A Study of O_2- versus H_2O_2-Supported Activation of Fe·Bleomycin

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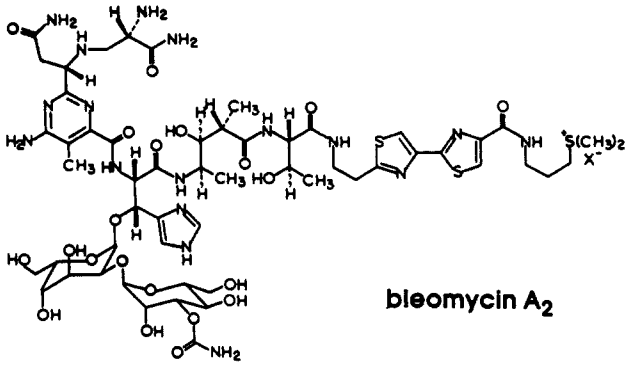
Abstract: The rates of formation of product resulting from the cleavage of d(CGCT$_3$A$_3$GCG) by Fe(II)·BLM + O_2 as well as Fe(III)·BLM + H_2O_2 have been measured under a variety of experimental conditions in order to permit comparison of drug activation with the appearance of DNA degradation products. The activation of bleomycin with Fe(II) under aerobic conditions was found to be fast at neutral pH but was inhibited by DNA. In the presence of reducing agents such as sodium ascorbate, however, activation was rapid even in the presence of DNA. Presumably this reflects the ability of external reductants to bypass an otherwise obligatory bimolecular collision of two Fe(II)·BLM's in the presence of O_2 to achieve activation. The decay of the activated Fe·BLM obtained by admixture of Fe(II)·BLM + O_2 was also found to occur quickly, with $t_{1/2} \sim 2$ min at 0 °C. The rates of product release, in contrast, were found to be much slower, although both free bases and base propenals were released on the same time scale. The activation of Fe(III)·BLM with H_2O_2 was found to be slow at pH 7.2 but rapid at both acidic and basic pH. These observations can be rationalized by a mechanism that posits a slow bimolecular reaction between Fe(III)·BLM and H_2O_2 at neutral pH. When activated at pH 5.8, the rates of product release from DNA obtained with activated Fe(III)·BLM were found to be comparable to the rates observed for aerobically activated Fe(II)·BLM at the same pH. The activation of Fe(III)·BLM by H_2O_2 was not inhibited by DNA. Both methods of Fe·BLM activation produced active species that behaved in the same fashion with regard to their reactivity toward DNA.

The bleomycins (BLM's) are glycopeptide-derived antibiotics having clinically useful antitumor activity.[1] It is believed that DNA represents the cellular locus at which bleomycin mediates its therapeutic effects. In the presence of metal ions such as Fe(II), bleomycin forms a binary complex [Fe(II)·BLM] that can activate



bleomycin A_2

molecular oxygen. The resulting unstable and reactive species can bind to and degrade DNA,[2] apparently leading to cell growth inhibition, loss of colony-forming ability, and lessening of cell viability[3] as a consequence.

Several studies have addressed the issue of the molecular composition and structural nature of activated Fe·BLM.[4] Of particular interest in this regard are the study of Burger et al.[4a] which demonstrated that the same oxygenated activated species was accessible by admixture of either Fe(II)·BLM + O_2 or Fe(III)·BLM + H_2O_2 and another report from the same laboratory which showed by titration with I^- and thio-NADH that activated Fe·BLM has two more oxidizing equivalents than Fe(III)·BLM.[5] On the basis of these data, activated Fe·BLM is probably best represented as a perferryl (Fe^V=O) species, a view that is supported by the ability of activated Fe·BLM to mediate chemical transformations like olefin epoxidation, hydroxylation of aromatic rings, and N-demethylation of *N,N*-dimethylaniline,[6] i.e., reactions characteristic of heme proteins like cytochrome P-450.[7]

