The Secondary Amine Group of Bleomycin Is Not Involved in Intramolecular Hydrogen Bonding in "Activated Bleomycin"

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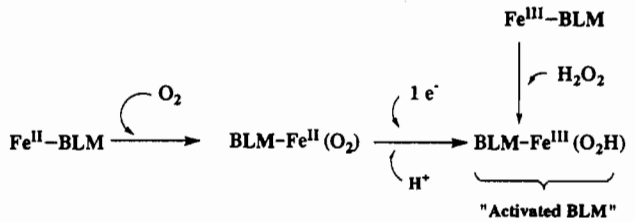
The iron complexes of two designed ligands, PMAH and PMCH, that mimic the metal-chelating portion of the antitumor drug bleomycin (BLM) react with dioxygen to afford low-spin hydroperoxo–Fe(III) species that exhibit EPR spectra very similar to that of the "activated bleomycin". Much like the Fe–BLMs, these active intermediates induce DNA damage via an oxidative pathway and also promote facile oxo transfer to olefinic substrates. A recent theoretical study concluded that activation of Fe–BLM might involve internal hydrogen bonding between the secondary NH group and the coordinated hydroperoxo unit. This work demonstrates that the O_2-activation capacity of the iron complex of PMCH, a ligand that contains a N–CH_3 group instead of the N–H group of PMAH, is identical to that of the iron complex of PMAH. It is, therefore, evident that the secondary amine group of PMAH (and BLM) does not assist the process of O_2 activation by forming an internal hydrogen bond.

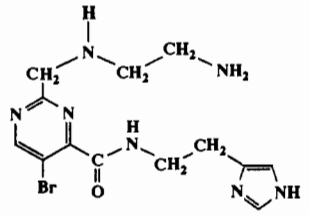
Introduction

The antineoplastic activity of the glycopeptide antibiotic *bleomycin* (BLM) is believed to arise from oxidative damage of cellular DNA by the iron complex of the drug.[1] One-electron reduction of the oxygenated Fe(II) chelate of BLM affords "activated bleomycin", a low-spin hydroperoxo–Fe(III) species[1a,2]

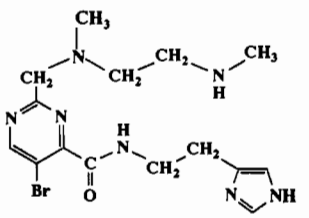


with a characteristic EPR spectrum.[3] This EPR-active species can be generated from reaction of the Fe(III)–BLM complex with H_2O_2 as well. Activated bleomycin induces DNA strand scission via an oxidative pathway in which the intermediacy of a hypervalent iron–oxo (ferryl (Fe^{IV}=O) or perferryl (Fe^{V}=O)) unit has often been implicated.[1] Formation of either of these two intermediates requires cleavage of the O–O bond of the coordinated hydroperoxo group. The monooxygenase activity of the Fe–BLMs toward olefinic substrates[1d,4] also provides evidence for such O–O bond cleavage. The O–O bond scission reaction at the iron center of the Fe–BLMs is unique in the sense that this event occurs at a non-heme iron site. Though the mechanism(s) of O_2 activation by non-heme iron-containing enzymes[5] as well as model complexes thereof[6] has been the subject of recent research activities, no definite mechanistic pathway has been suggested by any group.[7]

In a recent paper, Valentine and co-workers[8] have proposed that intramolecular hydrogen bonding between the secondary amine group of BLM and the coordinated hydroperoxide moiety could facilitate the O–O bond cleavage reaction at the iron center of Fe–BLMs. They arrived at this conclusion following a molecular mechanics (MM) study on metal complexes of PMAH (**1**, H is the dissociable amide H), which we reported during the



PMAH (**1**)



PMCH (**2**)

past few years.[9] The most stable calculated structures for the five- and six-coordinate complexes obtained from the MM study match extremely well with the X-ray structures of the corresponding Cu(II) and Co(III) complexes of PMAH. In these

