# Electrospray Mass Spectrometry of Iron Bleomycin: Demonstration That Activated Bleomycin Is a Ferric Peroxide Complex†

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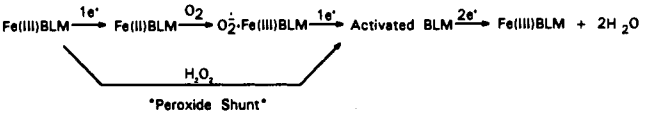
**Abstract:** The glycopeptide antibiotic bleomycin $A_2$ (BLM) possesses potent antineoplastic activity, presumably due to its ability to bind iron and activate oxygen, forming a species, activated BLM, that is kinetically competent to cleave DNA. Activated BLM may be formed from Fe(II)BLM and $O_2$ followed by single electron reduction or directly from Fe(III)BLM and $H_2O_2$ (Burger, R. M.; Peisach, J.; Horwitz, S. B. *J. Biol. Chem.* **1981**, *256*, 11636–11644). We have used electrospray mass spectrometry to study the activation of oxygen by FeBLM. Upon reacting Fe(III)BLM with $H_2O_2$ or Fe(II)BLM with $O_2$, we observe an intermediate that displays kinetics of formation and decay similar to those of activated BLM and a mass to charge ratio consistent with that of HOO–Fe(III)BLM. Formation of this species by reacting Fe(III)BLM with $H_2{}^{18}O_2$ and the observation of its increase in mass by 4 Da confirm that this species contains two oxygen atoms derived from hydrogen peroxide. These results strongly suggest that activated BLM is a ferric peroxide complex. Tandem mass spectrometry (MS/MS) of activated BLM was also performed, and the data indicate that the O–O bond is labile. The significance of these results to the activation of oxygen by FeBLM and other non-heme iron systems is discussed.

## Introduction

The relatively recent development of electrospray and related ionization techniques has fostered a remarkable growth in the application of mass spectrometry (MS) to the study of biomolecules.[1,2] Owing to the tendency of electrospray ionization (ESI) to produce multiply charged ions, accurate measurements of the molecular weight of large biomolecules such as proteins and nucleic acids can be performed routinely with conventional quadrupole mass spectrometers. Furthermore, the addition of coaxial nebulizing gas to the sprayer of an electrospray source, known as ionspray ionization, enhances droplet dispersion and ion evaporation, thereby allowing the use of high flow rates and buffered, aqueous solutions.[3] Hence, this technique has revolutionized the characterization of macromolecules by liquid chromatography mass spectrometry. However, the combined characteristics of gentle ionization and high throughput of sample should make the ionspray technique especially well suited to the analysis of transient intermediates of biologically relevant reactions. Yet, ionspray mass spectrometry has seen only limited utilization in this capacity. Presently, we report the application of ionspray mass spectrometry to the study of oxygen activation by bleomycin $A_2$ (BLM, Figure 1A), a member of the bleomycin family of antineoplastic glycopeptides.

It has been known for many years that BLM is able to form a complex with iron (cf. Figure 1B) and activate oxygen in a manner reminiscent of heme-bearing oxygenases and peroxidases (for reviews, see refs 6–8). For example, the ferrous complex of BLM (Fe(II)BLM) can bind oxygen[9] and, subsequent to single electron reduction,[10] form activated BLM, a species defined by a unique EPR spectrum ($g$ = 2.26, 2.17, 1.94) and the ability to cleave DNA.[11,13] Alternatively, ferric BLM (Fe(III)BLM) reacted with peroxide will also yield activated BLM.[13] Activated BLM contains the same number of oxidizing equivalents as compound I of horseradish peroxidase and the putative activated oxoiron(V) form of cytochrome P-450, i.e. two reducing equivalents are required to discharge activated BLM to Fe(III)BLM:[14]




The two electron equivalents may be provided by the oxidation of DNA,[12] RNA,[15] or other substrates. In the absence of these compounds, activated BLM is discharged through autoxidation, producing an FeBLM derivative incapable of activating oxy-

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(1) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64–71.
(2) McCloskey, J. A., Ed. *Methods in Enzymology*; Academic Press: New York, 1990; Vol. 193, pp 3–842.
(3) Bruins, A. P.; Covey, T. R.; Henion, J. D. *Anal. Chem.* **1987**, *59*, 2642–2646.
(4) Sugiura, Y. *J. Am. Chem. Soc.* **1980**, *102*, 5208–5215.
(5) Akkerman, M. A. J.; Neijman, E. W. J. F.; Wijmenga, S. S.; Hilbers, C. W.; Bermel, W. *J. Am. Chem. Soc.* **1990**, *112*, 7462–7474.
(6) Dedon, P. C.; Goldberg, I. H. *Chem. Res. Toxicol.* **1992**, *5*, 311–332.
(7) Stubbe, J.; Kozarich, J. W. *Chem. Rev.* **1987**, *87*, 1107–1136.
(8) Petering, D. H.; Byrnes, R. W.; Antholine, W. E. *Chem.-Biol. Interact.* **1990**, *73*. 133–182.
(9) Burger, R. M.; Horwitz, S. B.; Peisach, J.; Wittenberg, J. B. *J. Biol. Chem.* **1979**, *254*, 12299–12302.
(10) The single electron may be provided by a second molecule of Fe(II)BLM, yielding Fe(III)BLM as an additional product, or by other reducing agents, such as ascorbate, superoxide, or dithionite. See refs 11, 12, and the following: Ciriolo, M. R.; Magliozzo, R. M.; Peisach, J. *J. Biol. Chem.* **1987**, *262*, 6290–6295.
(11) Kuramochi, H.; Takahashi, K.; Takita, T.; Umezawa, H. *J. Antibiot.* **1981**, *34*, 576–582.
(12) Suzuki, H.; Nagai, K.: Yamaki, H.; Tanaka, N.; Umezawa, H. *J. Antibiot.* **1969**, *22*, 446–448.
(13) Burger, R. M.; Peisach, J.; Horwitz, S. B. *J. Biol. Chem.* **1981**, *256*, 11636–11644.
(14) Burger, R. M.; Blanchard, J. S.; Horwitz, S. B.; Peisach, J. *J. Biol. Chem.* **1985**, *260*, 15406–15409.
(15) Magliozzo, R. M.; Peisach, J.; Ciriolo, M. R. *Mol. Pharm.* **1989**, *35*, 428–432.