The mechanism of DNA degradation by activated Fe·BLM has also been the subject of intense scrutiny. This process involves the formation of two sets of products. One of these, which is accompanied by DNA strand scission, includes base propenals, a 3′-oligonucleotide terminating with a 5′-phosphate, and a 5′-oligonucleotide terminating with a 3′-(phospho-2″-*O*-glycolate) moiety.[1e,8] These products are apparently formed via the intermediacy of a C-4′ hydroperoxide derivative of deoxyribose, which undergoes cleavage of the C-3′–C-4′ bond by a Criegee-type rearrangement. The other set of products, which is apparently formed via hydroxylation of the C-4′ position of deoxyribose,

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(1) (a) Umezawa, H. In *Bleomycin: Current Status and New Developments*; Carter, S. K., Crooke, S. T., Umezawa, H., Eds.; Academic: New York, 1978. (b) Umezawa, H.; Maeda, K.; Takeuchi, T.; Okami, Y. *J. Antibiot.* **1966**, *19*, 200. (c) Umezawa, H. *Pure Appl. Chem.* **1971**, *28*, 665. (d) Umezawa, H. *Biomedicine* **1973**, *18*, 459. (e) Hecht, S. M. In *Bleomycin: Chemical, Biochemical and Biological Aspects*; Hecht, S. M., Ed.; Springer-Verlag: New York, 1979.

(2) (a) Ishida, R.; Takahashi, T. *Biochem. Biophys. Res. Commun.* **1975**, *66*, 1432. (b) Sausville, E. A.; Peisach, J.; Horwitz, S. B. *Biochem. Biophys. Res. Commun.* **1976**, *73*, 814. (c) Sausville, E. A.; Peisach, J.; Horwitz, S. B. *Biochemistry* **1978**, *17*, 2740. (d) Oppenheimer, N. J.; Chang, C.; Rodriguez, L. O.; Hecht, S. M. *J. Biol. Chem.* **1981**, *256*, 1514. (e) Ehrenfeld, G. M.; Shipley, J. B.; Heimbrook, D. C.; Sugiyama, H.; Long, E. C.; van Boom, J. H.; van der Marel, G. A.; Oppenheimer, N. J.; Hecht, S. M. *Biochemistry* **1987**, *26*, 931.

(3) (a) Barranco, S. C.; Humphrey, R. M. *Cancer Res.* **1971**, *31*, 1218. (b) Terasima, T.; Takabe, Y.; Katsumata, T.; Watanabe, M.; Umezawa, H. *J. Natl. Cancer Inst. (U.S.)* **1972**, *49*, 1093. (c) Hittelman, W. N.; Rao, P. N. *Cancer Res.* **1974**, *34*, 3433. (d) Barlogie, B.; Drewinko, B.; Schumann, J.; Freireich, E. J. *Cancer Res.* **1976**, *36*, 1182. (e) Clarkson, J. M.; Humphrey, R. M. *Cancer Res.* **1976**, *36*, 2345. (f) Berry, D. E.; Chang, L.-H.; Hecht, S. M. *Biochemistry* **1985**, *24*, 3207.

(4) (a) Burger, R. M.; Peisach, J.; Horwitz, S. B. *J. Biol. Chem.* **1981**, *256*, 11636. (b) Kuramochi, H.; Takahashi, K.; Takita, T.; Umezawa, H. *J. Antibiot.* **1981**, *34*, 576.

(5) Burger, R. M.; Blanchard, J. S.; Horwitz, S. B.; Peisach, J. *J. Biol. Chem.* **1985**, *260*, 15406.

(6) (a) Murugesan, N.; Ehrenfeld, G. M.; Hecht, S. M. *J. Biol. Chem.* **1982**, *257*, 8600. (b) Ehrenfeld, G. M.; Murugesan, N.; Hecht, S. M. *Inorg. Chem.* **1984**, *22*, 1496. (c) Murugesan, N.; Hecht, S. M. *J. Am. Chem. Soc.* **1985**, *107*, 493. (d) Heimbrook, D. C.; Mulholland, R. L., Jr.; Hecht, S. M. *J. Am. Chem. Soc.* **1986**, *108*, 7839. (e) Heimbrook, D. C.; Carr, S. A.; Mentzer, M. A.; Long, E. C.; Hecht, S. M. *Inorg. Chem.* **1987**, *26*, 3836.

