Reaction of DNA-Bound Ferrous Bleomycin with Dioxygen: Activation versus Stabilization of Dioxygen†

Patricia Fulmer and David H. Petering*

Department of Chemistry, University of Wisconsin—Milwaukee, Milwaukee, Wisconsin 53201

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ABSTRACT: The properties of binding of dioxygen to ferrous bleomycin [Fe(II)Blm] in the presence of DNA have been examined. Dioxygen reacts rapidly with Fe(II)Blm·DNA, forming an adduct that is increasingly stable to oxidation–reduction as the ratio of base pairs to drug becomes larger. This species has a spectrum similar to that reported for a proposed dioxygenated intermediate in the solution reaction of Fe(II)Blm with O_2. It contains Fe(II) as measured with bathophenanthroline disulfonate (BPS) and O_2 according to direct observation of stoichiometric release of O_2 upon chelation of Fe(II) by BPS. The dioxygenated form, O_2-Fe(II)Blm·DNA, is highly stable; bound O_2 dissociates upon purging with N_2 with a rate constant of 0.16 min^{-1}. Although Fe(II)Blm or Fe(II)Blm·DNA reacts almost completely with BPS within the time of mixing, O_2-Fe(II)Blm·DNA reacts much slower in a process that is first order in Fe(II) and in BPS at constant DNA concentration. The rate is inversely related to DNA concentration, reaching a limiting value at 50–100 base pairs per drug molecule. The kinetics of oxidation of Fe(II)Blm bound to DNA by O_2 are biphasic and depend on the base pair to drug ratio. After formation of O_2-Fe(II)Blm·DNA, another reaction occurs in which O_2 is released, which is second order in dioxygen donor, and in which the second-order rate constant declines with increasing base pairs to FeBlm ratio. The rate of this second-order process accelerates at higher ionic strength. DNA strand scission as measured by base–propenal formation or base release also decreases as the ratio of base pairs to drug becomes larger, with the second-order rate constants for malondialdehyde formation similar to those for dioxygen release from DNA-bound drug.

The antitumor glycopeptide, bleomycin, causes single- and double-strand cleavage of DNA (Byrnes *et al.*, 1990; Petering *et al.*, 1990). The latter is closely correlated with inhibition of cell proliferation of cancer cells. Chemical studies of the mechanism of strand cleavage originally identified iron as a necessary cofactor for this process (Sausville *et al.*, 1978a,b). The drug contains two domains: One binds metal ions and is comprised of a set of nitrogen ligands (Figure 1); the second contains the bithiazole group and a variable, positively charged tail that can bind to DNA. The two are linked through a series of peptides. Thus, a mechanism of efficient DNA damage has been envisioned in which interaction of the second domain with DNA brings the iron complex of the drug in close proximity to DNA to carry out DNA damage in a redox mechanism involving the reduction of dioxygen to species equivalent to hydroxyl radicals (Petering *et al.*, 1990).

According to previous work, DNA damage can be initiated by peroxy-Fe(III)Blm[1] or an equivalent species which is generated from Fe(II)Blm or from Fe(III)Blm and H_2O_2 (Burger *et al.*, 1981):

$$2\text{Fe(II)Blm} + O_2 + H^+ \rightarrow HO_2\text{-Fe(III)Blm} + \text{Fe(III)Blm} \quad (1a)$$

$$\text{Fe(III)Blm} + H_2O_2 \rightarrow HO_2\text{-Fe(III)Blm} + H^+ \quad (1b)$$

The peroxy-Fe(III) product then reacts with the DNA backbone and is thought to generate a C4′ carbon radical on deoxyribose that undergoes further reaction to produce strand cleavage (Wu *et al.*, 1983, 1985) (Figure 2). It has also been shown that Cu(I) can substitute for Fe(II) in the activation of Blm to cause DNA damage (Ehrenfield *et al.*, 1987). Nevertheless, the importance of the iron-chelated form of the drug for the cellular mechanism of action of bleomycin has been established by the recent observations that its activity is reduced or abolished in iron-deficient cells and can be restored by the use of Fe(III)Blm but not Cu(II)Blm (Radtke *et al.*, 1991).

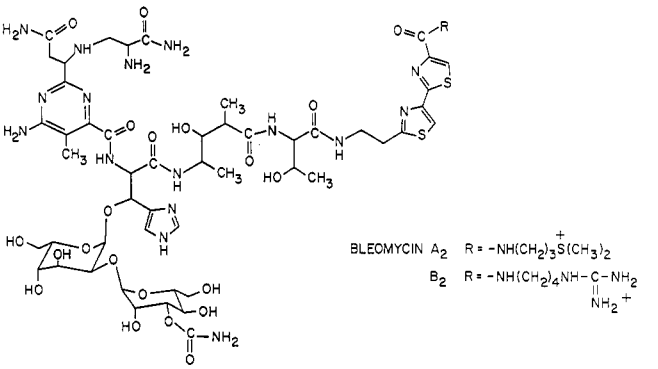

FIGURE 1: Structural bleomycin A_2 and B_2.

An understanding of the detailed mechanism of DNA cleavage by iron bleomycin is of interest because of the common use of this drug in tumor therapy. It is also important because Blm has served as the model DNA cleavage agent for the development of synthetic chemical nucleases that contain ligand binding sites for redox active metal ions tethered to DNA binding domains (Dervan, 1986; Sigman & Chen, 1989).

Several facets of the reaction of FeBlm with DNA particularly attract further study. First, FeBlm can carry out

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* Author to whom correspondence should be addressed.

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[1] Abbreviations: Blm, the clinical mixture of bleomycins containing mostly Blm A_2 and Blm B_2; MDA, malondialdehyde; TBA, thiobarbituric acid.

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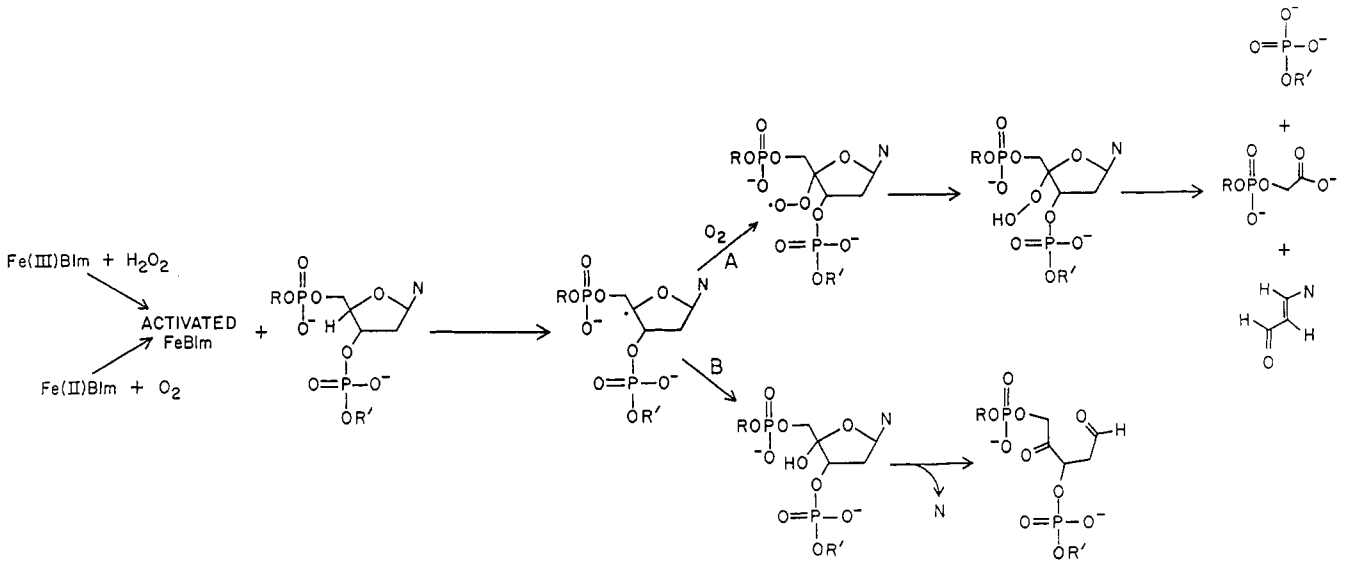