0002-7863/94/1516-5250$04.50/0 

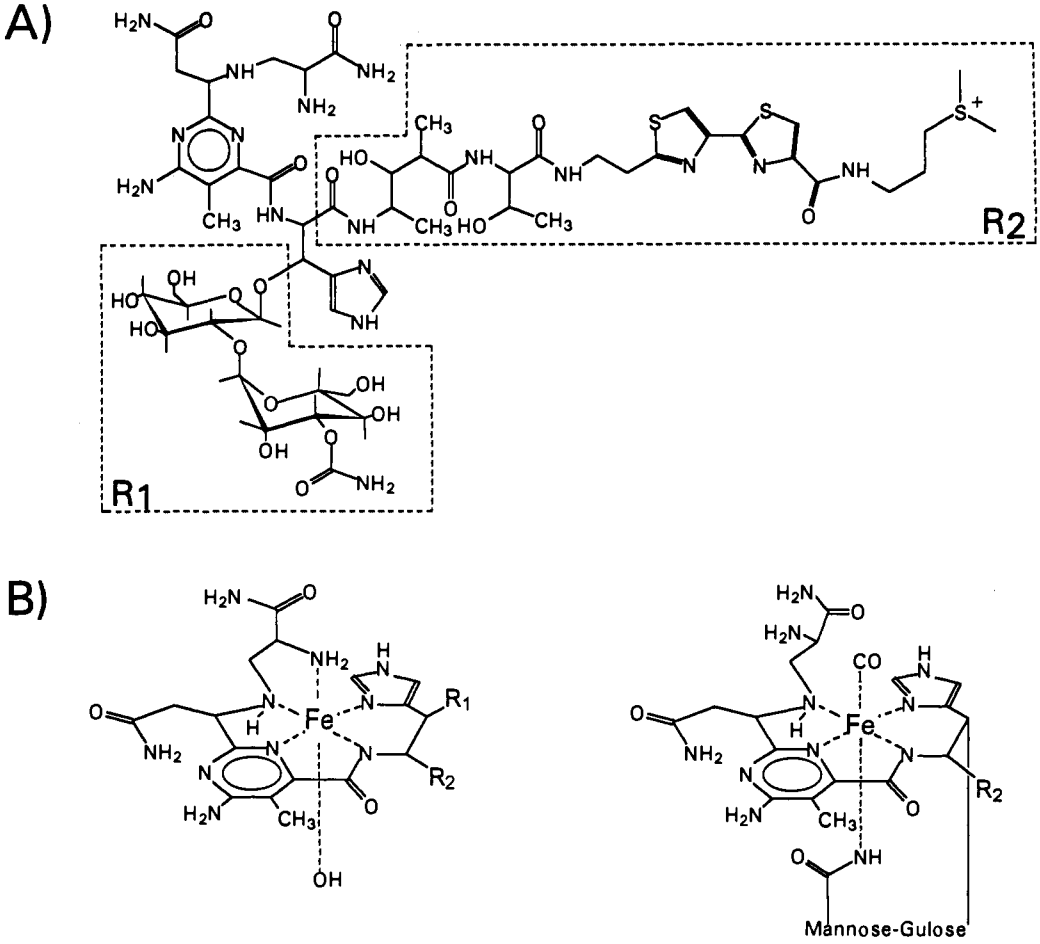


**Figure 1.** Structure of (A) metal-free bleomycin $A_2$ and the proposed structures of (B) Fe(III)BLM[4] and CO–Fe(II)BLM.[16]

gen.[16,17] Numerous spectroscopic and synthetic studies have been conducted to characterize activated BLM and probe its similarity to the activated forms of the aforementioned heme systems. Burger et al. demonstrated by optical, EPR, and Mössbauer spectroscopy that activated BLM is a low-spin, ferric species with at least one oxygen atom, derived from $O_2$ or $H_2O_2$, bound to iron.[9,13,18] However, whether two oxygen atoms as hydroperoxide (HOO–Fe(III)BLM)[19] or a single oxygen atom at the oxidation state of atomic oxygen ($O^0$–Fe(III)BLM) is bound to the ferric iron of activated BLM remains unknown, although some authors,[20] without direct evidence, subscribe to the former structure based on EPR similarities with low-spin iron hydroperoxide systems.[4]

We have used ionspray mass spectrometry to determine the number of oxygen atoms in activated bleomycin. Upon reacting Fe(III)BLM with $H_2O_2$, or Fe(II)BLM with $O_2$, we observe an intermediate that displays kinetics of formation and decay similar to those of activated BLM and a mass consistent with that of HOO–Fe(III)BLM. When we produce this species by reacting Fe(III)BLM with $H_2{}^{18}O_2$, we observe an increase in its mass by 4 Da, confirming that it contains two oxygen atoms derived from hydrogen peroxide. These results demonstrate that activated BLM is a ferric peroxide complex. Furthermore, our experiments provide no evidence for the formation of $O^0$–Fe(III)BLM, HO•–Fe(IV)BLM, or $H_2O$–Fe(V)BLM, although tandem mass spectrometry (MS/MS) indicates that the O–O bond of activated BLM is labile.

## Experimental Section

**Sample Preparation.** Bleomycin sulfate (Blenoxane, the generous gift of Bristol-Myers Co., Syracuse, NY) was purified over a mono-S FPLC column (Pharmacia, Piscataway, NJ), by eluting with a linear 10–50 mM gradient of $NH_4HCO_3$. Fractions containing bleomycin $A_2$ were pooled and dried using a speed-vac apparatus (Savant, Farmingdale, NY); their purity was confirmed by TLC, as previously reported,[21] and by the present ESI–MS experiments. Fe(III)BLM was produced by addition of $Fe(III)NH_4(SO_4)_2$ to a slight excess of purified bleomycin $A_2$ followed by >10 equiv of $NH_4CH_3CO_2$ to neutralize the solution. All other forms of the drug were produced with a rapid mixing device which directly interfaced with the mass spectrometer, as described below. $^{18}O$-labeled (90 atom%) hydrogen peroxide was obtained from Icon (Mt. Marion, NY). Hydrogen peroxide solutions were standardized by their optical absorbance ($\epsilon_{240}$ = 43.6 $M^{-1}$ $cm^{-1}$).[22]

