The Redox State of Activated Bleomycin*

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Richard M. Burger, a John S. Blanchard, b
Susan Band Horwitz, c and Jack Peisach d

From the Departments of *Molecular Pharmacology,
*Biochemistry, *Cell Biology, and *Molecular Biology, Albert
Einstein College of Medicine, Bronx, New York 10461

Activated bleomycin appears to have two more oxidizing equivalents than the Fe(III) • bleomycin to which it spontaneously decays. Activated bleomycin reacts with NADH and thio-NADH, two-electron reductants, and with KI, a one-electron reductant, to yield Fe(III) • bleomycin. The observed stoichiometries were 0.85 ± 0.07 eq of thio-NADH oxidized or 1.5 ± 0.25 eq of KI oxidized per mole of activated bleomycin. None of these reactions requires the presence of a redox mediator, as does the reduction of Fe(III) • bleomycin by NADH or thio-NADH. The oxidations of both pyridine nucleotide coenzymes and of KI are inhibited by DNA, the usual bleomycin target.

The bleomycins are members of a family of structurally similar glycopeptide antibiotics that oxidatively cleave DNA (1–3). Efficient DNA-cleaving activity in vitro (4) results from drug complexation with Fe(II) or Fe(III) (5) followed by activation with O2 or peroxides, respectively (6). The activated form of the drug, termed “activated BLM” (6), releases free nucleic bases (7, 8) and base propenals (9, 10) from DNA, the postulated structure of the oxyferrous precursor (11), and at least one atom of oxygen derived from O2. Considering the postulated structure of the oxyferrous precursor (14), which is either O2• Fe(II) • BLM or its preferred resonance form, O2• - Fe(III) • BLM (19), and assuming that the metal ion and its ligands are the only redox-active centers in the molecule, it has been inferred that activated BLM possesses two more oxidizing equivalents than Fe(III). Thus it is at the same redox potential level, V, as compounds 1 of horseradish and chine

O2• Fe(II) • BLM + Fe(II) • BLM → Fe(III) • BLM + activated BLM

Activated BLM is the species kinetically competent to attack DNA (6), and there is evidence that this attack begins with a hydrogen atom abstraction at deoxyribose carbon-4 (16). Plausible mechanisms have been proposed to account for the subsequent release of free nucleic base or base propenal following the insertion of either a hydroxyl radical or of O2, respectively, at the site of the initial attack (16–18).

The structure of activated BLM has not been unequivocally proven, although Mössbauer (19) and EPR studies with 57Fe and 17O (6) demonstrate that it contains low spin ferric iron and at least one atom of oxygen derived from O2. Considering the postulated structure of the oxyferrous precursor (14), which is either O2• Fe(II) • BLM or its preferred resonance form, O2• - Fe(III) • BLM (19), and assuming that the metal ion and its ligands are the only redox-active centers in the molecule, it has been inferred that activated BLM possesses two more oxidizing equivalents than Fe(III). Thus it is at the same redox potential level, V, as compounds 1 of horseradish and chine

The redox state of activated bleomycin has been demonstrated, involving a series of one-electron redox reactions analogous to those for cytchrome P-450 (12). Fe(III) • BLM, which is stable, can be reduced to Fe(II) • BLM by a single-electron reaction (E° = 129 mV (13)). Fe(II) • BLM reacts rapidly with O2 to form O2• - Fe(II) • BLM, an EPR-silent complex (14). This soon reacts to yield a 1:1 mixture of activated BLM and Fe(III) • BLM (15). A one-electron reduction of O2• - Fe(II) • BLM by Fe(II) • BLM has been proposed as the mechanism for this step (15).

In this present study, we report both one- and two-electron oxidations carried out by activated BLM, using KI, NADH, and thio-NADH as reductants. We demonstrate the discharge of activated BLM, leading to the formation of Fe(III) • BLM, by thio-NADH, a two-electron reductant, with a stoichiometry approaching one, which reinforces the hypothesis that activated BLM is at oxidation level V. We further show that the oxidation of iodide and thio-NADH are strongly inhibited in the presence of DNA, suggesting that DNA either alters the redox potential of activated BLM or renders the iron-nitrogen complex inaccessible to the reductants.