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(7) Unlike the Fe–BLM system, the enzymes contain diiron centers and hence the mechanism of O_2 activation could be different. Also, the model systems either consist of diiron centers ligated to tailored ligands or involve diiron–oxo species as intermediates.[6]

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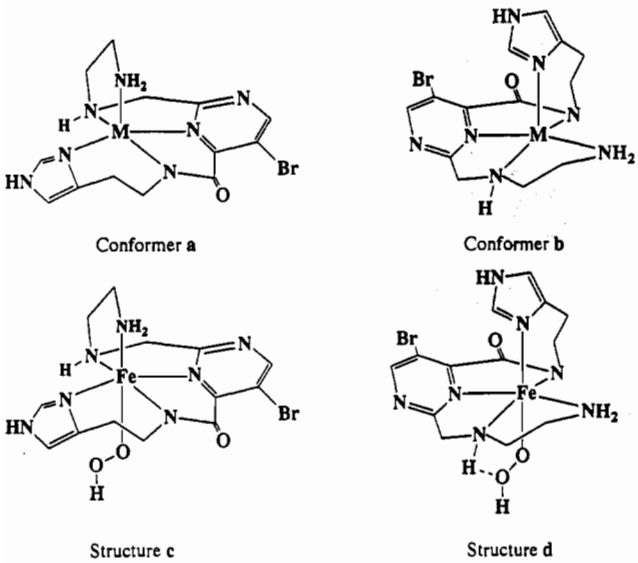

Figure 1. Two modes of chelation of the PMA^- framework around the metal center (ref 8): conformer **a**, most stable, NH_2-axial; conformer **b**, imidazole-axial. Strucutre **c** illustrates that, in conformer **a**, the N–H group is not properly positioned to form a hydrogen bond with the coordinated hydroperoxide group in "activated bleomycin" while, in structure **d**, intramolecular hydrogen bonding between the same two groups is favored with the PMA^- framework in the **b** conformation.

structures, the deprotonated PMA^- framework is ligated to the metal centers with the NH_2 group occupying the axial coordination site (Figure 1, conformer **a**). In addition, the theoretical modeling work indicated another structure with the imidazole group in the axial position (Figure 1, conformer **b**) which is several kcal/mol less stable than the NH_2-axial-conformer **a**. However, in conformer **b**, the N–H bond of the secondary amine is axial and is ideally situated for hydrogen bonding with the sixth O_2 or O_2H^- ligand (structure **d**) and appears to assist the O–O bond cleavage. When this idea is extended to the hypothetical iron complex of the metal-binding region of BLM, structure **d** also accommodates selected substrates near the iron center and promotes facile oxo transfer. These facts prompted Valentine and co-workers to suggest that activation of Fe–BLM by O_2 or H_2O_2 might involve conformational change from type **a** to type **b** to achieve internal hydrogen bonding.

We were rather intrigued by this suggestion of Valentine and co-workers. To date, all complexes of PMAH belong to structure **a**.[9] Even with smaller ligands, the three N donor centers of the pyrimidine ring, carboxamide group, and the imidazole ring are always coplaner (again structure **a**).[10] Since the iron complexes of PMAH, structure **a** by all spectroscopic parameters,[2a] give rise to EPR signals identical to that of the "activated bleomycin" in presence of O_2 or H_2O_2 and also promote facile oxo transfer to olefinic substrates, structure **b** does not appear to be involved even in the process of O_2 activation. We therefore, decided to address this issue more directly with the aid of the iron complex of the designed ligand PMCH (**2**, H is the dissociable amide H) that contains a $N–CH_3$ moiety in place of the secondary amine group of PMAH. The objective was to find out whether or not the removal of the possibility of hydrogen bonding with the N–H group alters the capacity of O_2 activation by these model complexes to a significant extent. Reported in this account are (a) the syntheses and spectral parameters of PMCH and $[Fe^{II}(PMC)]^+$, the Fe(II) complex of this ligand, (b) reactions of $[Fe^{II}(PMC)]^+$ with O_2, and (c) preliminary results of the DNA cleavage and oxo transfer reactions by $[Fe^{II}(PMC)]^+$. Since $[Fe^{II}(PMC)]^+$ is almost identical to $[Fe^{II}(PMA)]^+$ in every respect including the capacity of O_2 activation, we believe that intramolecular hydrogen bonding between the coordinated hydroperoxo moiety and the secondary amine group of BLM is not a requirement for the observed reactivity of BLM.

