EPR Spectroscopic Investigation of the Lability of Oxygen in Activated Bleomycin: Implications for the Mechanism of Bleomycin-Mediated DNA Degradation†

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ABSTRACT: Bleomycin (BLM), an antitumor antibiotic, is capable of degrading DNA through the formation of activated BLM, an activated iron–oxygen complex of the drug with a unique EPR spectrum. A recent study [Rabow, L. E., McGall, G. H., Stubbe, J., & Kozarich, J. W. (1990) *J. Am. Chem. Soc. 112,* 3203–3208] has cast doubt onto the "hydroxyl-radical-rebound" mechanism, commonly accepted for cytochrome P-450 [McMurray, T. J., & Groves, J. T. (1985) in *Cytochrome P-450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P., Ed.) pp 1–28, Plenum, New York] and proposed for the anaerobic nucleic base release reaction of BLM, by demonstrating that the source of oxygen in the products of this reaction is solvent molecules and not molecular oxygen. A central issue in this debate is whether the oxygen of activated BLM is available for exchange with that of solvent. The lability of oxygen in activated BLM has been investigated through the use of EPR spectroscopy to measure the exchange of ^{17}O ($I = 5/2$) between activated BLM and solvent. Evidence for the lack of oxygen exchange between activated BLM and solvent is presented, and the implications of this result for the mechanism of BLM-mediated DNA degradation are discussed.

The bleomycins (BLM) constitute a family of glycopeptide antibiotics originally isolated in 1966 from *Streptomyces verticillus* (Umezawa et al., 1966). Currently, BLM is an important member of our chemotherapeutic armamentarium. The drug is known to bind various transition metal ions, and when complexed with Fe, Cu, or Co, it is capable of activating oxygen and cleaving DNA [for recent reviews, see Stubbe and Kozarich (1987) and Petering et al., 1990)]. At present, the best understood metal–BLM complex is that with iron. Studies of Fe–BLM and the mechanism through which oxygen is activated and DNA is degraded has received much attention due to the usefulness of BLM not only as an antineoplastic agent but also as a model for non-heme iron-mediated oxygen activation.

Oxygen Activation. Through previous stopped-flow optical, EPR, and Mössbauer spectroscopic studies, it has been inferred that the activation of oxygen by BLM proceeds through the formation of a ternary complex with Fe(II) and O_2 (Burger et al., 1979a, 1981, 1983). This complex is then reduced by one electron equivalent, which may be provided by Fe^{II}–BLM (Burger et al., 1981; Kuramochi et al., 1981) or by other reducing agents such as ascorbate or superoxide anion (Sausville et al., 1978a; Ciriolo et al., 1987), giving rise to "activated BLM", an intermediate characterized by a unique EPR spectrum ($g = 2.26, 2.17, 1.94$; see Figure 2A) and the kinetic competence to degrade DNA (Burger et al., 1981). Alternatively, activated BLM may be formed by the addition of various oxidants, such as H_2O_2, to Fe^{III}–BLM (Burger et al., 1981). This reactivity is reminiscent of several proteins bearing a heme prosthetic group, such as horseradish peroxidase, chloroperoxidase, and cytochrome P-450, which are known to, or in the case of cytochrome P-450, are generally accepted to, activate oxygen through the formation of hypervalent iron intermediates (McMurray & Groves, 1985). Thus, activated BLM is commonly envisioned as an iron–oxene complex, BLM–Fe^{V}=O, similar to that proposed for cytochrome P-450 or, by analogy to compound I of horseradish peroxidase, as an Fe^{IV} species with an additional oxidizing equivalent stored at a site other than the iron atom, such as BLM–Fe^{IV}–•OH. However, neither of these structures is in accord with known EPR and Mössbauer spectroscopic studies which indicate that activated BLM is a low-spin ferric species (Burger et al., 1981, 1983). Furthermore, ribonucleotide reductase, another non-heme iron system capable of activating oxygen, had previously been assumed to involve hypervalent iron intermediates but has recently been demonstrated by EPR and Mössbauer studies to proceed through a high-spin ferric species (Bollinger et al., 1991). Thus, we prefer the representation of activated BLM as BLM–Fe^{III}–O_2^{2-} or BLM–Fe^{III}–O, as originally postulated (Burger et al., 1981), but also acknowledge that experimental evidence is sparse regarding the structure of activated BLM and the nature of the two oxidizing equivalents not located at the iron atom.