EXPERIMENTAL PROCEDURES

Materials—BLM sulfate (Blenoxane), a gift of Bristol Laboratories, was dissolved in water and standardized by means of its optical extinction, ε290 = 1.7 × 104 M–1 cm–1, based on a titration with Fe(II) (21). Solutions of Fe(II) • (NH4)2(SO4) • 6H2O were prepared daily and diluted with 10 mM H2SO4. Fe(III) • BLM was prepared by mixing 0.1 M Fe(III) • (NH4)2(SO4) • 6H2O with 0.10 mM BLM, washing 2 min, and adding sufficient Hepes buffer, pH 7.0, to neutralize and dilute to the desired concentration. Solutions of NADH (Sigma) were prepared immediately before use.

Thio-NADH was prepared from thio-NAD (Sigma) by enzymatic reduction with 80 mM ethanol in the presence of alcohol dehydrogenase (Boehringer Mannheim), aldehyde dehydrogenase (Boehringer Mannheim), 20 mM KCl, 0.1 mM dithiothreitol, and 20 mM Tris buffer, pH 8.5. The enzymes were removed by ultrafiltration, and the reaction mixture was chromatographed on a Pharmacia Mono Q anion-exchange column as described by Orr and Blanchard (22). The thio-NADH concentration was determined optically before each experiment, using ε290 = 1.13 × 104 M–1 cm–1 (23). For control experiments, the thio-NADH preparation in 0.1 M Hepes buffer, pH 7.0, was oxidized by 20 mM pyruvate in the presence of lactate dehydrogenase (Sigma).

Potassium ferricyanide (Fluka, parvis pro analysis grade) and water-soluble starch (Baker) were used to iodimetrically standardize a sodium thiostulate solution as described by Klothoff and Sandell (24), adapted to a 100-fold reduction in volume. The thiostulate was used to standardize a solution of 4.7 mM I2 in 24 mM KI (24), and this was used to calibrate the assay of I2 produced from KI by activated BLM. EPR Spectrometry—Reaction mixtures contained 0.2 mM Fe(II), 0.4 mM BLM, and 20 mM Hepes or sodium phosphate buffer, pH 7.0. In some experiments 1 mM DNA, 60 mM KI, or both, or 1 mM NADH,
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20 mM phenazine methosulfate, or both were added. Reactions were run at 4 °C with redox reagents added either before the Fe(II), 10 s afterward (when drug activation was complete (6)), or not at all. All reaction mixtures received 1 volume of ethylene glycol 40 s after Fe(II) addition, were then transferred to quartz EPR tubes, and immersed in liquid nitrogen at 1 min. Fe(III)-BLM and activated BLM were quantitated by double integration of EPR spectra as previously described (6).

**DNA Scission by BLM**—The single-turnover DNA-scission activities of Fe(II)/BLM reaction mixtures were measured at various times by adding DNA and permitting them to react to completion during 25 min at 4 °C. Reaction mixtures initially contained 0.45 mM BLM, 17 mM Hepes, or sodium phosphate buffer, pH 7.0, followed by 0.3 mM Fe(II) to initiate the reaction. After 15 s, some mixtures received small volumes of redox reagents such as NADH or thio-NADH, or the latter two with or without phenazine methosulfate. At subsequent times, 30-μl aliquots of these mixtures were added to 170 μl of 0.5 mM DNA in the same buffer. The final yield of base propenal, the stoichiometric product of bleomycin-induced DNA scission (11), was then assayed spectrophotometrically with 2-thiobarbituric acid as previously described (6).