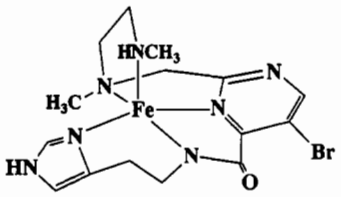

Figure 2. Structure of $[Fe^{II}(PMC)]^+$ as evidenced by its spectroscpic parameters.

Experimental Section

$[Fe^{II}(PMC)]Cl·MeOH$. The designed ligand PMCH was synthesized by following a procedure identical to that for PMAH[9d] except for the last step where 4 equiv of *N,N'*-dimethylethylenediamine was used instead of ethylenediamine. The pale yellow foam thus obtained was characterized by NMR spectroscopy.[11] Under dry N_2 atmosphere, addition of 163 mg (1 mmol) of $FeCl_2·2H_2O$ in 5 mL of methanol to a mixture of 475 mg (1.2 mmol) of PMCH and 46 mg (1.2 mmol) of LiOMe in 25 mL of methanol afforded a deep blue solution from which $[Fe^{II}(PMC)]Cl·MeOH$ was isolated upon removal of most of the solvent (yield of the blue solid: 65%). Selected IR bands (KBr pellet, cm^{-1}): 3350 (vs), 2940 (s), 1590 (s, ν_{CO}), 1460 (m), 1407 (m), 1180 (m), 1060 (m), 1025 (m), 796 (m).

Other Physical Measurements. Infrared spectra were obtained with a Perkin-Elmer 1600 spectrophotometer. Absorption spectra were recorded on a Perkin-Elmer Lambda-9 spectrophotometer. A Bruker ESP-300 spectrometer was used to record the EPR spectra at X-band frequencies.

DNA Cleavage Reactions. Reaction mixtures contained 1 μg of *ϕX174* DNA (replicative form, BRL) in 20 mM sodium cacodylate buffer (pH 7.2) and 10 mM sodium ascorbate in a total volume of 25 μL. These were incubated with 15 μM Fe^{2+} (final concentration) and a slight excess of PMCH for different reaction times in air. Agarose gel (1%) electrophoresis was run (3 h) on a BRL H-5 horizontal gel system. Photographs were taken with a Polaroid MP-4 system following ethidium bromide staining.

Oxo-Transfer Reactions. A 1-mL volume of an ~10 mM solution of $[Fe^{II}(PMC)]^+$ in methanol was mixed with 30 mg of sodium ascorbate and 50 μL of *cis*-stilbene or 15 mg of PPh_3 in 1.5 mL of aqueous methanol in an inert-atmosphere glovebox, and then the mixture was exposed to air and stirred for 1 h. Detection of the oxo-transfer products was done by HPLC (70:30 $CH_3CN:H_2O$ as the mobile phase, 250-mm C18 Econosphere column, UV detection at 240 (for stilbene) or 260 (for PPh_3) nm).

Results and Discussion

The structure of the complex ion $[Fe^{II}(PMC)]^+$ (shown in Figure 2) is discernible from its spectroscopic parameters which are very similar to those of $[Fe^{II}(PMA)]^+$.[2a] For example, in methanol, $[Fe^{II}(PMC)]^+$ exhibits absorption bands at 662 (1700), 615 (1400), and 450 (800) nm. Strong charge-transfer bands at similar λ_{max} values are also observed with $[Fe^{II}(PMA)]^+$ (see supplementary material). The similarity points to the fact that in both complexes the three nitrogens located in the pyrimidine and imidazole rings as well as the deprotonated peptido moiety are coordinated to the Fe(II) center in the basal plane.[2a,10a] Coordination of the deprotonated peptido nitrogen to Fe(II) in $[Fe^{II}(PMC)]^+$ is further evidenced by ν_{CO} at 1590 cm^{-1}.[2a]