FIGURE 2: Proposed mechanisms of FeBlm-induced damage to DNA (Worth *et al.*, 1993).

efficient double-strand scission despite its appearance as a monofunctional DNA cleavage compound having only one iron site at which to activate dioxygen (Byrnes *et al.*, 1990; Steigner & Povirk, 1990). Second, the iron site activates dioxygen with little production of diffusible radicals so that there are specific sites of cleavage instead of a distribution of neighboring sites as occurs with the EDTA-based chemical nuclease molecules (Dervan, 1986; Rodriguez & Hecht, 1982). Third, as noted previously with FeBlm and as described below, the redox behavior of metallobleomycins is substantially altered by adduct formation with DNA (Albertini *et al.*, 1982).

To explore facets for the activation process described by reaction 1a, Co(II)Blm has been substituted for Fe(II)Blm (Xu *et al.*, 1992a,b). It reacts with dioxygen with the same overall reaction as the iron complex, yielding stable products that do not degrade DNA or undergo self-degradation. Furthermore, the reaction kinetics are slower and amenable to detailed examination. In summary, oxidation–reduction occurs according to the following series of reactions:

$$\text{Co(II)Blm} + O_2 \rightleftharpoons O_2\text{-Co(II)Blm} \quad (2)$$

$$2O_2\text{-Co(II)Blm} \rightleftharpoons \text{BlmCo(II)-}O_2\text{-Co(II)Blm} + O_2 \quad (3)$$

$$\text{BlmCo(II)-}O_2\text{-Co(II)Blm} + H^+ \rightleftharpoons HO_2\text{-Co(III)Blm} + \text{Co(III)Blm} \quad (4)$$

When DNA was present, the rate of the overall reaction was slower and highly sensitive to the ratio of base pairs to Co(II)Blm; above a ratio of 3–4, the rate of reaction 3 declined precipitiously and O_2-Co(II)Blm was stabilized (Xu *et al.*, 1992b).

On the basis of these findings, it was hypothesized that FeBlm can participate in a series of reactions analogous to reactions 2–4 to form the activated species, HO_2-Fe(III)Blm, that is competent to initiate DNA cleavage. According to this scheme, O_2-Fe(II)Blm can be stabilized at large DNA to drug ratios, thereby preventing activation of dioxygen to cleave the DNA polymer.

The present investigation provides evidence for the operation of pathway 2–4 for the reaction of Fe(II)Blm bound to DNA with O_2 and for the stabilization of O_2-Fe(II)Blm·DNA at large ratios of DNA to base pairs. In addition, it describes some of the properties of the O_2-Fe(II)Blm·DNA adduct that must be reduced to form the activated form of FeBlm. As with the investigation of the reactions of Co(II)Blm, O_2, and DNA, the earlier observations of Albertini and Garnier-Suillerot on the reaction of Fe(II)Blm, DNA, and O_2, which indicated a stabilization of Fe(II)Blm in the presence of DNA, have provided a useful starting point (Albertini *et al.*, 1982).

EXPERIMENTAL PROCEDURES

Bleomycin sulfate (Blenoxane) was supplied by Bristol Laboratories, Syracuse, NY; it contains approximately 70% bleomycin A_2 and 30% bleomycin B_2. Calf thymus DNA was purchased from P-L Biochemicals, Inc. Bathophenanthrolinedisulfonic acid, disodium salt hydrate, and 1,10-phenanthroline were purchased from Aldrich Chemical Co. Fe-$(NH_4)_2(SO_4)_2$·$6H_2O$ from Mallinckrodt Chemical Co. was of reagent grade.

All plastic and glassware were washed with Alconox, then soaked in 10% nitric acid or 30 mM EDTA for approximately 1 day to remove any trace metals, and finally rinsed in deionized, glass-distilled H_2O. Both stock solutions were stored at 4 °C. All other stock solutions were prepared in glass-distilled water shortly before use.

UV–Visible Spectroscopy. All kinetic data were collected on a Hewlett-Packard 8451A diode array spectrophotometer at 25 °C, unless otherwise indicated. Spectral scans were stored for later examination on disk at desired time intervals using the computer program TIMESCAN.

Anaerobic Spectrum of Fe(II)Blm in the Presence of DNA. An anaerobic spectrum was taken of Fe(II)Blm in the presence of DNA. A heated catalyst was used to remove O_2 from the N_2 gas supply. An anaerobic cuvette was attached to the dinitrogen train, such that the reactants in the apparatus were alternatively evacuated and purged with dinitrogen. In the anaerobic cuvette, premixed calf thymus DNA (1.00 mM base pairs) and bleomycin (0.100 mM) were placed in the base and Fe^{2+} (0.100 mM) in 20 mM phosphate buffer, pH 7.4, was placed in a sidearm. Once the system was made anaerobic, the Fe^{2+} solution was tipped into the reaction mixture to form Fe(II)Blm·DNA.

Oxidation of Fe(II)Blm in the Presence of DNA: Spectrophotomeric Analysis. Reactions were carried out at DNA (base pairs):Fe(II)Blm ratios of 10:1 and 100:1 at 25 °C. Bleomycin and DNA were initially mixed under aerobic conditions; then, addition of Fe^{2+} (0.031 mM) initiated the reaction.

When the DNA:Fe(II)Blm ratio was 10:1, the reaction mixtures consisted of calf thymus DNA (0.313 mM base pair) and bleomycin (0.031 mM) in 20 mM phosphate buffer, pH 7.4. A final spectrum was taken at 60 min when the reaction was finished. For the DNA:Fe(II)Blm ratio of 100:1, the reaction mixture consisted of calf thymus DNA (3.13 mM base pairs) and bleomycin (0.031 mM) in 20 mM phosphate buffer, pH 7.4. The last spectrum was taken 24 h later.