**Mass Spectrometry (MS).** All mass spectra were acquired using an API III triple quadrupole mass spectrometer equipped with an ionspray interface (PE-Sciex, Thornhill, ON, Canada). Instrument settings, data acquisition, and data processing were controlled by a MacIntosh Quadra 950 computer (Cupertino, CA). Samples were introduced using a dual syringe pump (Harvard Apparatus, South Natick, MA) fitted with Hamilton syringes (Hamilton Co., Reno, NE) feeding into a low dead volume mixing tee (Upchurch, Oak Harbor, WA) that interfaced with the ionspray source. All connections were made using fused silica capillary

(16) Takita, T.; Muraoka, Y.; Nakatani, T.; Fujii, A.; Iitaka, Y.; Umezawa, H. *J. Antibiot.* **1978**, *31*, 1073–1077.
(17) Nakamura, M.; Peisach, J. *J. Antibiot.* **1988**, *41*, 638–647.
(18) Burger, R. M.; Kent, T. A.; Horwitz, S. B.; Münck, E.; Peisach, J. *J. Biol. Chem.* **1983**, *258*, 1559–1564.
(19) The oxidation states of iron in all oxoiron species described in this paper are explicitly labeled, i.e. Fe(III), Fe(IV), and Fe(V) are used to describe the $d^5$, $d^4$, and $d^3$ states of iron, respectively. Thus, Fe(III)–$O^0$, Fe(IV)–•OH, and Fe(V)–$OH_2$ represent three possible configurations of an oxoiron(V) species in which the oxidizing equivalents reside entirely on oxygen (Fe(III)–$O^0$), on both the Fe and O atoms (Fe(IV)–•OH), or entirely on the iron atom (Fe(V)–$OH_2$).
(20) Guajardo, R. J.; Hudson, S. E.; Brown, S. J.; Mascharak, P. K. *J. Am. Chem. Soc.* **1993**, *115*, 7971–7977.
(21) Roy, S. N.; Orr, G. A.; Brewer, F.; Horwitz, S. B. *Cancer Res.* **1981**, *41*, 4471–4477.
(22) Hoogland, H.; van Kuilenberg, A.; van Riel, C.; Muijsers, A. O.; Wever, R. *Biochim. Biophys. Acta* **1987**, *916*, 76–82.

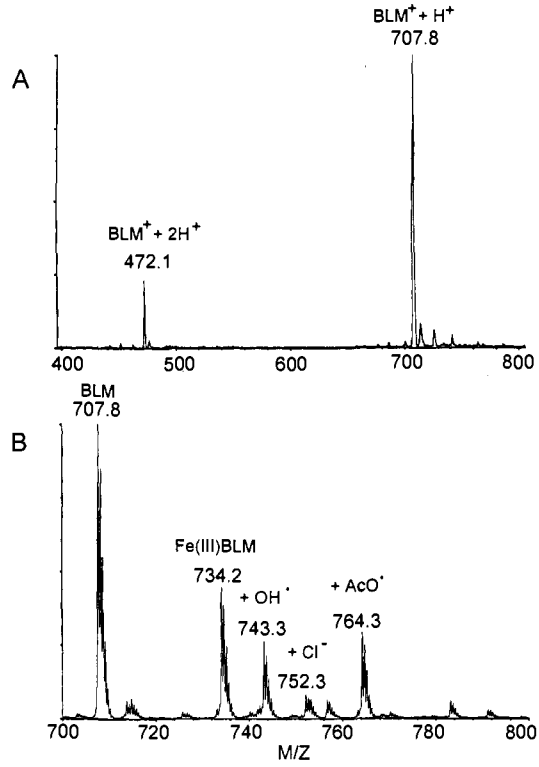


**Figure 2.** Mass spectra of (A) metal-free BLM and (B) Fe(III)BLM. Peak assignments are indicated and are also listed in Table 1. The *y*-axis indicates relative peak intensity.

tubing and Fingertight III PEEK unions and fittings (Upchurch, Oak Harbor, WA). For most of the experiments the mass resolution of the spectrometer was tuned to give a constant peak width of 0.5 Da (at 10% peak height) across the mass range of interest, so that the doubly charged species could be identified unambiguously. Typically, 10 scans of 10–20-s duration were acquired and added to yield a mass spectrum. Fragment ion (Tandem or MS/MS) mass spectra were obtained by collision-induced dissociation of precursor ions selected by their $m/z$ value in the first quadrupole. Collisional activation is accomplished by introducing argon into the second (rf-only) quadrupole, and the resulting fragment ions are analyzed in the third quadrupole of the instrument. In these experiments, a target gas thickness of $1.5 \times 10^{14}$ atoms/cm$^2$ and laboratory-frame collisional energies of 40–60 eV for the doubly charged BLM complexes were typically employed.

## Results

The mass spectrum of metal-free BLM is shown in Figure 2A. The peak at a mass to charge ratio ($m/z$) of 707.8 has been assigned to $[BLM^+ + H^+]^{2+}$,[23] whereas the smaller peak at $m/z$ = 472.2 corresponds to the doubly protonated, triply charged form of the drug ($m/z$ = (1414.5 + 2)/3 or 472.2).[24] The assignments of these peaks and those of the following spectra are compiled in Table 1. The addition of Fe(III) to a slight excess of BLM produces several new peaks in the mass spectrum, e.g. at $m/z$ = 734.3, 743.3, 752.3, and 764.4, as shown in Figure 2B. Furthermore, each of these peaks can be assigned to Fe(III)-BLM complexed with a different anion, which may be bound, for example, as a counterion to the dimethylsulfonium group or as an axial ligand to iron. The latter is supported, in part, by the recent demonstration by resonance Raman spectroscopy that $OH^-$ is an axial ligand in the ferric complex of the drug.[25]

Unfortunately, the ESI–MS technique provides no information about the three-dimensional structures or the redox states of the individual components of these species. However, the triple-quadrupole instrument does allow one to partially characterize these ions through tandem mass spectrometric measurements. In these experiments, the precursor ion of interest is selected in the first quadrupole and allowed to collide with argon gas introduced into the second (rf-only) quadrupole. The resulting fragment ions are then analyzed in the third quadrupole. Thus, when the MS/MS spectra of the various complexes of Fe(III)BLM were acquired, a distinct fragmentation pattern emerged (cf. Figure 3A–D). Namely, each of the complexes is capable of dissociating from its anionic ligand, leaving free Fe(III)BLM ($m/z$ = 734.3) which can, in turn, shed the terminal dimethylsulfonium group (yielding a fragment with $m/z$ = 703.4) and then, to a lesser extent, the C-terminal $\alpha$-aminoacetamide moiety (forming an $m/z$ = 666.8 fragment).[26] Thus, the similarity of the fragmentation patterns of the $m/z$ = 707.8, 734.3, 743.3, and 764.4 species supports the interpretation of these ions as complexes of Fe(III)BLM differing only in the bound anion.