The rates of DNA scission by Fe(III)-BLM reaction mixtures supplemented by additions of NADH and/or phenazine methosulfate were measured by adding DNA after 10 s, incubating at 25 °C for 15 to 60 min, and determining the rate of base propenal production (6).

**Iodimetry**—The oxidation of KI to I₂ by activated BLM was measured spectrophotometrically with stachar as indicator. Drug reaction mixtures (200 μl) containing 200 mM Fe(II), 300 μM BLM, and 20 mM Hepes or sodium phosphate buffers (pH 7.0) were added to 400 μl of 50 mM KI, 15 mM I₂, followed after 10 s by 50 μl of 0.3% soluble starch. The starchiodine absorption was monitored after 1 min at 575 nm in a 1-cm pathlength cuvette. The method was calibrated by substituting for the reaction mixture 200 μl of buffer plus I₂ solution, and titrating such mixtures with small volumes of Na₂S₂O₃. The dependence of Aₑ₆₆₅ on I₂ was 3.25 × 10⁻⁴ M⁻¹ cm⁻¹ and fulfilled Beer's law within the absorbance unit range of 0.1 to 0.7.

**Stoichiometry of Activated BLM and Thio-NADH Consumption**—For stoichiometry determinations, thio-NADH was added to Fe(II) initially supplied when BLM is in excess (6,21). Since activated BLM had decayed spontaneously and was no longer available for reduction, the redox mediator such as phenazine methosulfate (Fe(II)-bleomycin unless one provides a reductively recycled and that a sufficient excess of reductant is available to react with the activated BLM. In these experiments no more than 15% of the reductant was oxidized upon completion of the reaction with activated BLM. At concentrations of thio-NADH and NADH below 1 mM, Fe(III)-BLM is not significantly recycled via Fe(II)-bleomycin unless one provides a redox mediator such as phenazine methosulfate (Fig. 1 and Table I) or NADPH cytochrome P-450 reductase (28). When thio-NADH oxidation was assayed, 0.32 mM was added to reaction mixtures 15 s after the Fe(II). By this time, 6% of the activated BLM had decayed spontaneously and was no longer available for reduction, since the rates of its formation and decay in the absence of pyridine nucleotide coenzymes were respectively 30 (14) and 0.3 min⁻¹ (Fig. 2). Of the remaining activated BLM, 34% was reduced by the 0.32 mM thio-NADH, since this amount of thio-NADH increased the rate of decay to 0.74/0.49 min⁻¹ in a controlled reaction containing (thio-NADH instead: 0.74 - 0.49)/0.74 = 0.34.

**RESULTS AND DISCUSSION**

The activation of Fe(II)-BLM with O₂ leads to the formation of equal amounts of activated BLM and Fe(III)-BLM (Equation 1). The latter in phosphate buffer is an S = 5/2 species with a prominent EPR feature near g = 4.3 (15, 25).

Activated BLM decays to Fe(III)-BLM within minutes at 4 °C. When KI, a one-electron reductant, is added to activated BLM, I₂ is formed, and all the activated BLM is converted within seconds to Fe(II)-BLM (Fig. 1D). This reaction with KI is inhibited 90% by DNA. In control experiments, neither KI nor I₂ reacted with Fe(III)-BLM.

From the amount of I₂ formed, we sought to obtain a noninferential measurement of the redox state of activated BLM. In a series of kinetic experiments, the ability of BLM reaction mixtures to oxidize KI was found to decline in parallel with the amount of activated BLM present, but in six experiments the stoichiometry, extrapolated to the time of drug activation, was 1.5 ± 0.25 eq of KI oxidized per activated BLM reduced. Such an outcome could result if the one-electron reduction of activated BLM by KI formed an unstable drug intermediate which could yield Fe(III)-BLM either by a prompt disproportionation or by reacting with a second I⁻ equivalent. Such an intermediate would be EPR silent, but the net loss of EPR activity would be difficult to detect unless the intermediate constitutes a significant proportion of the iron-BLM species. The quantitative and rapid conversion of activated BLM to Fe(III)-BLM is evident in Fig. 1 spectra A and B. The reaction is complete when observed shortly after KI addition, which suggests that an intermediate must be short-lived if it exists at all, so this hypothesis was not further investigated.