(7) (a) White, R. E.; Coon, M. J. *Annu. Rev. Biochem.* **1980**, *49*, 315. (b) Guengerich, F. P.; Macdonald, T. L. *Acc. Chem. Res.* **1984**, *17*, 9. (c) Ortiz de Montellano, P. R., Ed. *Cytochrome P450: Structure, Mechanism and Biochemistry*; Plenum: New York, 1985.

(8) (a) Burger, R. M.; Berkowitz, A. R.; Peisach, J.; Horwitz, S. B. *J. Biol. Chem.* **1980**, *255*, 11832. (b) Giloni, L.; Takeshita, M.; Johnson, F.; Iden, C.; Grollman, A. P. *J. Biol. Chem.* **1981**, *256*, 8608. (c) Wu, J. C.; Kozarich, J. W.; Stubbe, J. *J. Biol. Chem.* **1983**, *258*, 4694. (d) Uesugi, S.; Shida, T.; Ikehara, M.; Kobayashi, Y.; Kyogoku, Y. *Nucleic Acids Res.* **1984**, *12*, 1581.

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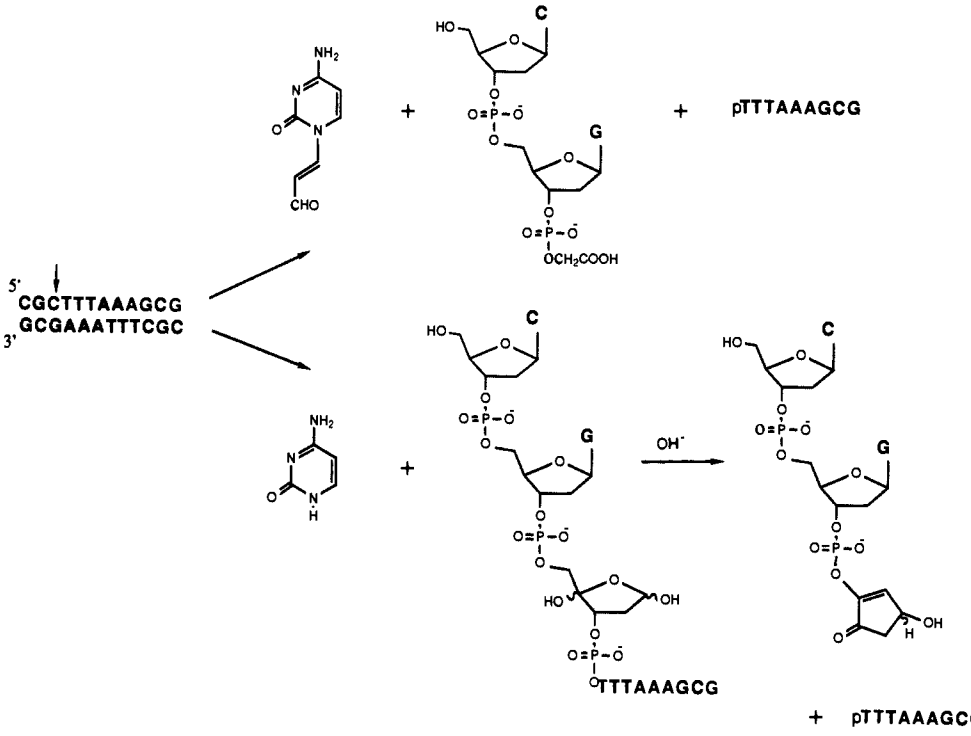
Scheme I. Degradation Products Resulting from Treatment of d(CGCT$_3$A$_3$GCG) with Activated Fe·BLM