DNA Degradation. Due to the efforts of numerous research groups, it is now well established that DNA degradation catalyzed by activated BLM proceeds along two pathways (Sausville et al., 1978b; Burger et al., 1980; Giloni et al., 1981; Sugiyama et al., 1985). In one, an oxygen-dependent strand scission takes place with the release of nucleic base propenal. The second is an oxygen-independent reaction which produces free nucleic base and imparts alkali lability to the DNA strand. Furthermore, in the absence of DNA or other substrates, activated BLM undergoes a much slower self-inactivation (Kuramochi et al., 1981; Nakamura & Peisach, 1988; Barr et al., 1990). In all three reactions, activated BLM is discharged to the ferric level of oxidation.

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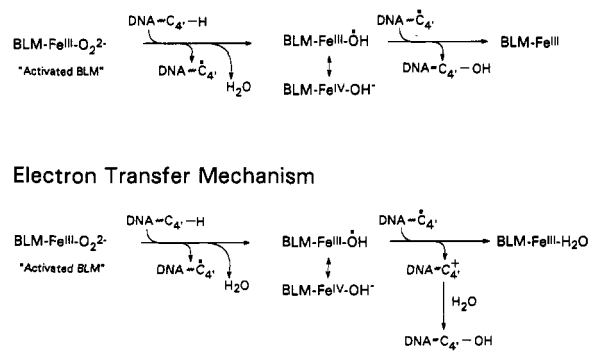

FIGURE 1: Proposed mechanisms for the oxidation of $C_{4'}$ of the deoxyribose moiety in the anaerobic base release reaction of activated BLM-mediated DNA degradation. See text for a description.

Recent studies by Rabow et al. (1990) on the oxygen-independent pathway of DNA degradation have established that the source of oxygen in the 4′ keto moiety of the product derived from the deoxyribose is solvent and not the O_2 initially complexed with Fe^{II}–BLM in the production of activated BLM. This result called into question the prevailing hypothesis for the mechanism of Fe–BLM-mediated DNA degradation, a "hydroxyl-radical-rebound" scheme [see Stubbe and Kozarich (1987)] proposed by analogy to cytochrome P-450 (see Figure 1). In this mechanism, activated BLM abstracts a hydrogen atom from the 4′position of the deoxyribose, generating a carbon-centered radical and BLM–Fe^{III}–•OH, a single-electron-reduced form of activated BLM. This is followed by "rebound" of the nascent hydroxyl radical from the Fe–BLM complex onto the 4′ carbon radical, yielding the C_4–OH product and BLM–Fe^{III}. Instead, Rabow et al. (1990) favored an "electron transfer" mechanism (see Figure 1) in which BLM–Fe^{III}–•OH abstracts a second electron from the 4′ position generating ferric BLM and a 4′-carbonium ion which is subsequently attacked by solvent to yield the $C_{4'}$ hydroxyl group. However, Rabow et al. (1990) acknowledge several alternative interpretations of their data, one of which retains the aforementioned radical-rebound scheme but requires that the oxygen of activated BLM exchange with solvent oxygen.