In order to determine the number of oxygen atoms in activated BLM, we rapidly injected buffered Fe(III)BLM and $H_2O_2$ into a mixing tee which directly interfaced with the ionization source of the mass spectrometer. The total internal volume of the mixing chamber and the ionspray source is approximately 5 $\mu$L; thus, by adjusting the flow rate (2–60 $\mu$L/min), we were able to analyze the reaction products formed from a few seconds to a few minutes after mixing. The spectrum obtained 20 s after mixing is shown in Figure 4C. When compared with the spectrum of Fe(III)-BLM (cf. Figure 4A,B), a new peak at $m/z$ = 751.3 is readily apparent; furthermore, this mass to charge ratio corresponds to a species with mass 16 Da greater than that of HO–Fe(III)BLM (743.3 + 16/2 = 751.3), which is exactly what one would predict for activated BLM formulated as a ferric peroxide (HOO–Fe(III)BLM). It should be stressed at this point that the location of the protons in the ions produced in these experiments cannot be determined by this technique, and hereafter, we will assume that the peroxide of activated BLM is protonated. The formation of this species by reacting Fe(III)BLM with $H_2{}^{18}O_2$ and the increase in its mass by 4 Da, i.e. a shift in the position of the peak to $m/z$ = 753.3 (cf. Figure 4D), demonstrates that this species contains two oxygen atoms, both derived from hydrogen peroxide. In addition, it is important to note that there was no evidence for the production of $O^0$–Fe(III)BLM, HO$^\bullet$–Fe(IV)BLM, or $H_2O$–Fe(V)BLM, all of which one would predict to give rise to a peak at $m/z$ = 742.3.

We sought to confirm that the $m/z$ = 751.3 species does in fact represent activated BLM by investigating the reaction of Fe(II)BLM with $O_2$. Upon mixing ferrous iron with a buffered solution of BLM in the presence of dissolved oxygen, we again observed a peak at $m/z$ = 751.3 in the mass spectrum of the reaction products (cf. Figure 4E), providing strong evidence for its assignment as activated BLM. It should be mentioned that this peak is not assigned to $O_2$–Fe(II)BLM, which would have $m/z$ = 750.7 and a lifetime[9] much shorter than that observed for the $m/z$ = 751.3 peak, nor was there any indication that $O^0$–Fe(III)BLM, HO$^\bullet$–Fe(IV)BLM, or $H_2O$–Fe(V)BLM was produced.

Unlike the activation of Fe(III)BLM with peroxide, the activation of Fe(II)BLM with molecular oxygen is rapid compared

(23) BLM, as depicted in Figure 1A, has a monoisotopic molecular weight of 1414.5 Da. Thus, the $m/z$ for singly protonated, doubly charged BLM is (1414.5 + 1)/2 or 707.8.

(24) In all spectra, the triply charged species represent a minor fraction of the BLM complexes. Hence, hereafter we refer only to the $z$ = 2 species, although the conclusions drawn in this paper are supported by data for the $z$ = 3 forms of the drug as well.

(25) Takahashi, S.; Sam, J. W.; Peisach, J.; Rousseau, D. L. *J. Am. Chem. Soc.* **1994**, in press.

(26) Interestingly, this finding may have implications to the structure(s) of FeBLM. Akkerman et al.[5] have proposed a structure (cf. Figure 1B) for the carbon monoxy ferrous form of the drug (CO–Fe(II)BLM) based on NMR experiments; their proposal is novel in that the primary amine of the $\beta$-aminoalanine moiety is not a ligand to iron. We observed that this region of Fe(III)BLM is fragmented in our MS/MS experiments. Since one would expect that the portions of the BLM molecule not anchored to iron would be relatively flexible and exposed and therefore more likely to dissociate upon collisional activation, the present data might be considered evidence in support of the structure of CO–Fe(II)BLM proposed by that study.

**Table 1.** Proposed Assignments of the $z = 2^+$ Ions Cited in the Paper

| m/z | assignment | comments |
|---|---|---|
| 664.2 | $BLM^+ - NHCH_2CH(NH_2)CONH_2 - H + O + H^+$ | autoxidation (N-dealkylation) product |
| 666.8 | $BLM^+ + Fe^{3+} - S(CH_3)_2 - CH(NH_2)CONH_2 - 2H^+$ | fragment of 703.4 |
| 676.8 | $BLM^+ - S(CH_3)_2 + H^+$ | fragment of 707.8 |
| 690.7 | $BLM^+ + Fe^{3+} - CH_2CH(NH_2)CONH - 2H$ | autoxidation (N-dealkylation) product |
| 702.3[a] | $BLM^+ + Fe^{5+} - 4H^+ - S(CH_3)_2$ | fragment of 751.3, 753.3 |
| 703.3 | $BLM^+ + Fe^{3+} - 2H^+ - S(CH_3)_2$ | fragment of 734.3 |
| 707.8 | $BLM^+ + H^+$ | |
| 713.3 | $BLMB_2^+ + H^+$ | |
| 715.7 | $BLM^+ - H + OH + H^+$ | autoxidation (N-dealkylation) product |
| 725.9[b] | $BLM^+ + Cl^- + 2H^+$ | |
| 733.3[a] | $BLM^+ + Fe^{5+} - 2H^+$ | fragment of 751.3, 753.3 |
| 734.3 | $BLM^+ + Fe^{3+} - 2H^+$ | |
| 742.3 | $BLM^+ + (Fe^V{-}O^{2-}, Fe^{IV}{-}{\cdot}O^-, Fe^{III}{-}O^0)^{3+} - 2H^+$ | not observed |
| 743.3 | $BLM^+ + Fe^{3+} + OH^- - H^+$ | |
| 750.7 | $BLM^+ + Fe^{2+} + O_2 - H^+$ | not observed |
| 751.3 | $BLM^+ + Fe^{3+} + OOH^- - H^+$ | |
| 752.3[b] | $BLM^+ + Fe^{3+} + Cl^- - H^+$ | |
| 753.3 | $BLM^+ + Fe^{3+} + {}^{18}O_2H^- - H^+$ | |
| 764.3 | $BLM^+ + Fe^{3+} + CH_3CO_2^- - H^+$ | |
| 783.3 | $BLM^+ + Fe^{3+} + SO_4^{2-}$ | |

[a] An alternative assignment for this ion is one in which a lower valence iron atom is bound to a BLM moiety having increased positive charge (cf. the discussion of this experiment in the Results. [b] The chloride ion in this species derives, presumably, from trace impurities in the solutions used. This assignment was confirmed by observing an increase in the amplitude of the $m/z$ = 725.9 and 752.3 peaks in the mass spectrum of FeBLM produced with $FeCl_3$ (data not shown).