Kinetics of Removal of Fe(II) from O_2-Fe(II)Blm·DNA by BPS. All reaction mixtures consisted of bleomycin (0.050 mM) in 20 mM phosphate buffer, pH 7.4 at 25 °C, and various concentrations of calf thymus DNA ranging from 0.10 to 5.00 mM base pairs. The aerobic reaction between calf thymus DNA and Fe(II)Blm was initiated by the addition of Fe(II) (0.050 mM) to the solution containing DNA and Blm. The reactions were then allowed to incubate for 2 min. The addition of 4,7-phenylsulfonyl-1,10-phenanthroline (bathophenanthroline disulfonate, BPS) (1.00 mM) initiated reactions with O_2-Fe(II)Blm·DNA. This chelating agent forms a tris complex with Fe(II) that has an intense absorbance maximum at 534 nm (ϵ_{534nm} = 22 140 M^{-1} cm^{-1}) (Demant, 1984). The absorbance of each reaction mixture was monitored at 534 nm. Data was analyzed using the computer program LOTUS 123 to generate first-order $\ln(A - A_\infty)_{534nm}$ *vs* time plots. The slopes of the straight lines obtained were calculated by least-squares linear regression analyses, leading to the definition of pseudo-first-order rate constants. All kinetic runs were done in triplicate. The standard deviations were also calculated for each set of rate constants.

A series of reactions was carried out to determine the dependence of the observed rate constant on the BPS concentration for removal of Fe(II) from O_2-Fe(II)Blm·DNA by BPS. All reaction mixtures were composed of calf thymus DNA (2.50 mM base pair), Fe(II)Blm (0.025 mM), and various concentrations of BPS varying from 0.250 to 2.50 mM which reacted at 25 °C.

A parallel experiment measured the kinetics of reaction of FeBlm·DNA with BPS in 20 mM phosphate buffer, pH 7.4 at 25 °C, under anaerobic conditions. The base of the cuvette contained calf thymus DNA (2.50 mM base pairs) and bleomycin (0.050 mM); Fe^{2+} (0.050 mM) and BPS (1.00 mM) were placed separately in the two sidearms. Once the reaction mixture and all of the components were anaerobic, the solution containing Fe^{2+} was tipped into the solution of Blm·DNA to form Fe(II)Blm·DNA. The system was allowed to equilibrate for 2 min. The BPS solution was then tipped into the reaction mixture to initiate the reaction between Fe(II)Blm·DNA and BPS. The reaction was monitored at 534 nm for 60 min.

Liberation of Dioxygen from O_2-Fe(II)Blm·DNA. Release of dioxygen from O_2-Fe(II)Blm·DNA was monitored with a Yellow Springs Instrument Model 53 biological oxygen monitor system. It was calibrated with air-saturated water and the probe checked for membrane integrity. Prior to use, solutions of 20 mM phosphate buffer, pH 7.4, and glass-distilled water were air-saturated by stirring under atmospheric conditions for several hours. It was assumed that the 20 mM phosphate buffer solution had the same dioxygen solubility as water (0.24 mM at 0 °C and 760 mmHg). The average atmospheric pressure and temperature at the time of experimentation were 746 mmHg and 25 °C, respectively, resulting in an average dioxygen solubility in H_2O of 0.22 mM.

Mixtures of calf thymus DNA (1.00 mM base pair) and bleomycin (0.040, 0.020, and 0.010 mM) in 20 mM phosphate buffer, pH 7.4, were allowed to equilibrate with each other for 5 min at 25 °C. Then, reactions were initiated by injecting microliter amounts of Fe^{2+} (0.040, 0.020, and 0.010 mM) into the sample chamber. After 14 min of reaction time to form the dioxygenated adduct, each mixture was purged with N_2 for various time intervals (5, 10, 20, 30, and 50 min). Free O_2 was lost from the solution within 10 s under the conditions of vigorous bubbling with N_2. After each period of purging, BPS was added to release quantitatively the residual dioxygen bound to Fe(II)Blm. From these data and knowledge of the initial concentrations of dioxygenated Fe(II)Blm·DNA adducts present, the concentrations of O_2 that had dissociated from the adducts at each time point could be calculated.

Kinetics of Reaction of O_2 with Fe(II)Blm·DNA. Changes in dioxygen concentration were measured as described above. Samples consisted of calf thymus DNA and bleomycin in 20 mM phosphate buffer, pH 7.4. Each reaction was initiated by injecting microliter amounts of fresh Fe^{2+} [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$] into solutions containing DNA and Blm. The Fe^{2+} solution was washed into the sample chamber by carefully lifting and replacing the injector. This was considered the start of the reaction, which continued for 15 min. After this time, bathophenanthrolinedisulfonic acid (BPS) was introduced into the sample chamber in the same manner as the Fe^{2+} solution. Ten minutes after the addition of BPS, an ultraviolet–visible spectrum was taken to measure the amount of $Fe(BPS)_3^{2+}$ in each sample solution.

Kinetics of Formation of Malondialdehyde Product upon Reaction of O_2 with Fe(II)Blm·DNA. In the presence of DNA, dioxygen-activated Fe(II)Blm initiates a reaction with DNA that produces free bases and a malondialdehyde-like product which react to form a chromophoric adduct with thiobarbituric acid (Petering *et al.*, 1990; Templin *et al.*, 1992). The thiobarbituric acid addition product has an extinction coefficient at 532 nm of 1.6 × 10^5 M^{-1} cm^{-1}. All DNA strand scission reactions were conducted at 22 ± 2 °C. At time points of 30 s and 1, 2, 5, and 15 min, a 0.1-mL aliquot of the DNA strand scission reaction mixture was added to 0.9 mL of a solution consisting of TBA (40.00 mM) and EDTA (1.00 mM). This solution has a pH of 2, which rapidly dissociates FeBlm, preventing further reaction, and EDTA, to prevent iron precipitation. The final mixtures were covered with aluminum foil, incubated for 30 min at 90 °C, cooled to room temperature, and assayed spectrophotometrically for the MDA–TBA adduct.

Chromatographic Analysis of Free Base Release during Reactions of FeBlm with DNA. HPLC analysis of free base release from DNA was done on a Bio-Rad reversed-phase C_{18} column (250 × 10 mm). A linear gradient of 5–65% acetonitrile in 0.1 M sodium acetate, pH 6.5, was employed over a 45-min time period with a flow rate of 3.0 mL/min. Effluent was monitored at 260 nm. In one reaction, the reactants consisted of calf thymus DNA (5.0 mM base pair), FeBlm (1.0 and 0.50 mM), and hydrogen peroxide (2.0 mM) in 20 mM sodium phosphate buffer, pH 7.4, which were incubated anaerobically for 1 h. In the second, calf thymus DNA (5.0 mM base pair), Blm (1.0 and 0.50 mM), and Fe^{+2} (1.0 and 0.5 mM) in 20 mM sodium phosphate buffer, pH 7.4, were reacted in the presence of air for 1 h.

