-CH (the site of the *O*-carbamoyl moiety). The chemical shifts induced by metal binding are less than 0.1 ppm for the remaining resonances. As observed for bleomycin, there is no evidence for coordination of the bithiazole moiety to the metal.

Four ligands are clearly implicated by the $^1H$ NMR data: (1) the secondary amine, (2) the pyrimidine N-1, (3) the imidazole N-1, and (4) the carbamoyl moiety. These assignments are based on the following evidence. (a) The chemical shifts of the propionamide and aminoalanine $CH_2$ resonances in the Zn(II) complex at pD 6.5 are similar to those observed in Ac-bleomycin A2 at pD 2.9. This result reflects the mimicking of protonation at the secondary amine by coordination to Zn(II). (b) There is no observable dependence on pD for any resonances over the range from 4.8 to 9.5 (data not shown). This result is consistent with the participation of both the imidazole and secondary amine as ligands, groups that normally have their $pK_a$ in this range. (c) The pyrimidine methyl is strongly deshielded and the mannose 3-CH resonance is strongly shielded in Zn(II)–Ac-bleomycin A2. The observed shifts are comparable to those of the Zn(II)–bleomycin complex, thus implicating by analogy both of these groups as ligands. It should be noted that the shift of the pyrimidine methyl cannot be accommodated solely by the electrostatic effects that would accompany coordination to the secondary amine since the methyl resonance is insensitive to the protonation of the secondary amine.

In addition, we observe that the largest shift resulting from coordination of Zn(II) (0.75 ppm) occurs for the α-CH of the valerate moiety. This result directly parallels the extreme sensitivity of the α-CH resonance in the Zn(II)–bleomycin complex and suggests a similar intimate association between the valerate moiety and Zn(II)–imidazole. The question as to whether the valerate carbonyl is a ligand, however, remains to be answered conclusively.

(*4*) *Analysis of Coupling Constants.* Bleomycin has potentially many degrees of torsional freedom around the backbone linkages. The distribution of rotamers around these bonds can be calculated from the angular dependence of vicinal coupling constants (Karplus, 1959, 1963) by using the analysis of Blackburn et al., (1970). The coupling constants for selected resonances in Ac-bleomycin A2 and Zn(II)–Ac-bleomycin A2 are compared in Table III. Rotamer populations were calculated by using 10–12 Hz as an estimate for trans coupling

constants and 2.5 Hz as an estimate for gauche coupling constants.

The change in the vicinal coupling between the $\alpha$ and $\beta$ protons of the $\beta$-hydroxyhistidine moiety caused by zinc binding to Ac-bleomycin A2 is comparable to that observed for bleomycin. The value of 3.2 Hz is consistent with a strongly preferred gauche rotamer. Other changes in coupling constants between metal-free Ac-bleomycin A2 and its zinc complex are less dramatic. The vincinal coupling between the $\alpha$-CH and $\beta$-$CH_2$ of the $\beta$-aminoalanine moiety indicates that the gauche–gauche rotamer is strongly disfavored whether or not metal is bound. Comparable changes (in hertz) are observed for the vincinal coupling constants of the propionamide moiety; however, this group now shows a strong preference for one of the gauche–trans populations. It is not clear whether the coupling constants reflect a fixed, although slightly nonstaggered conformation or that significant torsional flexibility remains around this bond. The results do not preclude any further coordination site(s) in this portion of the molecule. The vincinal coupling constants for the valerate moiety become nearly equal upon zinc binding, a result similar to that observed for the formation of the Zn(II)–bleomycin complex (Oppenheimer et al., 1979a). Therefore, this portion of the molecule retains considerable torsional flexibility. The coupling constants of the sugar moieties are little altered by the binding of zinc; hence, the effect on the distribution of conformers for these groups is minimal.

(*5*) *Exchangeable $^1H$ Resonances.* $^1H$ NMR spectra of Ac-bleomycin A2 and Zn(II)–Ac-bleomycin A2 were obtained in 90% $H_2O$–10% $D_2O$. Assignments of the amide NH resonances have been made by homonuclear spin decoupling of the vicinal coupling to the previously assigned, nonexchangeable proton resonances. The proton resonances for the 4-amino group of the pyrimidine and the carbamoyl group are assigned by comparison with the model compounds $\beta$-(4-amino-6-carboxamido-5-methylpyrimidin-2-yl)propionamide and 3-carbamoyl-$\alpha$-methyl-D-mannose, respectively.