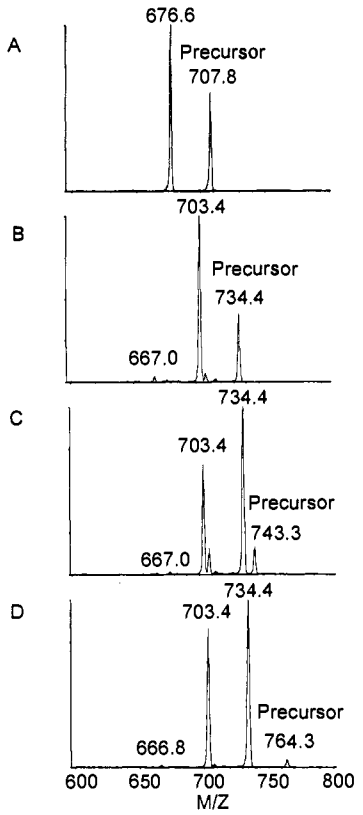


**Figure 3.** Tandem mass spectra of (A) metal-free BLM, (B) Fe(III)-BLM, (C) HO–Fe(III)BLM, and (D) $CH_3CO_2$–Fe(III)BLM. The precursor ion is indicated in each spectrum. The $y$-axis indicates relative peak intensity.

to the rate of disappearance of activated BLM;[13] thus, we were able to follow the formation and decay of activated BLM produced in this manner. Figure 5 shows the $m/z$ = 751.3 peak height versus time after mixing Fe(II) with BLM at room temperature. The peak amplitude is maximal after approximately 10 s and decays to baseline within 2 min. This behavior agrees well with the kinetics of formation and autoxidation of activated BLM[27] and lends further support to the formulation of activated BLM as HOO–Fe(III)BLM.

In all of the above reactions, activated BLM is discharged by autoxidation, by either an intermolecular (bimolecular in BLM) or intramolecular autoxidation resulting in a BLM derivative able to bind iron but incapable of activating oxygen.[16,17] Furthermore, it is probable that there are several competing reactions leading to the observed inactive BLM products since several lesions in the inactive species have been identified.[16,17,28] The mass spectrum of the products formed upon completion of the reaction of Fe(III)BLM with 1 equiv of $H_2O_2$ is shown in Figure 6 (identical products were obtained in the reaction of Fe(II)BLM with $O_2$, data not shown). Several new peaks are observed, e.g. at $m/z$ = 664.2, 690.7, and 715.7. We contend that all three species result from oxidation of the $\beta$-aminoalanine secondary amine, as proposed by Owa et al.[28] Interestingly, in that study, the authors observed N-dealkylation of PYML, a synthetic BLM analog, which occurred exclusively at the 2-pyridylmethylene carbon, yielding the corresponding aldehyde, hemiacetal, and acetal. In contrast, while we observe similar oxidation at the corresponding 2-pyridinylmethylene position in BLM, yielding the 2-pyridinylformaldehyde derivative ($m/z$ = 664.3), we also detect the 2-pyridinylmethylamine product ($m/z$ = 690.7), resulting from oxidation at the $\beta$ carbon of the $\beta$-aminoalanine residue, as well as hydroxylated BLM ($m/z$ = 715.8), which presumably precedes formation of the other two products.

Finally, the $m/z$ = 751.3 ion was analyzed by tandem mass spectrometry and the resulting fragment ion spectrum is shown in Figure 7A. We contend that the peak at $m/z$ = 742.6 results from cleavage of the bound peroxide with loss of $^{\cdot}OH$. In order to test this assignment, we performed the same experiment on activated BLM produced with $^{18}O$-labeled hydrogen peroxide. The spectrum we obtained is shown in Figure 7B and is nearly identical with Figure 7A, with the exception that the peak at $m/z$ = 742.6 has shifted by 2 Da to $m/z$ = 743.8. This indicates that the $m/z$ = 742.6 and 743.8 peaks represent fragment ions in which the peroxide bond of activated BLM has been cleaved with loss of $^{\cdot}OH$. Hence, these data may indicate, as proposed by Wu

(27) Due to the dead time of the experimental apparatus, the initial binding of oxygen to Fe(II)BLM is not observed. Thus, the rate of formation of the $m/z$ = 751.3 peak is representative of the reduction of oxyferrous BLM by a second molecule of Fe(II)BLM to yield activated BLM. This reaction has been shown to occur with a "$t_{1/2}$" of 2 s at 20 °C,[9] whereas the self-inactivation of activated BLM has been shown to occur with a "$t_{1/2}$" of 17 s at 23 °C.[13] Both values are comparable to the rates of formation and disappearance of the $m/z$ = 751.3 feature.

(28) Owa, T.; Sugiyama, T.; Otsuka, M.; Ohno, M.; Maeda, K. *Tetrahedron Lett.* **1990**, *31*, 6063–6066.