In methanol, $[Fe^{II}(PMC)]^+$ reacts readily with dioxygen to generate an oxygenated species that exhibits a strong rhombic EPR signal (g = 2.30, 2.18, 1.93). This spectrum (Figure 3b) is very similar to that of "activated bleomycin"[3] and [(PMA)-

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(11) ^{13}C NMR spectrum ($(CD_3)_2SO$, 298 K, 300 MHz; δ, ppm from TMS): 26.61, 33.73, 38.95, 41.86, 46.82, 53.08, 61.89, 115.04, 117.09, 134.78, 135.13, 159.72, 160.82, 164.12, 166.15. The product also contained trace amount of *N,N'*-dimethylethylenediamine. PMCH is sensitive to both moisture and oxygen. When kept at 253 K under dry N_2, the compound is stable for weeks.

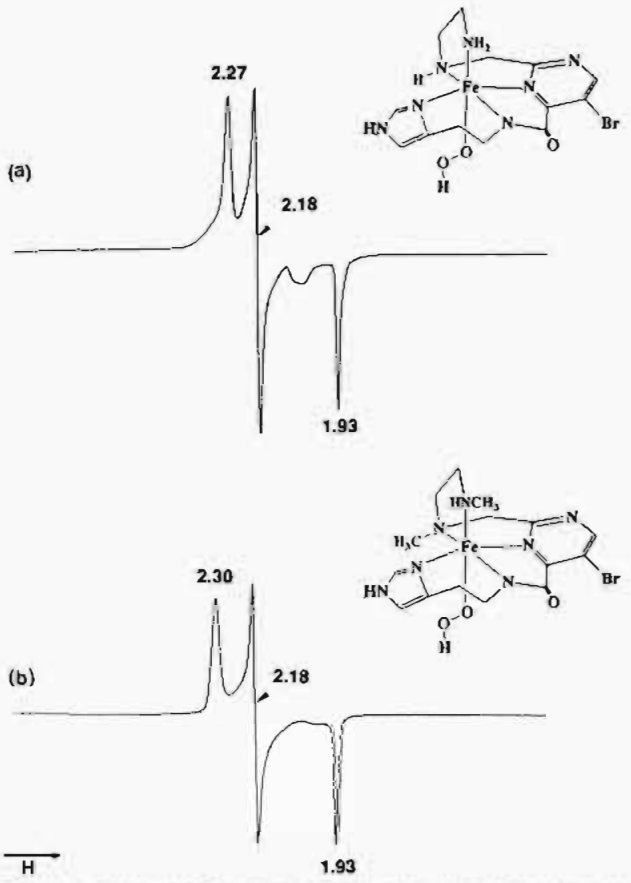

Figure 3. X-band EPR spectra of the oxygenated products from (a) $[Fe^{II}(PMA)]^+$ and (b) $[Fe^{II}(PMC)]^+$ in methanol glass (100 K). Selected g values are shown. Spectrometer settings: microwave frequency, 9.43 GHz; microwave power, 13 mW; modulation frequency, 100 kHz; modulation amplitude, 2 G.

$Fe^{III}OOH]^+$ (Figure 3a).[2a] As is the case with $[Fe^{II}(PMA)]^+$, the second electron needed for the formation of $[(PMC)Fe^{III}OOH]^+$ is provided by a second $[Fe^{II}(PMC)]^+$ ion which in turn is converted into a high-spin Fe(III) species ($g = 4.3$, signal ratio 1:1, not shown in Figure 3). Spin integration experiments[12] confirm that with samples of the same concentration, both $[Fe^{II}(PMA)]^+$ and $[Fe^{II}(PMC)]^+$ give rise to the *same* amounts of the hydroperoxo–Fe(III) species in solution. Clearly, replacement of the N–H group with an $N–CH_3$ moiety in the BLM analogues does not modulate the extent of the formation of active oxygenated species by their iron complexes.