RESULTS

Oxidation of Aerobic Fe(II)Blm·DNA as a Function of the Base Pair to Drug Ratio. The anaerobic spectrum of Fe(II)Blm in the presence of a 10-fold excess of DNA base pairs is shown in Figure 3. This spectrum displays a broad weak absorbance at 476 nm corresponding to a characteristic pink

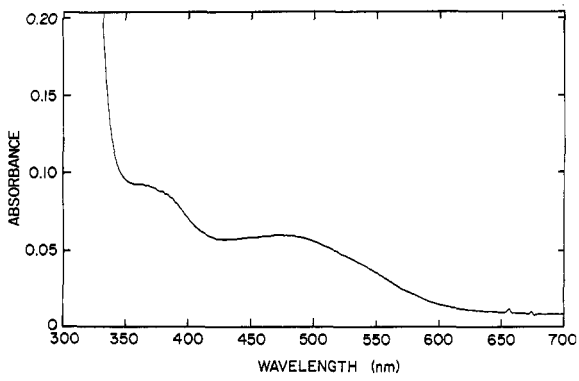

FIGURE 3: Anaerobic spectrum of Fe(II)Blm in the presence of DNA. The reaction mixture contained 1.00 mM DNA (base pairs) and 0.100 mM bleomycin and Fe^{2+} in 20 mM phosphate buffer, pH 7.4, 25 °C.

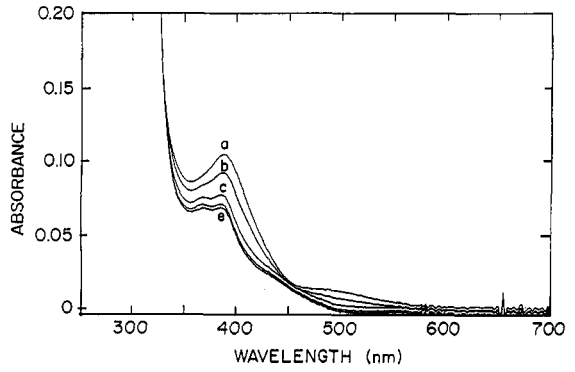

FIGURE 4: Fe(II)Blm oxidation in the presence of DNA. All reaction mixtures contained 0.313 mM DNA (base pairs) and 0.031 mM bleomycin and Fe^{2+} in aerated 20 mM phosphate buffer, pH 7.4, 25 °C: (a) 0 min; (b) 1 min; (c) 5 min; (d) 10 min; (e) 15 min.

color of Fe(II)Blm (Burger *et al.*, 1979a; Antholine & Petering, 1979).

Absorbance spectra in Figure 4 show the time course of reaction of Fe(II)Blm with O_2 in the presence of a 10-fold excess of DNA base pairs. The initial spectrum was not that of Fe(II)Blm bound to DNA shown in Figure 3. Instead, it was quite similar to the kinetic difference spectrum of the first observable intermediate in the oxidation of Fe(II)Blm by dioxygen constructed by Peisach and co-workers using stopped-flow spectroscopy (Burger *et al.*, 1979b). Studied under aerobic conditions, the complex exhibited a maximum at 384 nm ($\epsilon = 5000\ M^{-1}\ cm^{-1}$) with an additional broad weak absorbance near 500 nm. This complex was thought to be dioxygenated Fe(II)Blm bound to DNA. Over time, it gradually lost its absorbance at 500 nm and changed to a complex having maxima at 365 and 384 nm, characteristic of the absorbance spectrum of Fe(III)Blm (Burger *et al.*, 1979b).

At a DNA:Fe(II)Blm ratio of 10:1, the appearance of the Fe(III)Blm spectrum was complete in approximately 15 min. When the DNA:Fe(II)Blm ratio was increased to 100:1, the time course of reaction greatly lengthened. After 60 min of reaction time, the absorbance at 384 nm had decreased by only 9%. Production of the Fe(III)Blm spectrum took approximately 24 h. Apparently, the DNA-bound dioxygenated Fe(II)Blm complex became kinetically stabilized at higher DNA:Fe(II)Blm ratios.

Kinetics of Removal of Fe(II) from O_2-Fe(II)Blm·DNA by BPS. To demonstrate that the initial species observed in Figure 4 was dioxygenated Fe(II)Blm, reaction mixtures were mixed with BPS to remove Fe(II). Fe(II) was then spectrophotometrically quantitated as $Fe(BPS)_3$ and the O_2 released monitored with a dioxygen analyzer. In early experiments

Table 1: Kinetics of Reaction of BPS with O_2-Fe(II)Blm·DNA: Dependence on O_2-Fe(II)Blm:DNA Ratio[a]

DNA:Fe(II)Blm	DNA (mM)	k_{obs} (min^{-1})	% total as Fe(II)
2:1	0.10		2.60 ± 0.7
4:1	0.20		4.00 ± 3.0
8:1	0.40	1.3 ± 0.7	42.40 ± 16.7
10:1	0.50	0.8 ± 0.3	60.80 ± 6.3
12:1	0.60	0.48 ± 0.03	69.80 ± 6.3
16:1	0.80	0.32 ± 0.04	73.00 ± 2.8
20:1	1.00	0.24 ± 0.01	78.80 ± 2.1
50:1	2.50	0.15 ± 0.03	84.20 ± 4.1
100:1	5.00	0.12 ± 0.02	84.60 ± 6.7

[a] All reaction mixtures contained 0.05 mM Fe(II)Blm, 1.00 mM BPS, and various concentrations of calf thymus DNA (base pairs) pH 7.4, 25 °C. Reaction of BPS with Fe(II) started 2 min after addition of Fe^{2+} to Blm·DNA.

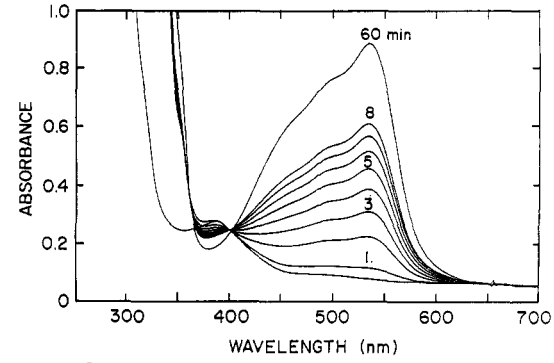

FIGURE 5: Spectral changes during aerobic reaction of O_2-Fe(II)-Blm·DNa with BPS. All reaction mixtures contained 2.50 mM DNA (base pairs), 0.050 mM bleomycin and Fe^{2+}, and 1.00 mM BPS in 20 mM phosphate buffer, pH 7.4, 25 °C.

1,10-phenanthroline was used as the chelating agent in this experiment. This reagent has a large thermodynamic affinity for Fe(II) (log apparent stability constant, pH 7.0, log K = 21.1) (Sillén & Martell, 1964). However, it was necessary to use large concentrations of the ligand to get efficient reaction [100:1 with respect to Fe(II)], possibly because it can bind to DNA. Thus, a negatively charged analog, BPS, was substituted which was thought not to interact with DNA. At pH 7.4, the addition of BPS led to the formation of the $Fe(BPS)_3$ complex, indicating the presence of Fe(II)Blm remaining in the reaction mixture. Table 1 shows the percent of total Fe(II) left in the reaction mixture after 2 min of reaction time as a function of the DNA base pair:FeBlm ratio. It was evident that the extent of conversion of Fe(II) to Fe(III) was systematically decreased as the base pair:Fe(II)-Blm ratio increased.