includes free bases and C-4′ hydroxyapurinic acids.[8c,9] This lesion does not result in DNA strand scission directly but is labile to a number of chemical reagents (e.g., alkali).[10] These observations have been rationalized on the basis of a mechanism involving an initial bleomycin-mediated, rate-limiting abstraction of C-4′ H from a subset of the deoxyribose moieties in DNA. The derived radical is then presumed to partition to the aforementioned C-4′ hydroperoxide via capture of O_2 or to the C-4′ hydroxy intermediate via hydroxylation of the C-4′ radical. In support of the postulated abstraction of C-4′ H as the rate-limiting step, Wu et al. have measured a tritium isotope effect, k_H/k_T, of 7.2 for Fe·BLM-mediated degradation of poly(dA[4′-^{3}H]dU) at 25 °C.[11]

The overall process of bleomycin-mediated DNA degradation can be subdivided broadly into two major events: drug activation and subsequent DNA degradation. Each of these events undoubtedly represents the sum of a number of simpler events, a complete and precise description of which has not yet been achieved. Peisach and co-workers have shown that Fe(II)·BLM binds O_2 rapidly and reversibly to yield a ternary complex, which collapses in a slower step to afford activated Fe·BLM.[4a] Subsequent chemical reaction of activated bleomycin was shown to be slower still. Povirk et al. have measured the kinetics of binding of Fe(III)·BLM and Cu(II)·BLM to DNA; for Fe(III)·BLM, DNA complexes having lifetimes of up to 22 s were observed.[12] Thus, the rate of appearance of BLM-mediated degradation products could be limited by the intrinsic nature of the degradation chemistry, by the rate of binding of the drug to DNA, or by the rate of dissociation of Fe(III)·BLM from the DNA subsequent to the creation of a DNA lesion. Clearly, an understanding of the processes that limit the rate of BLM activation and DNA degradation can contribute importantly to our understanding of the mode of action of bleomycin.

Reported herein is a study of the kinetics of BLM activation and DNA oligonucleotide degradation that employed Fe(II)·BLM + O_2 as well as Fe(III)·BLM + H_2O_2 for the degradation of d(CGCT$_3$A$_3$GCG), a self-complementary dodecanucleotide shown previously[13] to be a highly efficient substrate for activated BLM. By the use of this system, we demonstrate that the conversion of Fe(II)·BLM + O_2 to activated Fe·BLM was fast at neutral pH but was subject to inhibition by DNA. Product release from DNA was slow, but both base and base propenal were released at the same approximate rate. The formation of activated Fe·BLM by admixture of Fe(III)·BLM + H_2O_2 was found to be slow at neutral pH but rapid at acidic or basic pH. Activation was not inhibited by DNA. Both modes of activation produced species that were of equal competence in mediating DNA oligonucleotide destruction and that quickly underwent self-inactivation in the absence of substrate.

Results and Discussion

The ability of activated Fe·BLM to degrade DNA was studied by the use of the synthetic dodecanucleotide d(CGCT$_3$A$_3$GCG). In earlier work, this oligonucleotide was shown to act as a highly efficient substrate for Fe·BLM, producing lesions amenable to direct characterization and quantitation by analytical reverse-phase HPLC.[13] Because most of the BLM-mediated damage occurred at cytidine$_3$ and cytidine$_{11}$ (i.e., at the two 5′-GC-3′ sites), the product analysis was particularly straightforward. The actual products obtained were of the same type produced upon degradation of DNA (vide supra); these are summarized in Scheme I for lesions produced at cytidine$_3$. As is clear from the scheme, each lesion resulted in the formation of one cytosine or cytosine propenal. Since the same was also true of damage at cytidine$_{11}$ and damage at these two positions constituted the majority of all Fe·BLM-mediated damage to d(CGCT$_3$A$_3$GCG),[13] simple summation of the amount of cytosine and cytosine propenal formed provided a reasonably good quantitative estimate of the total amount of damage to d(CGCT$_3$A$_3$GCG).