Analysis of the spectrum of Zn(II)–Ac-bleomycin A2 shows that a total of 14 NH and 4 CH resonances are present in the region from 13 to 6 ppm. Therefore, all of the expected NH resonances are observable, including the resonance for the imidazole NH at 12.73 ppm (when observed at −3 °C in $H_2O$–$CD_3OD$), with the exception of the $N^{\alpha}$ of the $\beta$-hydroxyhistidine resonance. Consequently, none of these nitrogens can serve as a ligand *where Zn(II) has replaced a proton in the neutral species*. The comparative effect on chemical shifts of the NH resonances in $H_2O$ of the Zn(II)–Ac-bleomycin A2 complex at pH 5.1 to Ac-bleomycin A2 at pH 2.9 is also shown in Figure 6. Especially large changes may be noted in the chemical shift for the $\alpha$-NH of the threonine and the $\gamma$-NH of the valerate moieties, as well as for the pyrimidine $NH_2$. The absence of the NH resonance for the $N^{\alpha}$-hydroxyhistidine may not necessarily reflect its participation as a ligand, as found in the copper complex with P-3A (Iitaka et al., 1978) and suggested for other metal–bleomycin complexes (Takita et al., 1978b). We have previously shown for Zn(II)–bleomycin and Fe(II)–bleomycin–CO (Oppenheimer et al., 1979a,b) that coordination of the metal can be accounted for without invoking the $N^{\alpha}$ of the $\beta$-hydroxyhistidine moiety as a ligand. Furthermore, as we have noted (Oppenheimer et al., 1979a), this NH exchanges more rapidly with $H_2O$ than any of the other amide resonances in metal-free bleomycin and is barely observable at pH 5. The instability of metal–bleomycin complexes below pH 5 precludes acidification as a means of slowing the exchange of this resonance, and the solubility of metal–bleomycin complexes in $H_2O$–methanol mixtures limits the minimum temperature that can be reached to about −20 °C. Thus, the question of coordination of this nitrogen in Ac-bleomycin A2 to zinc remains unresolved.

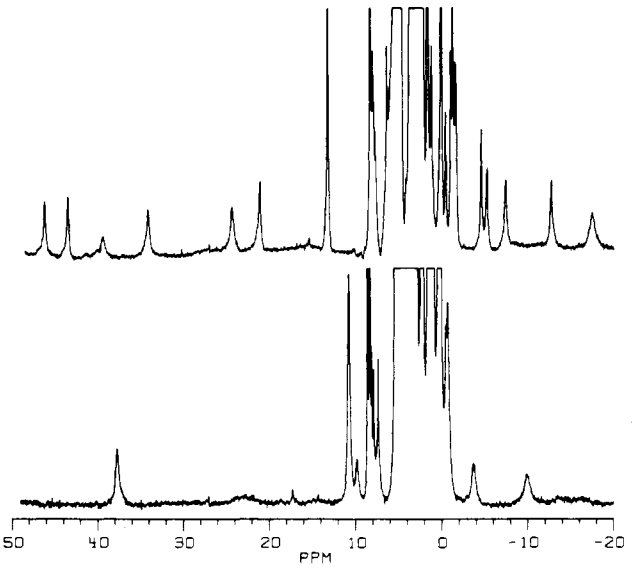


FIGURE 7: Comparison of the $^1H$ NMR spectra of Fe(II)–bleomycin (top) and Fe(II)–Ac-bleomycin A2 (bottom). The spectrum of Fe(II)–Ac-bleomycin A2 is unaltered in the presence of either $O_2$ or CO.

(*6*) *Fe(II)–Ac-Bleomycin.* We have reported previously (Oppenheimer et al., 1979b) that the chemical shifts of the $^1H$ resonances of the paramagnetic binary Fe(II)–bleomycin complex cover a range of 70 ppm (see Figure 7). There are seven resonances downfield from 10 ppm (integrated area, 9 protons) and eleven resonances upfield from 0 ppm (integrated area, 12 protons) for this complex. The spectrum for the binary Fe(II)–Ac-bleomycin A2 complex differs drastically, containing only three resonances that are downfield of 10 ppm (integrated area, 5 protons) and two that are upfield from 0 ppm (integrated area, 6 protons). Since the contact and pseudocontact interactions responsible for the large shifts have a strong dependence on the distance and angle to the unpaired electrons, as well as on the spin state of the iron, the large differences in the $^1H$ NMR spectrum indicate either a large reordering of the ligands with respect to the internal coordinates of the Fe(II) atom must occur or N-acetylation results in the stabilization of the iron in an alternated spin state.