To clarify the reaction mechanism, we have undertaken a study of the lability of oxygen in activated BLM. Previously Burger et al. (1981) had shown that the EPR spectrum of an activated BLM sample produced with $^{17}O_2$ exhibits broadening of the $g = 2.17$ and $g = 1.94$ features due to the hyperfine interaction with the nuclear spin, $I = 5/2$, of bound ^{17}O. Our present strategy was to activate BLM–Fe^{II} with $^{17}O_2$ in $H_2{}^{17}O$ or $H_2{}^{16}O$ (henceforth referred to as $^{17}O_2/H_2{}^{17}O$ and $^{17}O_2/H_2{}^{16}O$) and compare their EPR spectra with BLM–Fe^{II} samples activated with $^{16}O_2$ in $H_2{}^{17}O$ or $H_2{}^{16}O$ (referred to as $^{16}O_2/H_2{}^{17}O$ and $^{16}O_2/H_2{}^{16}O$). If exchange with solvent oxygen were to occur, one would expect to see narrowing of the spectral features of the $^{17}O_2/H_2{}^{16}O$ sample and broadening of the spectral features of the $^{16}O_2/H_2{}^{17}O$ sample as the reactions progress.

MATERIALS AND METHODS

Materials. Bleomycin sulfate (blenoxane) was generously supplied by Bristol-Myers and was used without further purification. Solutions of the drug were standardized using EPR spectroscopy by titration with Fe^{III} to produce the low-spin complex ($g = 2.45, 2.18, 1.89$) (Burger et al., 1979b). $H_2{}^{17}O$ (36.4 atom %) was purchased from Cambridge Isotope Laboratories and $^{17}O_2$ (43.7 atom %) was a product of Isotec, Inc.

Sample Preparation. Activated BLM samples were prepared by the sequential addition of $Fe(II)(NH_4)_2(SO_4)_2$, BLM, and Na_2HPO_4, pH = 6.9, solutions to $H_2{}^{16}O$ or $H_2{}^{17}O$ and initiated with $^{16}O_2$- or $^{17}O_2$-saturated ethylene glycol. All solutions except the ethylene glycol were sparged with argon and kept on ice at 4 °C. Final concentrations: Fe(II) = 500 μM, BLM = 700 μM, $NaHPO_4$ = 10 mM, ethylene glycol = 50% (v/v); transfers of solutions were accomplished with gas-tight Hamilton syringes. For each solution, a 500-μL aliquot was transferred to a quartz EPR tube and immersed into liquid nitrogen within 30 s of initiation of the reaction. After the EPR spectrum of a reaction mixture was acquired, further time points were obtained by rapidly thawing the sample by immersing the EPR tube in a 80% 2-methylbutane: 20% methylcyclohexane solution kept on ice and then refreezing in liquid nitrogen. This procedure was repeated for each sample over the lifetime of activated BLM as measured by EPR spectroscopy and allowed the observation of 8–12 time points over a period of 6–8 min of thawing time [>80% completion; $t_{1/2}$ for activated BLM at 4 °C is approximately 2 min (Burger et al., 1981)]. It is noteworthy that the conditions and time scale of these reactions are similar to those of standard BLM-mediated DNA degradation assays (Burger et al., 1986). To test for artifactual introduction of oxygen due to vessel leakage or manipulation of the solutions, non-oxygenated controls were thawed to room temperature, allowed to react to completion anaerobically, and assayed for BLM–Fe^{III} by EPR spectroscopy. These controls showed O_2 contamination to be less than 1% (data not shown).

EPR Spectroscopy. EPR spectroscopy was conducted at 77 K on an X-band Varian E112 spectrometer equipped with a Systron-Donner frequency counter and a Varian NMR gaussmeter with a rectangular TE101 cavity at 10-mW power and 10-G modulation amplitude. Control spectra (not shown) were acquired over a range of microwave powers and modulation amplitudes to ensure that the observed broadening was due to neither saturation nor overmodulation of the samples. Peak widths were measured at half-peak height with greater than ±1-G precision. Furthermore, the widths were also assessed at 2/3 peak height to minimize any artifact due to the poorly resolved $g = 1.95$ shoulder in the spectral feature at $g = 1.94$; the extent of broadening as determined by this method was identical to the more conventional width at half-height measurement.