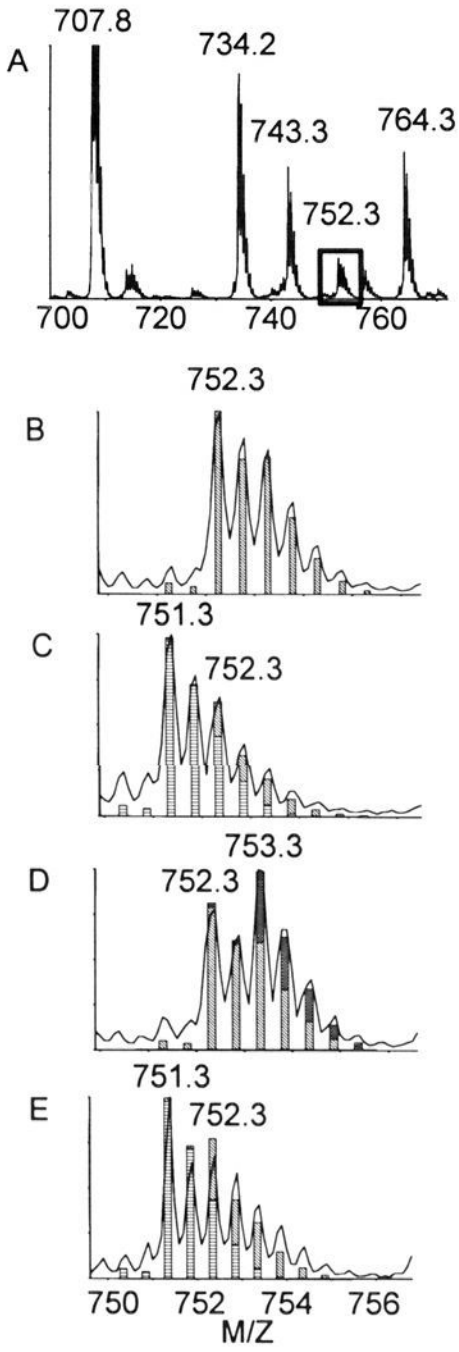


**Figure 4.** Mass spectra of Fe(III)BLM (A and B) and activated BLM produced by reacting (C) Fe(III)BLM with $H_2O_2$, (D) Fe(III)BLM with $H_2{}^{18}O_2$, and (E) Fe(II)BLM with $O_2$. Spectra B–E are expanded views of the spectral region indicated by the box in A. The *y*-axis indicates relative peak intensity. Experimental data are indicated by the solid lines, whereas the theoretical isotopic distributions for the $m/z$ = 751.3 (horizontal lines), 752.3 (diagonal lines), and 753.3 (hatched) ions were calculated using the COMP routine of the ICIS data system (Finnigan MAT, San Jose, CA) and are indicated by bars.

et al.,[29] that the peroxide bond of activated BLM is made labile by the FeBLM molecule. Secondly, upon collisional activation, activated BLM, like the other ferric complexes of BLM (cf. Figure 3C,D), dissociates from its anionic ligand ($HOO^-$) and then fragments again, losing the dimethylsulfonium moiety (cf. Figure 7A). However, the fragment ions from activated BLM, as indicated by their $m/z$ values of 733.4 and 702.2, respectively, are 2 Da lower in mass than the fragments of the other ferric complexes of BLM. The simplest explanation for this discrepancy is that the oxygen atoms of the peroxide ligand dissociate from activated BLM as water molecules, with the electrons used in reducing the peroxide to $H_2O$ coming from either ferric iron or the BLM molecule. In fact, in tandem mass spectrometric studies of peptides, such losses of water molecules, formed by reduction of a side chain alkyl or acyl hydroxide with concommitant oxidation of another side chain or the peptide backbone, are common.[30] It is, however, important to stress that these data should not be taken as evidence for the formation of a hypervalent form of FeBLM in solution.

(29) Wu, Y.-D.; Houk, K. N.; Valentine, J. S.; Nam, W. *Inorg. Chem.* **1992**, *31*, 718–720.
(30) Tang, X.-J.; Thibault, P.; Boyd, R. K. *Org. Mass Spectrom.* **1993**, *28*, 1047–1052.

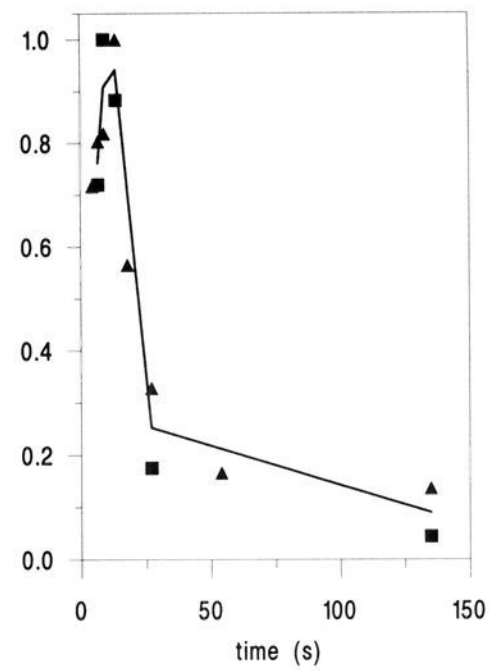


**Figure 5.** Time dependence of the $m/z$ = 751.3 feature produced by reacting Fe(II)BLM with $O_2$. The line indicates the average of the data obtained from two separate experiments, represented by (■) and (▲).

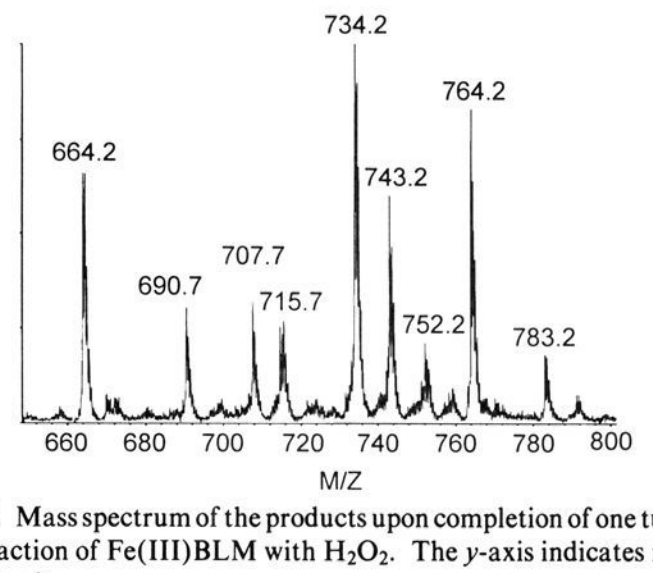


**Figure 6.** Mass spectrum of the products upon completion of one turnover of the reaction of Fe(III)BLM with $H_2O_2$. The *y*-axis indicates relative peak intensity.