The reaction between DNA-bound Fe(II)Blm and BPS involves a ligand substitution reaction.

$$DNABlmFe(II) + 3BPS \rightarrow Fe(BPS)_3 + DNA{\cdot}Blm \quad (5)$$

Under anaerobic conditions, this reaction went to completion rapidly with a burst of $Fe(BPS)_3$ formation observed immediately after the addition of BPS. The kinetics were fast whether or not DNA (50:1) was present. Before the first spectrum could be taken, the reaction was more than 95% complete in the presence or absence of DNA.

The rate of $Fe(BPS)_3$ formation was also observed in the presence of air as a function of the DNA:Fe(II)Blm ratio. Unlike anaerobic conditions, $Fe(BPS)_3$ did not form quickly but instead developed over a 60-min time period (Figure 5). The presence of an isosbestic point at 400 nm indicated the existence of two interconverting absorbing species in the

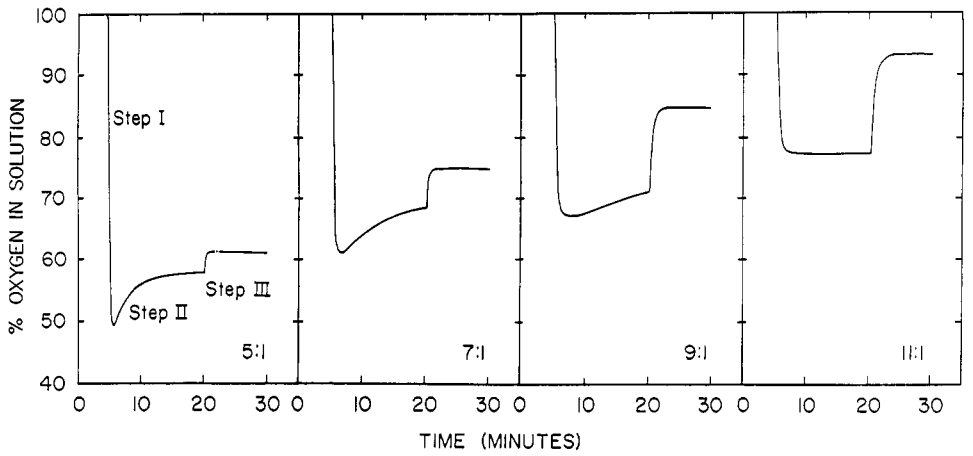

FIGURE 6: Dioxygen uptake and release during reaction of Fe(II)Blm·DNA and O_2. All reaction mixtures contained 1.00 mM DNA base pairs, 0.22 mM O_2, and various concentrations of Blm plus Fe^{2+} to start the reactions in 20 mM phosphate buffer, pH 7.4, 25 °C. Step III shows O_2 released upon addition of 4 mM BPS. Base pair to FeBlm ratios are shown in the lower right of each panel.

Table 2: Kinetics of Reaction of BPS with O_2-Fe(II)Blm·DNA: BPS Concentration Dependence[a]

BPS:Fe(II)Blm	BPS (mM)	k_{obs}[b] (min^{-1})
10:1	0.50	0.040
15:1	0.75	0.050 ± 0.003
20:1	1.00	0.057 ± 0.003
25:1	1.25	0.066 ± 0.002
100:1	5.00	0.225 ± 0.028

[a] All reaction mixtures contained 2.50 mM DNA (base pairs), 0.025 mM Fe(II)Blm, and various concentrations of BPS, pH 7.4, 25 °C. [b] Average ± standard deviation of three trials.

Table 3: Kinetics of Dissociation of O_2 from O_2-Fe(II)Blm·DNA

DNA:Fe(II)Blm[a]	k[b] (min^{-1})	O_2-Fe(II)Blm remaining[c] (μM)
25:1[d]	0.156 ± 0.018	9.0 ● 0.6
50:1[e]	0.158 ± 0.013	8.0 ± 0.1
100:1[e]		8.8 ± 0.3

[a] All reactions contained 1.00 mM DNA (base pairs), 0.22 mM O_2, and 0.040, 0.020, and 0.010 mM Fe(II)Blm, respectively pH 7.4, 25 °C. [b] First-order rate logarithim constants derived from plots of $[O_2]_t - [O_2]_\infty$ vs t. [c] Concentration of deoxygenated sites remaining after completion of first-order release of O_2. [d] Three trials. [e] Two trials.

reaction, a species of Fe(II)Blm and $Fe(BPS)_3$. All reactions were observed under pseudo-first-order conditions for BPS concentration and proceeded with simple first-order kinetics. The observed rate constants at each concentration of DNA were directly proportional to the BPS concentration (Table 2). Accordingly, the reaction between DNA-bound O_2-Fe(II)Blm and BPS (DNA base pair:FeBlm = 100) is first order in Fe(II) and BPS, having a second-order rate constant of 0.13 ± 0.03 mM^{-1} min^{-1}.

The concentration of DNA was allowed to vary at a constant concentration of Fe(II)Blm, and the observed rate constants for the formation of $Fe(BPS)_3$ were determined. The dependence of k_{obs} upon DNA (base pairs) indicated an inverse dependence of the ligand substitution reaction on DNA concentration at constant pseudo-first-order excess of BPS (Table 1).

A final characteristic of the reaction is a release of dioxygen that is observed after the reaction of BPS with O_2-Fe(II)Blm·DNA (Figure 6). Prior to this, when Fe^{2+} was originally added to the aerobic solution of Blm·DNA, there was a rapid loss of O_2 from the solution (step I). Then, when BPS was added after 15 min of reaction time, dioxygen reappeared slowly in solution as BPS reacted with Fe(II) (step III). The amount of dioxygen released from FeBlm·DNA was equivalent to the amount of $Fe(BPS)_3$ formed (data not shown). Aerobically, the overall reaction considered may be

$$\text{DNA·BlmFe(II)-O}_2 + 3\text{BPS} \rightarrow \text{Fe(BPS)}_3 + \text{O}_2 + \text{DNA·Blm} \quad (6)$$

From the combined results it was evident that O_2-Fe(II)Blm formed and was stable on DNA and that it was the initial species observed spectrophotometrically in Figure 4. As dioxygenated Fe(II)Blm molecules were bound to DNA at increasingly larger DNA:Fe(II)Blm ratios, they were kept far enough apart to stabilize the dioxygenated adduct and prevent oxidation–reduction to produce the products of reaction 1a. Under anaerobic conditions, Fe(II)Blm was readily accessible for interaction with BPS whether DNA was present or absent. In the presence of molecular dioxygen and DNA, accessibility was dramatically decreased.

Dissociation of Dioxygen from O_2-Fe(II)Blm·DNA under Anaerobic Conditions. Efforts to remove O_2 reversibly bound to O_2-Fe(II)Blm·DNA by putting reaction mixtures under vacuum were not effective. As an alternative, samples were incubated with O_2 for 14 min to form O_2-Fe(II)Blm·DNA and then vigorously purged with N_2 for fixed times, which rapidly ($t_{1/2} \sim 10$ s) removed measurable free O_2 from solution. Then, dinitrogen purging was halted and the rate of reappearance of O_2 in solution examined either in the absence or in the presence of BPS. No detectable equilibration of O_2 occurred in the absence of BPS. However, the purging procedure did drive the irreversible loss of O_2 bound as O_2-Fe(II)Blm·DNA from the solution. This was quantitated using BPS to liberate residual O_2 associated with Fe(II)Blm·DNA.