The observation that activated BLM oxidizes more than 1 eq of KI prompted us to try the two-electron reductants, NADH and thio-NADH. The EPR spectrum C in Fig. 1 shows that NADH, like KI, converts activated BLM to high spin Fe(III)-BLM, but more slowly. The possibility that NADH or thio-NADH might reduce the product Fe(III)-BLM to Fe(II)-BLM, and thus initiate additional cycles of drug activation by reaction with O₂, was ruled out by control experiments. Table I shows that Fe(III)-BLM, which is inactive in DNA scission, remains practically inactive in the presence of NADH. This occurs even though NADH has a sufficiently low redox potential to reduce Fe(III)-BLM. The addition of phenazine methosulfate in substoichiometric quantities to iron-BLM reaction mixtures containing NADH converts essentially all the BLM to activated BLM (Fig. 1D) and dramatically enhances the scission of DNA (Table I). This is because phenazine methosulfate acting as a redox mediator permits the NADH to furnish single electrons to Fe(III)-BLM. The Fe(II)-BLM thus produced reacts promptly with O₂ and goes on to form more activated BLM as shown in Table I.
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**Figure 1.** Effects of reductants on activated BLM and Fe(III)-BLM. These EPR spectra were obtained from reaction mixtures originally containing Fe(III)-BLM and frozen in liquid nitrogen after incubation for 1 min at 4°C. Samples were prepared as described under “Experimental Procedures,” with the indicated additions made at 0 s: A, no addition; B, 50 mM KI; C, 1 mM NADH; D, 1 mM NADH plus 20 μM phenazine methosulfate (PMS). The EPR species seen here are activated BLM (g = 2.26, 2.17, and 1.94 (6, 26)) and Fe(III)-BLM, which exists in an equilibrium mixture of high spin (g = 4.3) and low spin (g = 2.42, 2.18, and 1.89) forms. The presence of phosphate buffer favors the high spin form, and the presence of iodide shifts the g = 4.3 feature of low spin Fe(III)-BLM upfield from its usual position (g = 2.45, not shown) to g = 2.42. When samples A and C were thawed for periods of 1–6 min at 0°C and refrozen, the activated BLM signals were found to decay at rates of 0.3 min⁻¹ in the presence of NADH and 0.2 min⁻¹ in its absence.

Equation 1. In the absence of a redox mediator the only constituent of the iron/BLM reaction mixture capable of reacting with NADH or thio-NADH is activated BLM.

When NADH or thio-NADH is added to Fe(II)-BLM mixtures without a redox mediator, the pseudo-first order decay of DNA scission activity is accelerated as activated bleomycin is reduced. This is shown in Fig. 2 wherein the effect of thio-NADH is compared to that of its oxidation product, thio-NAD. Although thio-NAD, like other phosphate-containing compounds (21), accelerates the spontaneous decay of activated bleomycin, reduction with the same concentration of thio-NADH results in a much greater acceleration. Thus the spontaneous decay rate of bleomycin activity, 0.31 s⁻¹, is increased to 0.44 s⁻¹ by 0.5 mM thio-NAD, and to 0.92 s⁻¹ by 0.5 mM thio-NADH. This greater effect of the thio-NADH is nullified by prior enzymatic oxidation with pyruvate in the lactate dehydrogenase reaction (data not shown), so this effect is due to the redox activity of thio-NADH and not to some contaminant of the preparation. Although thio-NAD is used as a control to estimate the nonreductive effect of thio-NADH in activating accelerated BLM decay, it is possible that the reduced and oxidized coenzymes may differ slightly in this effect despite their close structural similarity. The stoichiometry calculation for thio-NADH oxidation described under “Experimental Procedures” is subject to this uncertainty and must to this extent be considered an approximation.