Fe(II)·BLM + O_2. The rates of formation of cytosine and 3-(cytosin-1′-yl)propenal resulting from Fe(II)·BLM-mediated

(9) (a) Burger, R. M.; Peisach, J.; Horwitz, S. B. *J. Biol. Chem.* **1982**, *257*, 8612. (b) Wu, J. C.; Stubbe, J.; Kozarich, J. W. *Biochemistry* **1985**, *24*, 7569. (c) Sugiyama, H.; Xu, C.; Murugesan, N.; Hecht, S. M. *J. Am. Chem. Soc.* **1985**, *107*, 4104. (d) Rabow, L. E.; Stubbe, J.; Kozarich, J. W.; Gerlt, J. A. *J. Am. Chem. Soc.* **1986**, *108*, 7130.

(10) Sugiyama, H.; Xu, C.; Murugesan, N.; Hecht, S. M.; van der Marel, G. A.; van Boom, J. H. *Biochemistry* **1988**, *27*, 58.

(11) (a) Wu, J. C.; Kozarich, J. W.; Stubbe, J. *Biochemistry* **1985**, *24*, 7562. (b) Kozarich, J. W.; Worth, L., Jr.; Frank, B. L.; Christner, D. F.; Vanderwall, D. E.; Stubbe, J. *Science* **1989**, *245*, 1396.

(12) Povirk, L. F.; Hogan, M.; Dattagupta, N.; Buechner, M. *Biochemistry* **1981**, *20*, 665.

(13) (a) Sugiyama, H.; Kilkuskie, R. E.; Hecht, S. M.; van der Marel, G. A.; van Boom, J. H. *J. Am. Chem. Soc.* **1985**, *107*, 7765. (b) Sugiyama, H.; Kilkuskie, R. E.; Chang, L.-H.; Ma, L.-T.; Hecht, S. M.; van der Marel, G. A.; van Boom, J. H. *J. Am. Chem. Soc.* **1986**, *108*, 3852.

Table I. Rates of Product Formation from d(CGCT$_3$A$_3$GCG) by Fe(II)·BLM A$_2$ + O$_2$[a]

buffer	incubation time,[b] s	rate of product formation,[c] μM/min
50 mM sodium cacodylate, pH 7.2	15	9.5
	20	9.7
	30	19.2
	45	15.6
	60	14.7
	120	9.8
	180	6.0
	300	2.4
50 mM sodium cacodylate, pH 5.8	5	13.4
	15	20.6
	30	10.4
	60	5.6
50 mM sodium carbonate, pH 8.6	15	9.5
	30	12.5
	60	7.7

[a] Carried out at 0 °C with 0.4 mM Fe(II)·BLM A$_2$ and 2.5 mM d(CGCT$_3$A$_3$GCG) (final nucleotide concentration). [b] Refers to time of incubation of Fe(II) + BLM A$_2$ prior to the addition of DNA. [c] Rates of formation of cytosine + cytosine propenal.

degradation of d(CGCT$_3$A$_3$GCG) are summarized in Table I. In a typical experiment, 0.4 mM $Fe^{II}(NH_4)_2(SO_4)_2$ was mixed with 0.4 mM bleomycin A$_2$ in the appropriate buffer, and the dodecanucleotide was then added. The amounts of products released as a function of time were measured by withdrawing aliquots periodically over a period of 1 h and carrying out HPLC analysis. Rates of product formation were maximal during early stages of the reaction and were measured following the incubation of Fe(II) and BLM A$_2$ alone for varying lengths of time prior to the addition of d(CGCT$_3$A$_3$GCG). The ratio of cytosine propenal:cytosine remained essentially constant at 3–4:1 throughout the time course of individual incubations. As described below, the rates of product formation were measured at three different pH's.