For each ratio of DNA to FeBlm the percent of dioxygen remaining decreased as the time of purging was extended, indicating that this procedure did facilitate O_2 release. Each reaction was completed within 30 min but left a fraction of O_2 bound to Fe(II)Blm·DNA that was insensitive to purging. First-order plots of these results indicated that at each ratio of DNA to O_2-Fe(II)Blm, similar first-order kinetics for loss of dioxygen from its bound form were observed, which were characterized by closely similar rate constants (Table 3). The measured first-order rate process is thought to be the dissociation reaction of dioxygen from O_2-Fe(II)Blm·DNA (k_{-1} of reaction 7). Interestingly, at each ratio of DNA to

$$\text{Fe(II)Blm·DNA} + O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} O_2\text{-Fe(II)Blm·DNA} \quad (7)$$

Fe(II)Blm, the same concentration of O_2 remained bound to Fe(II)Blm·DNA after 30 min. Extended intense purging with N_2 did not drive off any more O_2. Thus, a fraction of the sites containing O_2-Fe(II)Blm were not in equilibrium with the solution pool of O_2 under these vigorous conditions.

Kinetics of Reaction of O_2 with Fe(II)Blm·DNA as a Function of the Base Pair to Drug Ratio. The reaction of dioxygen with Fe(II)Blm·DNA was measured with an O_2 analyzer as a function of the DNA:Fe(II)Blm ratio (Figure 6). At a ratio of 5:1 changes in concentration of solution dioxygen were clearly multiphasic. The first step of removal of O_2 from solution proceeded rapidly, going to completion in approximately ¼ min (step I). During the next 15 min, dioxygen reappeared in solution (step II). This suggested that the reaction mechanism for the oxidation of Fe(II)Blm by O_2 in the presence of DNA was similar to that for Co(II)Blm (reactions 2–4), in which O_2 initially binds to the metal center and then is partially displaced as a dimeric intermediate forms to accomplish electron transfer between the metal and dioxygen. As the base pair:FeBlm ratio increased to 11:1, the second step gradually disappeared, consistent with the stabilization of O_2-FeBlm·DNA.

Rate constants were calculated from the rate of dioxygen release during the second step of the reaction. They were second order as shown in Table 4. The second-order character of this rate process also stands in agreement with its definition as a dimerization process as described in reaction 3. As the base pair:Fe(II)Blm ratio increased from 5:1 to 8:1, the rate constant sharply declined from 1180 to 160 M^1 s^{-1}.

Fifteen minutes after the start of the reaction, BPS was added to each reaction mixture to bind any Fe(II) remaining in solution and to release O_2 from residual O_2-Fe(II)Blm·DNA (Figure 6, step III). It is evident that the amount of dioxygen that was released increased as the base pair to drug ratio became larger. In turn, this quantity correlated well with the amount of $Fe(BPS)_3^{3+}$ that had formed (data not shown). Clearly, as the average distance between base pairs became larger, less Fe(III)Blm was generated through reaction with dioxygen and O_2 + Fe(II)Blm·DNA became stable.

Effects of Ionic Strength on the Reaction of Dioxygen with Fe(II)Blm·DNA. It has been previously established that both the bithiazole and positively charged tail of Blm are involved in binding the drug to DNA (Dabrowiak, 1982). Increasing the ionic strength reduces the apparent equilibrium constant for this binding reaction (Chien *et al.*, 1977). To see what effect ionic strength has on the reaction of O_2 with Fe(II)Blm·DNA, the kinetics of changes in solution dioxygen concentration were determined as a function of the concentration of NaCl. The initial rate of dioxygen loss from solutions increased as the salt concentration was elevated. Similarly, the rate constant for step II rose with the ionic strength from 290 to 630 M^{-1} s^{-1} over the range 0–40 mM NaCl. At 200 mM NaCl the second step could not be observed (Table 4).

DNA Strand Scission Associated with the Reaction of Fe(II)Blm·DNA with O_2. To correlate the properties of reaction of the ferrous drug complex bound to DNA with dioxygen and the extent of DNA damage caused by its activated form, the formation of malondialdehyde product was determined as a function of base pair:drug ratio. Appearance of MDA is indicative of the pathway of strand scission that generates base propenal as a product (Figure 2). Figure 7 shows the kinetics of formation of MDA for a series of reactions varying in Fe(II)Blm concentration. Each of these can be described as a second-order process. The associated rate constants are given in Table 4, showing that as the base pair:drug ratio increased, the second-order rate constant for formation of one of the products of DNA damage sharply decreased. It is noted that the rate constants are similar to those for the dimerization reaction listed in the same table, suggesting that dimerization is rate limiting in MDA formation.

Efficiency of formation of MDA product is summarized in Table 5. It shows generally that as the base pair to FeBlm rates increased, the amount of DNA damage, assayed as MDA

Table 4: Rate Constants for Dioxygen Reaction with Fe(II)Blm·DNA and MDA Product Formation after Strand Scission[a]

base pairs: Fe(II)Blm	step II rate constant[b] (M^{-1} s^{-1})	MDA product formation rate constant[b,d] (M^{-1} s^{-1})
4:1		
5:1	1180 ± 20	1518 ± 33
6:1	490 ± 23	446 ± 93
7:1	280 ± 44	265 ± 72
	290 (0 mM NaCl)[c]	
	540 (10)	
	560 (20)	
	630 (40)	
8:1	160 ± 21	289 ± 49
9:1		224 ± 78
10:1		

[a] Reaction mixtures contained 1.00 mM DNA base pairs, various concentrations of Blm plus Fe^{2+}, and 20 mM phosphate buffer, pH 7.4, 25 °C. [b] Data in Figure 5, average ± standard deviation of at least three runs. Second-order rate constants were determined from plots of eq $1/[O_2\text{-FeBlm·DNA}] = kt + C$, where t is time and C is a constant. $[O_2\text{-FeBlm·DNA}] = [O_2]_{t=\infty} - [O_2]_t$. [c] Initial rate of O_2 uptake (step I): 0.12 mM·min^{-1} (0 mM NaCl), 0.18 (10 mM), 0.19 (20 mM), 0.22 (40 mM). [d] Data from Figure 6, average ± standard deviation of at least two runs. Second-order rate constants were determined as in *b*, except for $[O_2\text{-FeBlm·DNA}] = [MDA]_{t=\infty} - [MDA]_t$.