Activated BLM oxidizes thio-NADH more slowly than it does KI, so it was possible to compare the rate of thio-NADH oxidation, measured spectrophotometrically, to the rate of BLM activity loss seen in drug reaction mixtures containing thio-NADH, as measured by base propenal formation. Fig. 2 shows that the reduction of activated bleomycin by 0.5 mM thio-NADH more than doubles the rate of loss of drug activity. We were able to demonstrate equivalent kinetics of drug activity destruction (Fig. 2) and thio-NADH oxidation (Fig. 3). The oxidation of thio-NADH was observed optically during all but the initial mixing period of the reaction (Fig. 3). Mixtures lacking DNA oxidize about four times the thio-NADH consumed by otherwise identical mixtures containing 2 mM DNA (not shown). The kinetics of oxidation are pseudofirst order (Fig. 3, right panel), and extrapolating to the time of thio-NADH addition indicates that 37 μM was oxidized in a reaction mixture receiving 300 μM Fe(II), and 22 μM was oxidized in another mixture receiving 150 μM Fe(II).

The proportion of activated BLM reacting with thio-NADH rather than undergoing spontaneous decay was calculated as described under “Experimental Procedures” from the data on acceleration of decay shown in Fig. 2. This proportion is 34% of the activated bleomycin present when thio-NADH was added, which in 0.34 × 0.94 × 150 μM = 47 μM, and 0.34 × 0.94 × 75 μM = 24 μM, respectively. Thus, the observed stoichiometric ratio of thio-NADH oxidation to activated BLM reduction is 0.79 and 0.92, respectively, based on the amount of Fe(II) used to generate the activated bleomycin. In electron equivalents per activated bleomycin these are 1.6 and 1.8, respectively, comparable to the amount obtained by KI oxidation (1.5 ± 0.25) but also less than two. These values of more than one, taken together with the ability of thio-NADH without a redox mediator to participate in the reaction

Activated BLM + thio-NADH → Fe(III)-BLM + thio-NAD²⁻ (2)

Table I

<table>
<thead>
<tr>
<th>Addition</th>
<th>Initial rate of base propenal production</th>
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<tr>
<td>None</td>
<td>None</td>
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<tr>
<td>0.3 mM NADH</td>
<td>0.03</td>
</tr>
<tr>
<td>0.01 mM phenazine methosulfate</td>
<td>0.2</td>
</tr>
<tr>
<td>Both NADH and phenazine methosulfate</td>
<td>1200</td>
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**Table I**

Enhancement of Fe(III)-BLM activity by NADH and phenazine methosulfate

Reaction mixtures (200 μl) contained 0.05 mM Fe(III)-BLM plus 0.02 mM BLM and 20 mM Hepes buffer, pH 7.0, with or without 0.01 mM phenazine methosulfate, and with 0.4 mM DNA added last. Where indicated, 0.3 mM NADH was added 10 s before the DNA. Incubations were conducted for 15 s to 1 h at 25°C and terminated by the addition of the 2-thiobarbituric acid reagent for the assay of base propenal (6).
in two successive one-electron reactions such as those proposed to account for the deoxyribose C-4 hydroxylation of DNA (16, 17). The initial hydrogen atom abstraction, yielding the C-4 radical, would reduce the drug to a one-electron oxidizing species. This could then react with the sugar radical by radical recombination to yield Fe(III)-BLM and a 4'-hydroxylated deoxyribose, in a manner analogous to the mechanisms proposed for hydroxylations mediated by cytochrome P-450 (12) and butyrobetaine hydroxylase (29). Should this second reaction fail to occur, it would leave the deoxyribose radical available for attack by O₂, leading to DNA scission and base propenal release (16, 17). These hypotheses are consistent with the finding that free base production is independent of O₂ once BLM has been activated, but that additional O₂ is necessary for base propenal formation (6, 18).

REFERENCES

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