## Discussion

The activation of oxygen by FeBLM is commonly considered a model for heme bearing monoxygenases and peroxidases. Accordingly, activated BLM is often formulated as a hypervalent iron intermediate, Fe(V)–$OH_2$ or Fe(IV)–•OH. This was first suggested by the discovery that, like cytochrome P-450, Fe(II)-BLM can be activated by molecular oxygen followed by single electron reduction,[9,11,31] while Fe(III)BLM, again analogous to cytochrome P-450, may be activated by hydrogen peroxide[13] or single oxygen atom donors, such as iodosobenzene.[32] Moreover, activated BLM is capable of catalyzing many of the reactions typical of heme systems, such as olefin epoxidation and N-dealkylation.[33,34] Furthermore, FeBLM is, in many ways, spectroscopically similar to heme proteins; for instance, FeBLM displays EPR and Mössbauer spectroscopic parameters comparable to those of cytochrome P-450.[13,18,35] More recently, Takahashi et al.[25] have characterized FeBLM by resonance Raman spectroscopy. Their results indicate that BLM may function as an electron buffer for Fe, a role proposed for the porphyrin macrocycle in heme proteins, thereby potentially explaining the common reactivity of the structurally unrelated porphyrin and BLM systems.

However, there are several indications that the chemistry of FeBLM deviates from that typical of heme systems. Most

(31) Sausville, E. A.; Peisach, J.; Horwitz, S. B. *Biochem. Biophys. Res. Commun.* **1976**, *17*, 814–822.
(32) Murugesan, N.; Hecht, S. M. *J. Am. Chem. Soc.* **1985**, *107*, 493–500.
(33) Padbury, G.; Sligar, S. S. *J. Biol. Chem.* **1985**, *260*, 7820–7823.
(34) Hecht, S. M. *Acc. Chem. Res.* **1986**, *19*, 383–391.
(35) Sligar, S. G.; Murray, R. I. In *Cytochrome P-450, Structure Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1986; pp 479–503.

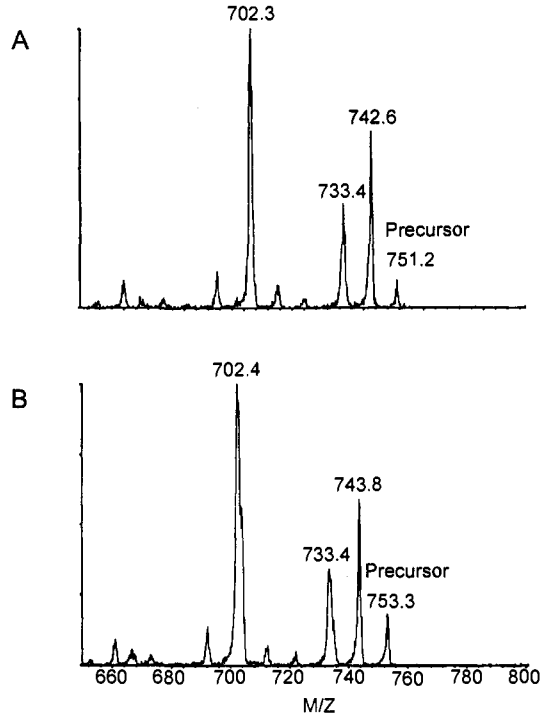


**Figure 7.** Tandem mass spectra of activated BLM produced by reacting Fe(III)BLM with (A) $H_2O_2$ or (B) $H_2{}^{18}O_2$. The precursor ion is indicated in each spectrum. The *y*-axis indicates relative peak intensity.

significant, perhaps, are the results of Burger et al.,[13,18] whoe demonstrated unambiguously, using EPR and Mössbauer spectroscopy, that activated BLM is not a ferryl species but a ferric species with at least one atom of oxygen bound to iron. Hence, it was proposed that activated BLM is either a ferric peroxide or an iron(III) oxene, Fe(III)–$O^0$, in which the oxygen atom is at the oxidation state of molecular oxygen. Furthermore, mechanistic departures from heme chemistry are surfacing from investigations of the degradation of DNA by FeBLM. Through studies primarily by Rabow, et al.,[36,37] it has been shown that FeBLM-mediated DNA degradation resulting in nucleic base release does not occur via a radical rebound mechanism, as postulated by analogy to cytochrome P-450, but instead, through a two-electron oxidation of C4′ of deoxyribose.[38] Similarly, Sugiyama et al.[39] have reported the FeBLM-mediated dehydrogenation of 2′-deoxyaristeromycin, a carbocyclic model of deoxyadenosine. This type of reaction, presumed to involve $2e^-$ oxidation at the C4′ position, is not catalyzed by cytochrome P-450.[39] Thus, it appears that the framework of heme systems and hypervalent iron chemistry are not sufficient for understanding the unique reactivity of FeBLM.

Interestingly, recent studies of several non-heme proteins and model compounds, proposed to involve hypervalent iron intermediates, indicate that the preceding statement is true for these systems as well. Using stopped-flow optical, and rapid freeze–quench EPR and Mössbauer spectroscopy, Bollinger et al.[40] have shown that the dinuclear iron enzyme, ribonucleotide reductase, generates a $\mu$-peroxodiferric intermediate which oxidizes tyrosine, either directly or subsequent to $1e^-$ reduction, to form a tyrosyl radical. More important, perhaps, is that no hypervalent iron species were identified in this study. Similarly, investigations of the mononuclear non-heme iron containing dihydrobenzoate dioxygenases have implicated ferric and ferrous peroxide complexes as the forms of activaged oxygen in the reactions of these enzymes.[41] Again, no expansion of the oxidation state of Fe is indicated in any of the intermediates studied. Finally, several mononuclear iron complexes have been shown to activate oxygen without the formation of hypervalent intermediates. Balasubramanian and Bruice[42,43] report that, in the reaction of *m*-chloroperbenzoic acid with Fe(III)EDTA in $CH_3OH$, the activated species is one in which the oxidizing equivalents reside mainly on the oxygen and not the iron atom, i.e. an iron oxene in which the iron atom is ferric and the oxygen atom is at the oxidation state of atomic oxygen (Fe(III)–$O^0$) and not an Fe(V)–$OH_2$ or Fe(IV)–$^{\bullet}OH$ intermediate, as predicted for the reaction with iron(III) tetraphenylporphyrin. Furthermore, several groups[44,45] have recently shown that the reaction of Fe(II)EDTA with peroxide, the so-called "Fenton reaction", does not produce hydroxyl radicals, as is widely believed, but instead the ferrous peroxide, HOO–Fe(II)EDTA. Similarly, Valentine and coworkers[46,47] have proposed that olefin epoxidations catalyzed by iron–cyclam complexes involve iron hydroperoxides as the activated intermediates. Thus, it appears that many non-heme iron systems which activate oxygen do not form Fe(V) or Fe(IV) intermediates but, instead, produce Fe(II)- and Fe(III)-bound peroxides as the active oxygen species.