RESULTS

EPR spectra of activated BLM and the g_{min} features of the four reactions are shown in Figure 2. The width at half-height of the $g = 1.94$ feature of the $^{16}O_2/H_2{}^{16}O$ sample was 20 G, whereas that of the $^{16}O_2/H_2{}^{17}O$ sample was 21 G.[1] In those samples activated with $^{17}O_2$, the $^{17}O_2/H_2{}^{16}O$ sample displayed a peak width of 24 G compared to 25 G for the doubly labeled $^{17}O_2/H_2{}^{17}O$ sample. More important, however, was the finding that these peak widths were constant within experimental error over the lifetime of activated BLM in all

[1] We excluded the $g = 2.17$ peak from our analysis since trace amounts of low-spin Fe^{III}–BLM ($g = 2.45, 2.18, 1.89$), i.e., not converted to the high-spin state ($g = 4.3$) by the presence of phosphate buffer (Burger et al., 1981), would result in artificial broadening of the $g = 2.17$ peak width due to spectral overlap. In contrast, the g_{min} features of the activated and low-spin ferric BLM EPR spectra are sufficiently removed from one another that the $g = 1.94$ feature of the activated drug complex can be resolved without complication.

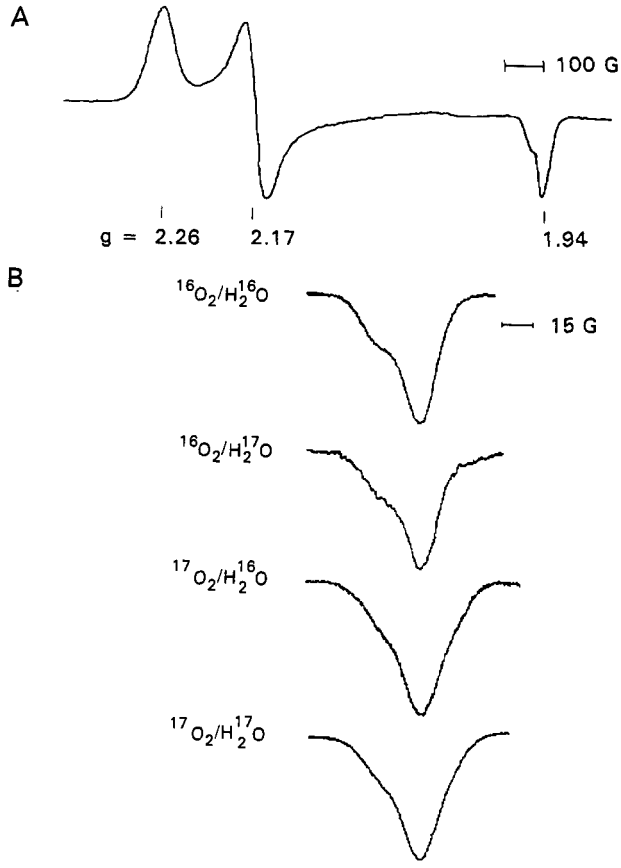

FIGURE 2: (A) X-band EPR spectrum of activated BLM. Fe^{II}–BLM activated with $^{16}O_2$ in $H_2{}^{16}O$. (B) EPR spectral features of activated BLM at g_{min}. Activation with $^{17}O_2$ or $^{16}O_2$ in $H_2{}^{17}O$ or $H_2{}^{16}O$ are as indicated. In both (A) and (B), EPR spectroscopy and sample preparation were performed as described in the text.

samples studied (data not shown). These results provide strong evidence that bound oxygen in activated BLM does not exchange with solvent.

DISCUSSION

We have acquired EPR spectra of BLM–Fe^{II} activated with and without ^{17}O-labeled molecular oxygen in solutions made with and without ^{17}O-labeled water. In the samples produced in $H_2{}^{17}O$, we observed minor broadening of the $g = 1.94$ feature by 1 G, reasonably due to a dipolar interaction with the nuclear spin of unbound solvent. When the samples were activated with $^{17}O_2$, we observed a broadening of 4 G presumably due to the hyperfine interaction with ^{17}O directly coordinated to activated BLM, and with the doubly labeled sample we observed a broadening of 5 G, the sum of these two effects. Furthermore, these values did not vary throughout the lifetimes of the activated BLM samples. We therefore conclude that exchange does not occur between oxygen bound to activated BLM and that of solvent.