The difference in properties for these two binary complexes are also reflected with respect to addition of CO. Whereas the Fe(II)–bleomycin complex readily forms a stable, diamagnetic Fe(II)–bleomycin–CO ternary complex, the $^1H$ NMR spectrum of Fe(II)–Ac-bleomycin A2 is unaffected by addition of CO. There is no evidence for any coordination of CO to generate either a diamagnetic or a paramagnetic complex even under 1 atm of CO. Furthermore, the Fe(II)–Ac-bleomycin A2 complex is stable in the presence of $O_2$ in contrast to the rapid oxidation observed for the Fe(II)–bleomycin or Fe(II)–bleomycin–CO complexes (Oppenheimer et al., 1979b).

## Discussion

Admixture of bleomycin and Fe(II) in the presence of $O_2$ has been reported to result in the transfer of one or more electrons to $O_2$ (Sugiura & Kikuchi, 1978; Sugiura, 1979a,b; Takita et al., 1978b; Burger et al., 1979a,b; Hecht, 1979; Oppenheimer et al., 1979b; Umezawa, 1979; Dabrowiak et

al., 1979; Lown, 1979). This affords species, detectable by spin trapping, that are believed to represent the "active" form of bleomycin. Analogous species are apparently formed in the presence of CO, NO, and ethyl isocyanide (Oppenheimer et al., 1979b; Burger et al., 1979a) and have been investigated as structural analogues of the putative active form of bleomycin. The absence of DNA degradation by Ac-bleomycin A2, a modified bleomycin that binds to DNA with approximately the same affinity as bleomycin A2 itself (Table I), has prompted an effort to determine the molecular basis for the inactivation of bleomycin.

The results of the $^1H$ NMR experiments show Ac-bleomycin A2 to interact with either Zn(II) or Fe(II) to yield a complex with a unique spectrum. There is no evidence for multiple coordination modes, multiple binding sites, or the binding of more than one metal atom per molecule. As observed for bleomycin, the stoichiometry of metal to Ac-bleomycin A2 is 1:1. The rate of dissociation of the metal from the binary complex is also slow on the NMR time scale and is not significantly faster than that for the comparable binary complexes involving the unmodified antibiotic. Thus, inactivation cannot be attributed to the inability of the metal to bind. Similarly, measurement of bleomycin fluorescence quenching in the presence of DNA establishes that whether or not bleomycin has been acetylated, the magnitude of quenching is unaltered. Therefore, to the extent that the quenching directly monitors interaction of the bithiazole moiety with DNA, acetylation of the primary amine causes no significant change in the interaction with DNA.

The $^1H$ NMR investigations show that the Fe(II)–Ac-bleomycin A2 complex is not redox active. Exposure to $O_2$ did not alter the $^1H$ NMR spectrum. Carbon monoxide, a compound that has been suggested to form a complex analogous to the "active" complex of Fe(II)–bleomycin (Oppenheimer et al., 1979b; Burger et al., 1979a,b), also showed no affinity for Fe(II)–Ac-bleomycin A2. These results indicate that the Fe(II) complex with the modified bleomycin is no longer redox competent. We therefore conclude that the functional deficiency of Fe(II)–Ac-bleomycin A2 is the inability of the Fe(II) complex to bind or reduce $O_2$.

The NMR studies provide information concerning the molecular basis for the observed inactivation. Contact and pseudocontact shifts induced by the paramagnetic Fe(II) atom have strong dependence on distance and geometry relative to the internal coordinates of the iron. Therefore, the large differences in the $^1H$ NMR spectra of the Fe(II)–Ac-bleomycin A2 complex compared to Fe(II)–bleomycin indicate a major change in the spatial relationships between the ligands of the two species. The exact nature of this spatial reorganization remains to be established. The data indicate, however, that the Zn(II)–Ac-bleomycin A2 complex retains at least four of the same ligands that have been implicated for the Zn(II)–bleomycin complex: the secondary amine, the imidazole, the pyrimidine, and the carbamoyl. In addition, the $\alpha$-CH resonance of the valerate moiety shows an analogously large sensitivity to the binding of Zn(II) so that some structural features are also conserved.

The analysis of the vicinal coupling constants among the $\beta$-aminoalanine resonances in Zn(II)–Ac-bleomycin A2 indicates that considerable changes in the conformation around this bond have occurred relative to the Zn(II)–bleomycin complex. These alterations may in part reflect the reorganization of the binding site that must occur as well as a loosening of the constraints that had formerly been imposed by coordination to the $\alpha$-nitrogen of the $\beta$-aminoalanine moiety.