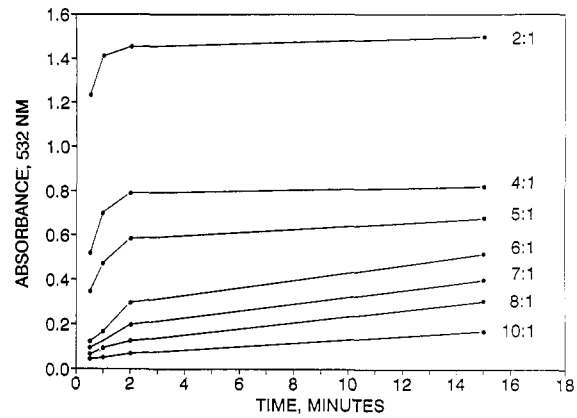

FIGURE 7: Kinetics of MDA formation in the DNA strand scission of Fe(II)Blm·DNA with O_2. All reaction mixtures contained 1.00 mM DNA base pairs and various concentrations of Blm plus Fe^{2+} to start the reactions in 20 mM phosphate buffer, pH 7.4, 25 °C.

Table 5: Reaction of DNA, Blm, Fe^{2+}, and O_2: Formation of DNA Damaged Product[a]

base pairs: Fe(II)Blm	MDA:Fe(II)Blm	base pairs: Fe(II)Blm	MDA:Fe(II)Blm
2:1	0.18 ± 0.01	8:1	0.16 ± 0.02
4:1	0.21 ± 0.01	9:1	0.16 ± 0.02
5:1	0.21 ± 0.01	10:1	0.13 ± 0.03
6:1	0.20 ± 0.01	11:1	0.12 ± 0.02
7:1	0.18 ± 0.01	12:1	0.11 ± 0.02

[a] All reaction mixtures contain 1.00 mM DNA (bp) and various concentrations of Fe(II)Blm, 20 mM phosphate buffer, pH 7.4, 25 °C.

production, declined from a maximum of 0.21 to 0.11. This agrees with the decrease in rate constant for dimerization (Table 4) and of O_2-FeBlm·DNA, which demonstrated that stabilization occurs at larger base pair:drug ratios. The slightly lower efficiency of MDA production at the 2:1 ratio compared with that observed at relative concentrations of DNA to FeBlm of 4:1 to 6:1 may be due to the probability that at the ratio of 2:1 some of the FeBlm reacted in solution, assuming a binding site size of 2–3 as seen with CoBlm (Xu *et al.*, 1992b). As the ratio increased toward 12:1, less MDA was produced during the 15-min period because O_2-Fe(II)Blm had attained some stability on the DNA as described above.

The relative amount of base release (G, C, and T) was also determined at base pair:Fe(II)Blm ratios of 5:1 and 10:1. At the larger ratio the concentration of free bases in the reaction mixture normalized to FeBlm concentration was 0.16 of that recovered at the 5:1 ratio. Thus, a doubling of the ratio had a large negative effect on base release, which was even greater than its impact on base propenal (MDA) production (Table 5). In contrast, when these same concentrations of DNA and Fe(II)Blm were reacted with 2 mM H_2O_2 under anaerobic conditions, 7 times as many bases were released with the smaller ratio as seen in the aerobic reaction above. At the 10:1 ratio, somewhat more bases (1.3 times) were liberated on a normalized basis. Thus, the base pair:FeBlm ratio had no negative impact on base release when activation of FeBlm did not require interaction of two Fe(II)Blm molecules.

DISCUSSION

The present series of experiments has examined the reaction of Fe(II)Blm with DNA under aerobic conditions in which the ratio of DNA base pairs to drug has been varied. The impetus for this work was the recognition that a variety of experimental means of achieving DNA strand scission *in vitro* with this drug had been used without much explicit discussion about their relevance to cellular DNA strand scission. Thus, at low base pair:DNA ratios (1–10 base pairs/Blm molecule), Fe^{2+}, Blm, and DNA have been mixed to obtain large amounts of cleavage products (Templin *et al.*, 1992). Under similar conditions or larger ratios of DNA to drug, H_2O_2 and Fe(III)Blm react with DNA to cause strand scission (Burger *et al.*, 1981). At ratios of DNA to Blm much larger than 1 (*ca.* 40:1), the mixture of Fe(III)Blm, DNA, and reducing agents produces DNA breaks (Steigner & Povirk, 1990). In cells, the ratio of base pairs to Blm in the nucleus is much larger (10^5–10^8:1) than used in any of these model reactions (Byrnes *et al.*, 1990; Roy & Horwitz, 1984).

One appreciates the dramatic effect of increasing base pair ratio on the redox chemistry of FeBlm in Table 1, in which the amount of Fe(II) remaining in an aerobic solution of Fe^{2+}, Blm, and DNA after 2 min of incubation increases from 3 to 84% of its initial concentration as the ratio increases from 2:1 to 50:1. Corresponding measurements of DNA strand scission confirm that as Fe(II) is progressively stabilized, there is a decreasing amount of DNA damage (Table 4). It has been recognized for years that DNA strand scission caused by the reaction of Blm, O_2, and Fe^{2+} is inhibited as the ratio of DNA to drug increases (Burger *et al.*, 1979; Ciriolo *et al.*, 1987). The present study provides a mechanistic understanding of this effect.

These experiments have defined properties of adduct formation between dioxygen and Fe(II)Blm·DNA. In the absence of O_2, Fe^{2+} bound stoichiometrically to Blm·DNA to form a complex with spectrophotometric features similar to those of Fe(II)Blm in solution. Evidently, the affinity of Blm for Fe^{2+} is large enough to exclude the competitive association of Fe^{2+} with the DNA structure. When O_2 was added to Fe(II)Blm·DNA, dioxygenated product was formed.

The rate of subsequent oxidation–reduction involving O_2-Fe(II)Blm·DNA was inversely related to the DNA base pair:FeBlm ratio (Tables 1 and 4; Figure 6). Under the conditions of these reactions and assuming the association constant of the Fe(II)Blm species to DNA is 10^5 or larger, virtually all of the drug was bound to the polymer (Petering *et al.*, 1990). Thus, the reason for the decline in redox activity must relate to differential interaction between O_2-Fe(II)Blm molecules associated with DNA in the different reaction mixtures.

The kinetics of the reaction of O_2 with Fe(II)Blm·DNA have been studied to explore this hypothesis. In analogy to studies with Co(II)Blm·DNA and O_2, changes in O_2 concentration were biphasic and consistent with the operation of reactions 2–4 in the case of FeBlm (Figure 6; Table 4) (Xu *et al.*, 1992b).

$$\text{Fe(II)Blm·DNA} + O_2 \rightarrow O_2\text{Fe(II)Blm·DNA} \quad (8)$$

$$2O_2\text{Fe(II)Blm·DNA} \rightarrow \text{DNA·BlmFe(II)-}O_2\text{Fe(II)Blm·DNA} + O_2 \quad (9)$$

$$\text{DNA·BlmFe(II)-}O_2\text{Fe(II)Blm·DNA} + H^+ \rightarrow HO_2\text{-Fe(III)Blm·DNA} + \text{Fe(III)Blm} \quad (10)$$

That the activated species was one of the final products of the redox process was shown by the fact that the MDA product characteristic of DNA strand scission by activated FeBlm appeared during the reaction (Figure 7) (Burger *et al.*, 1981). Interestingly, the kinetics of its formation paralleled those for the dimerization reaction (Table 4), suggesting that reaction 9 was rate limiting for the aerobic pathway of DNA strand scission as well as for activation of the drug. As expected from the dual mechanisms of DNA cleavage shown in Figure 2, both base propenal (MDA) (pathway A) and free base production (pathway B) declined as the base pair:Fe(II)Blm ratio increased because the same initial reaction of activated FeBlm with DNA must occur for both pathways.