The activation of oxygen by Fe–BLM is commonly conceptualized within the framework of hypervalent iron intermediates and the "hydroxyl-radical-rebound" mechanism for cytochrome P-450. The studies of Rabow et al. (1990) have cast considerable doubt onto this mechanism as applied to Fe–BLM-mediated DNA degradation. However, the "hydroxyl-radical-rebound" mechanism could still be operative if the oxygen of activated BLM were available for exchange with that of solvent. Previous studies by Burger et al. (1981) are often cited as evidence for the lack of oxygen exchange in activated BLM. Yet, these studies reported only the broadening of the EPR signal of activated BLM produced initially with $^{17}O_2$ and frozen immediately after activation; the kinetics of oxygen exchange were not addressed. Another study frequently referred to in discussions of oxygen exchange by activated BLM is that by Heimbrook et al. (1987), who reported the production of ^{18}O-labeled *cis*-stilbene oxide from Fe^{III}–BLM reacted with $H_2{}^{18}O_2$ for 1 h at room temperature. However, it remains unknown whether the ^{18}O was derived from activated BLM or subsequent O_2-mediated chemistry; furthermore, the lifetimes of the intermediates involved in this reaction are unknown, and therefore it is difficult to propose a rate of oxygen exchange. To our knowledge the experiments described in this present study are the first to conclusively show the lack of oxygen exchange by activated BLM.

However, arguments supporting the "hydroxyl-radical-rebound" mechanism may still be maintained by postulating a BLM intermediate subsequent to activated BLM which can exchange oxygen with solvent. Rabow et al. (1990) proposed that a "real activated BLM", BLM–Fe^{III}–O, might be produced from activated BLM, envisioned in their scheme as the ferric peroxide species, through heterolytic cleavage of the oxygen–oxygen bond and that this species undergoes exchange with solvent. We consider this unlikely since the so-called "real activated BLM" should be EPR active and despite the numerous EPR studies of activated BLM, no evidence for an intermediate produced subsequent to activated BLM has to our knowledge been noted.

It may also be argued that the oxygen of a proposed single electron reduced form of activated BLM, BLM–Fe^{III}–($^{\bullet}$OH), is labile. Again, we tend to disfavor this possibility since this intermediate has never been observed, and if it does exist, it must be short-lived, given that the initial hydrogen atom abstraction by activated BLM is largely rate determining as shown by the primary tritium kinetic isotope effect experiments of Wu et al. (1985). Hence, to maintain the radical-rebound scheme, one would have to postulate that this intermediate exchanges oxygen with solvent at a rate several orders of magnitude greater than activated BLM.

The remaining mechanisms for BLM-mediated DNA degradation are notable in that they involve a two-electron oxidation at the $C_{4'}$ position. Precedence for the ability of activated BLM to carry out two-electron in addition to single-electron oxidations is provided by studies by Burger et al. (1985), who demonstrated the approximately stoichiometric titration of activated BLM with the obligate two electron reducing agents, NADH and thio-NADH, and the work of Sugiyama et al. (1991) which reports a BLM-mediated dehydrogenation of a carbocyclic DNA analog, proposed to proceed through a 4′-carbonium ion intermediate which loses the 6′-proton to generate a cyclopentene.

In conclusion, we believe that the experiments reported in this paper provide strong evidence that the oxygen in activated BLM is not available for exchange with solvent. Thus, assuming that the oxidizing species of the 4′ position in DNA is in fact activated BLM and not another transient and heretofore undetected intermediate, and that the kinetics of oxygen exchange are the same in the absence of DNA as in its presence, we contend that the radical rebound mechanism of BLM-mediated DNA degradation may not be operative.

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