To this group of non-heme iron systems we now add activated BLM. The observation, upon activating Fe(III)BLM with $H_2O_2$ or Fe(II)BLM with $O_2$, of an intermediate that (i) has a mass consistent with that of HOO–Fe(III)BLM, (ii) exhibits kinetics of formation and decay similar to those of activated BLM, and (iii) increases in mass by 4 Da when produced with $H_2{}^{18}O_2$, demonstrates conclusively that activated BLM is a ferric peroxide. Furthermore, there was no evidence for the formation of hypervalent iron intermediates.

It has been suggested by Rabow et al.[36] that, although activated BLM may be a ferric peroxide, the true reactive species is "real activated BLM", $O^0$–Fe(III)BLM, a structure originally proposed by Burger et al.,[18] which would result from heterolytic cleavage of the peroxy bond of activated BLM and loss of $^-OH$. We could find no evidence for this species in our ESI–MS experiments. Furthermore, the resonance Raman study of CO–Fe(II)BLM by Takahashi et al.[25] may provide insight as to why the peroxy bond of activated BLM is intact, whereas in the activated forms of the aforementioned heme systems it is cleaved. Numerous studies of CO-bound heme proteins have discovered an inverse linear correlation between $\nu_{Fe-CO}$ and $\nu_{C-O}$ for systems of comparable *trans*-ligand electron donating ability.[48,49] The $\nu_{Fe-CO}$ and $\nu_{C-O}$ of CO–Fe(II)BLM[25] indicate that this complex, like cytochrome oxidase, is best characterized as a weak *trans*-ligand system. Thus, according to the "push–pull" model generally accepted for peroxide cleavage by heme proteins,[50,51] activated BLM, like other weak

(36) Rabow, L. E.; McGall, G. H.; Stubbe, J.; Kozarich, J. W. *J. Am. Chem. Soc.* **1990**, *112*, 3203–3208.
(37) Rabow, L. E.; Stubbe, J.; Kozarich, J. W. *J. Am. Chem. Soc.* **1990**, *112*, 3196–3203.
(38) Sam, J. W.; Peisach, J. *Biochemistry* **1993**, *32*, 1488–1491.
(39) Sugiyama, H.; Sera, T.; Dannoue, Y.; Marumoto, R.; Saito, I. *J. Am. Chem. Soc.* **1991**, *113*, 2290–2295.
(40) Bollinger, J. M., Jr.; Edmondson, D. E.; Huynh, B. H.; Filley, J.; Norton, J. R.; Stubbe, J. *Science* **1991**, *253*, 292–298.
(41) Lipscomb, J. D.; Orville, A. M. In *Metal Ions in Biological Systems*; Sigel, H., Sigel, A., Eds.; Marcel Dekker: New York, 1992; Vol. 28, pp 243–298.
(42) Balasubramanian, P. N.; Bruice, T. C. *J. Am. Chem. Soc.* **1986**, *108*, 5495–5503.
(43) Balasubramanian, P. N.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A* **1987**, *84*, 1734–1738.
(44) Sawyer, D. T.; Kang, C.; Llobet, A.; Redman, C. *J. Am. Chem. Soc.* **1993**, *115*, 5817–5818.
(45) Yamazaki, I.; Piette, L. H. *J. Am. Chem. Soc.* **1991**, *113*, 7588–7593.
(46) Valentine, J. S.; Nam, W.; Ho, R. Y. N. In *The Activation of Dioxygen and Homogeneous Catalytic Oxidation*; Barton, D. H. R., Martell, A. E., Sawyer, D., Eds.; Plenum Publishing Co.: New York, 1993; pp 183–198.
(47) Nam, W.; Ho, R.; Valentine, J. S. *J. Am. Chem. Soc.* **1991**, *113*, 7052–7054.
(48) Yu, N.-T.; Kerr, E. A.; Ward, B.; Chang, C. K. *Biochemistry* **1983**, *22*, 4534–4540.
(49) Nagai, M.; Yoneyama, Y.; Kitagawa, T. *Biochemistry* **1991**, *30*, 6495–6503.
(50) Dawson, J. H. *Science* **1988**, *240*, 433–439.
(51) Poulos, T. L. *Adv. Inorg. Biochem.* **1987**, *7*, 1–36.

*trans*-ligand systems, lacks sufficient electron donating ability to form hypervalent oxoiron intermediates.

However, this argument assumes that the coordinations of Fe in activated and carbon monoxy ferrous BLMs are similar. Indeed, this line of reasoning implies that the stability of the O–O bond of activated BLM requires that the electron donating ability of the *trans*-ligand in activated BLM can be similar to that of CO–Fe(II)BLM. Interestingly, a change in ligand geometry concommitant with the discharging of activated BLM has been recently proposed by Valentine and co-workers.[29] In their proposal, activated BLM can adopt a conformation in which the $\beta$-hydroxyhistidyl imidazole nitrogen atom is the *trans*-ligand and which allows intramolecular hydrogen bonding between the secondary amine of the $\beta$-aminoalanine residue and the remote oxygen of the bound peroxide; thus, in this conformation, the FeBLM molecule may facilitate O–O bond cleavage through an increase in *trans*-ligand electron donation as well as an anchimeric Lewis acid effect. Indeed, that this bond is labile is supported by the present MS/MS experiments which demonstrate cleavage of the peroxy bond of activated BLM by collisional activation.

In summary, we have provided strong evidence for the formulation of activated BLM as HOO–Fe(III)BLM and have indications that the O–O bond of activated BLM is labile. However, we cannot at the present time conclude at what point in the discharging of activated BLM cleavage of the peroxy bond occurs. We are currently performing a time-resolved resonance Raman investigation of the activation of oxygen by FeBLM which may shed light on this intriguing question.

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