The chemistry of the "activated oxygen" in $[(PMC)Fe^{III}OOH]^+$ [13] is also remarkably similar to that of "activated bleomycin" (and $[(PMA)Fe^{III}OOH]^+$). For example, in presence of sodium ascorbate and dioxygen, $[Fe^{II}(PMC)]^+$ induces rapid

(12) $Na[Fe(EDTA)]\cdot 4H_2O$ ($S = {}^5/_2$) and $[Cu(PMA)]BF_4$ ($S = {}^1/_2$) were used as standards in the spin integration measurements. Authentic samples were dissolved in aqueous glycerol (7:3), and the EPR signals at 100 K were integrated by using the software package on the Bruker ESP-300 spectrometer.

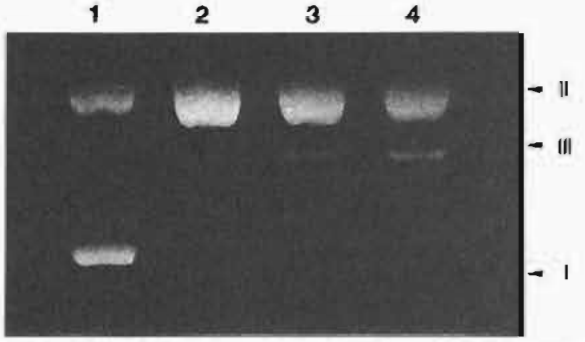

Figure 4. $\phi X174$ DNA (1 μg in a total volume of 25 μL) cleavage experiments with Fe^{2+} (15 μM final concentration) and 1.2 equiv of PMCH in 20 mM sodium cacodylate (pH 7.2) buffer. Reaction mixtures also contained 10 mM Na–ascorbate. Key: Lane 1, DNA only; lane 2, DNA + $[Fe^{II}(PMC)]^+$, 15 min incubation; lane 3, DNA + $[Fe^{II}(PMC)]^+$, 30 min incubation; lane 4, DNA + $[Fe^{II}(PMC)]^+$, 60 min incubation. Forms I–III refer to the covalently closed circular supercoiled, nicked circular, and linear DNA, respectively.

oxidative DNA cleavage (Figure 4) with the production of base propenals (indicated by a thiobarbituric assay[16]). Furthermore, the $[Fe^{II}(PMC)]^+$ + Na ascorbate + O_2 system promotes facile oxo transfer to stilbenes and PPh_3.[17] These reactions clearly indicate that the cleavage of the O–O bond of the coordinated hydroperoxide unit in $[(PMC)Fe^{III}OOH]^+$ occurs without the assistance of any hydrogen bonding.

In summary, model complexes of two designed ligands (PMAH and PMCH) that mimic the metal-binding domain of BLM activate dioxygen toward a variety of substrates including DNA and this capacity of O_2 activation is *not* attenuated by the possibility of intramolecular hydrogen bonding. This result indicates that geometric change in the coordination sphere of iron in "activated" Fe–BLM to achieve internal hydrogen bonding between the secondary amine group and the coordinated hydroperoxo unit, a conceptually elegant suggestion by Valentine and co-workers, is not required for its reactivity.

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Supplementary Material Available: A figure showing electronic absorption spectra of $[Fe^{II}(PMA)]^+$ (solid line) and $[Fe^{II}(PMC)]^+$ (broken line) in methanol (Figure S1) (1 page). Ordering information is given on any current masthead page.

(13) The strongly oxidizing nature of $[(PMC)Fe^{III}OOH]^+$ is evident by the rapid formation of the $^{\bullet}CH_2OH$ radical (detected by spin trapping, $A_H = 22.7$ G; $A_N = 16$ G)[14] and formaldehyde (detected by Nash reagent[15]) in methanolic solutions at room temperature. A solution of $[(PMC)Fe^{III}OOH]^+$ is stable for hours at liquid-N_2 temperature.

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(17) With *cis*-stilbene and PPh_3 as substrates, 12% *cis*-stilbene oxide and 108% PPh_3O (based on metal content) were obtained as products. For more discussion on these reactions, see ref 2a.