The further apart the molecules of FeBlm are dispersed on DNA, the slower the redox reaction and the smaller the amount of DNA strand cleavage. Recognizing that dioxygenated Fe(II)Blm molecules must be in physical contact to carry out reaction 9 and assuming that the size of the binding site for FeBlm is 2–3 base pairs, similar to that for CoBlm, it is clear that some process must occur to bring other molecules of O_2-FeBlm into contact to account for the actual ratio of MDA product per Fe(II)Blm reactant (Xu *et al.*, 1992b). This might involve sliding or dissociation and rebinding of FeBlm molecules. The further apart the reacting molecules are, the more extensive these events must be to bring them together. While these cannot be distingushed at present, the experiment which examined dioxygen kinetics as a function of ionic strength clearly showed that as the ionic interaction between the positively charged tail of the drug and the negatively charged phosphodiester of DNA was weakened, the rate of dimerization increased, presumably because the dioxygen adducts become more mobile with respect to DNA (Table 4). So, in some fashion the electrostatic interaction contributes to the localization of the drug to particular sites on the DNA molecule.

A number of additional observations contribute to this picture. First, the kinetic availability of Fe(II) for BPS was

substantially different in Fe(II)Blm·DNA and O_2-Fe(II)-Blm·DNA. In the former, nearly complete ligand substitution occurred within the time of mixing as in solution (Lyman *et al.*, 1989). Once the dioxygen adduct formed, the rate of reaction dramatically decreased. This reaction was first order in both O_2-Fe(II)Blm and BPS, consistent with direct rate-limiting interaction of the dioxygenated drug with the ligand; dissociation of O_2 was not the rate-determining step. Thus, it is apparent that dioxygen binding in some fashion masked the iron center from direct bimolecular reaction with BPS.

The environment of the metal center also seemed to be affected by the relative concentration of adduct bound to DNA because the rate of reaction of O_2-Fe(II)Blm with BPS was strongly dependent on this ratio (Table 1). The structure of each O_2-Fe(II)Blm·DNA site must, therefore, be perturbed over long distances along the double helix by the binding of other drug molecules, for the rate constant for Fe(II) removal by BPS changed markedly as the base pair to FeBlm ratio varied between 8:1 and 50:1. It was only when bound bleomycin molecules were 5–10 double-helical turns apart on the average that they were effectively isolated from one another in this reaction.

An explanation for the effect of bound O_2 on the reactivity of Fe(II)Blm·DNA with BPS derives from a previous finding, that in O_2-Co(II)Blm·DNA dioxygen apparently interacts strongly with DNA and is rotationally constrained to a plane perpendicular to the helix axis (Chikira *et al.*, 1989). In contrast, the metal centers of Cu and Fe(III)Blm bound to DNA have more rotational freedom (Chikira *et al.*, 1991). Close association of the metal center of O_2-Fe(II)Blm·DNA with the polymer could provide significant steric hindrance to BPS as it reacts with Fe(II). Long distance perturbation of the rate of this reaction by O_2-Fe(II)Blm binding might also be a reflection of changes in accessibility of the iron center to the competing phenanthroline ligand.

That both the metal- and DNA-binding domains of FeBlm interact with the DNA polymer was further supported by results showing the very slow first-order dissociation rate constant of O_2 bound to the complex. Though probably larger than that for O_2-Co(II)Blm·DNA, which does not dissociate over the course of many hours, it was, nevertheless, orders of magnitude smaller than O_2 dissociation rate constants determined for isolated dioxygenated α (28 s^{-1}) or β chains (16 s^{-1}) of hemoglobin and for the model dioxygen adduct with pyrroheme-*N*-[3-(1-imidazoyl)propyl]amide iron(II) (35 s^{-1}), all determined at pH 7–7.3 and 20–22 °C (Brunori & Schuster, 1969; Chang & Trayler, 1975; Collman *et al.*, 1980). This small dissociation rate constant is consistent with additional interaction of O_2 with DNA as well as with its binding site on FeBlm. An intriguing finding was that there were two classes of Fe(II)Blm·DNA binding sites for O_2. One equilibrated with solution O_2 under the conditions of N_2 purging. Another did not. At large ratios of DNA to Fe(II)Blm this comprises most of the bound O_2-Fe(II)Blm. This result suggests that there may be uncommon but preferential DNA sequences for O_2-Fe(II)Blm binding which need to be identified.

These results provide strong support for a model in which both metal- and DNA-binding domains of O_2-Fe(II)Blm interact strongly with DNA. This is a hypothesis that was originally suggested by the marked perturbation of the ESR spectrum of NO-Fe(II)Blm when bound to DNA and subsequently supported in other studies (Antholine & Petering, 1979; Antholine *et al.*, 1981; Carter *et al.*, 1990; Petering *et al.*, 1990). If the metal-binding domain is also in contact with DNA, perhaps through specific interactions, this would help explain the highly localized cleavage sites for cleavage by this drug in comparison with synthetic chemical nucleases based on FeEDTA redox sites, which are rotationally mobile at the end of tethers to DNA binding moieties and damage a set of adjacent sites (Dervan, 1986).

The relevance of this work on the redox behavior of Fe(II)Blm associated with DNA for the cellular mechanism of action of bleomycin may be seen in an examination of the pathway of reaction of the metal-free drug with cells. Bleomycin causes cytotoxicity if it can interact with cellular iron (Radtke *et al.*, 1991). Assuming that it is required to form FeBlm, then DNA damage results from the reaction of HO_2-Fe(III)Blm, the activated species, with the polymer backbone (Burger *et al.*, 1981). If FeBlm is reduced and activated before it binds to DNA, it has about a 15-s half-time in which to diffuse to the DNA before it is deactivated. This estimate is based upon a measured half-time of inactivation of 2 min at 6 °C and a cell culture temperature of 37 °C (Burger *et al.*, 1981). During this process, it reacts with itself, causing an ill-defined alteration in the bleomycin structure such that it loses its capacity to cause DNA damage (Nakamura & Peisach, 1988; Buettner & Moseley, 1992; Templin *et al.*, 1992). Thus, if HO_2-FeBlm does not reach the DNA or perhaps an RNA molecule before this reaction occurs, it cannot be reactivated to attack DNA (Holmes *et al.*, 1993). Once in the nucleus, the ratio of DNA bases to Blm is at least 10^5:1, precluding the reaction sequence 2–4 as an effective pathway for activation of FeBlm if the drug binds randomly to DNA (Byrnes *et al.*, 1990). Cellular experiments show that the single- and double-strand scission by Blm continues for at least 4 h (Byrnes & Petering, 1991). Thus, it is likely that most of the damage is caused by drug that diffuses into the nucleus, binds to DNA, and then is converted to the activated form of the drug with the aid of another cellular reducing agent.

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