One should note that in the absence of the availability of a ligand that would ordinarily participate in complex formation with a metal ion (the primary amine), a substantially altered complex has been obtained. This bears formal analogy to the situation that exists for P-3A and bleomycin itself. The former, which lacks many of the potential ligands of bleomycin, forms a crystalline complex with Cu(II). Although it has been tempting to conclude that this complex is similar to that formed by bleomycin, the additional ligands available in the antibiotic provide alternative binding modes.

The fact that both bleomycin and Ac-bleomycin A2 appear to be equally competent chelators clearly establishes that the amine-terminal end of the molecule represents far more than a simple metal chelating site. Bleomycin appears to be an exquisitely poised structure designed to accommodate Fe(II) in such a fashion as to promote binding of $O_2$ and its reduction. Therefore, one cannot reasonably hope to make analogues of bleomycin having improved therapeutic properties by random alterations of structure.

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# Structure of Myosin Subfragment 1 from Low-Angle X-ray Scattering†

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ABSTRACT: The X-ray scattering pattern produced by a solution of myosin subfragment 1 has been measured to a resolution (Bragg spacing) of 2 nm. We find that for subfragment 1 (S1) prepared by limited papain digestion in the presence of ethylenediaminetetraacetate the radius of gyration is 3.28 ± 0.06 nm, the volume is 151 ± 6 nm$^3$, the surface area is 330 ± 15 nm$^2$, and the length of the maximum chord is 12.0 ± 1.0 nm. The theoretical scattering patterns from several objects of uniform electron density have been calculated and compared with the observed scattering produced by S1. The recent three-dimensional electron micrograph reconstruction of S1-decorated actin by J. Seymour and E. O'Brien (private communication) generated the calculated pattern that best fit the observed scattering. This fit strongly suggests that this reconstruction resembles subfragment 1. The good correspondence between an S1 structure derived when S1 is attached to actin and a study of free S1 in solution strongly suggests that binding to actin does not grossly distort the shape of S1. This is consistent with the notion that S1 changes its orientation on actin, rather than its shape, in order to generate the contractile force in muscle.

The shape of the globular "head" portion of the myosin molecule (subfragment 1) has been a subject of considerable interest and controversy. Since the cross-bridges seen in electron microscopy of muscle consist primarily of these heads, even a low-resolution knowledge of their structure would greatly aid the analysis of X-ray diffraction by muscle fibers (Miller & Tregear, 1972; Squire, 1975; Lymn & Cohen, 1975; Haselgrove et al., 1976) and fluorescence polarization (Tregear & Mendelson, 1975; Mendelson & Morales, 1977) of muscle fibers. We report here the results obtained from low-angle X-ray scattering by the isolated subfragment 1 portion of myosin in solution.

Lowey et al. (1969) used rotary shadowing to establish that myosin has two "heads" and modeled these as spheres approximately 7 nm in diameter. Moore et al. (1970), studying the three-dimensional reconstruction of electron micrographs of negatively stained individual thin filaments "decorated" with subfragment 1 (S1),[1] found the heads to be asymmetric objects resembling a cupped hand. Similar results have been found by J. Seymour and E. O'Brien (private communication) on examining image reconstructions of S1 decorating thin filament paracrystals. Mendelson et al. (1973) found using fluorescence depolarization experiments that S1 in solution was elongate; when considered as a prolate ellipsoid of revolution, its axial ratio was 3.5 or greater. Other hydrodynamic measurements of Yang & Wu (1977) attributed an oblate shape to S1. Takahashi (1978), using a negative staining method on myosin, found that the heads were 21 nm long, while Elliott & Offer (1978) using a new rapid freeze rotary shadowing method found the heads of isolated myosin molecules to be about 19 nm long.

X-ray scattering from S1 in solution allows models to be tested with a resolution of up to 2 nm (Bragg spacing), and has the advantage that the shape is inferred from the native, enzymatically active particle.

In the first phase of this work, Kretzschmar et al. (1978) found that S1 had a radius of gyration of 3.24 nm, bearing out our earlier contention that the particle is quite asymmetric. Here we report wider angle (higher resolution) data, which permits us to select from among various proposed models of S1.

## Materials and Methods

*Protein Preparation.* Myosin subfragment 1 was prepared from insoluble rabbit dorsal skeletal muscle myosin, using

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[1] Abbreviations and symbols used: S1, subfragment 1 of myosin; fwhm, full width at half-maximum; $h = (4\pi/\lambda) \sin \theta$; $\lambda$, the X-ray wavelength; $2\theta$, the scattering angle; $V$, the molecular volume; $S$, the molecular surface area; $R_g$, the radius of gyration; LC1, LC2, and LC3, the light chains of myosin in descending order of molecular weight; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; AMPPNP, adenylylimidodiphosphate.

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