Burger et al.[4a] have reported that the formation of the Fe(II)·BLM-O$_2$ ternary complex was rapid at 0 °C ($t_{1/2} \sim 0.2$ s), whereas the formation of activated Fe·BLM was somewhat slower ($t_{1/2} \sim 6$ s). The decay of activated BLM was slower than either of these activation steps ($t_{1/2} \sim 2$ min at 0 °C). The results in Table I are consistent with these observations. At pH 7.2 in sodium cacodylate buffer, the velocity of product formation was maximal (19.2 μM/min) when ferrous ammonium sulfate and BLM A$_2$ were incubated together under aerobic conditions for at least 30 s prior to the addition of d(CGCT$_3$A$_3$GCG). When the length of the incubation was decreased to 15 s, product formation was only 9.5 μM/min, suggesting incomplete activation. Likewise, longer incubations of Fe(II) + BLM prior to oligonucleotide addition led to progressively smaller amounts of product; the observed rate of 2.4 μM/min after 5 min of incubation suggested that no more than 10–15% of the activated Fe·BLM formed initially was still present.

It is clear from the table that, under our experimental conditions, maximal aerobic activation of Fe(II)·BLM required ~30 s, suggesting a $t_{1/2}$ value of activation in reasonably good agreement with the value measured spectroscopically in unbuffered solution.[4a] It is also apparent from Table I that activated Fe·BLM was not stable in solution in the absence of substrate but decayed to other species incapable of effecting DNA oligonucleotide degradation.[14] If it is assumed that the rates of product formation in the various incubation mixtures in Table I are directly proportional to the concentrations of activated Fe·BLM present upon admixture of d(CGCT$_3$A$_3$GCG), then the data in Table I permit the derivation of a quantitative estimate of the half-life of activated Fe·BLM. When the fraction of activated Fe·BLM calculated by using the above assumption is plotted as a function of time (Figure 1), the half-life of activated Fe·BLM is found to be ~1.5 min at pH 7.2 and 0 °C. This value is in excellent agreement with the reported $t_{1/2}$ value of ~2 min determined spectroscopically in unbuffered solution at 6 °C, with 0.2 mM Fe(II) + 0.26 mM bleomycin.[4a] If it is assumed that there is a single activation rate constant k_1 and a single inactivation rate constant k_2, the latter of which is first order with respect to activated Fe·BLM, then k_2 = 0.46 min^{-1}. Consistent with this analysis, it was found that the half-life of activated Fe·BLM was not a function of Fe·BLM concentration. When the calculated value of k_2 was used to derive the first-order activation rate constant at t_{max} (30 s; cf. Table I), k_1 was found to be 5.40 min^{-1} (0.09 s^{-1}). This value was in excellent agreement with that determined earlier by direct spectroscopic measurement.[4a]

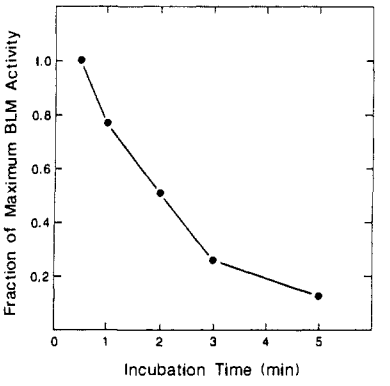


Figure 1. Fraction of activated Fe·BLM as a function of the time of incubation of Fe(II) and BLM A$_2$ (0.4 mM each) at pH 7.2 prior to addition of DNA substrate.

Also shown in Table I are the effects of pH on the rate of Fe·BLM activation and decay. When the aerobic activation of Fe(II)·BLM was carried out in sodium cacodylate at pH 5.8, maximum product formation from d(CGCT$_3$A$_3$GCG) was realized after incubation of Fe(II) + BLM + O$_2$ for only 15 s. A shorter incubation time of 5 s was insufficient for maximal activation, while longer incubation times of 30 or 60 s led to decreased velocities of product formation. Presumably, in the latter two cases decay of activated bleomycin through self-inactivation[14] or other chemical processes was responsible for the observed rates. When the results at pH 5.8 are compared with those at neutral pH, it is seen that both the formation and decay of activated bleomycin are accelerated at low pH. If it is assumed that the maximum observed velocity (20.6 μM/min) corresponds to the maximum concentration of activated Fe·BLM achievable with 0.4 mM Fe(II), then the $t_{1/2}$ for Fe·BLM activation is <5 s (Table I). Likewise, since the velocity of product formation following incubation of Fe(II) and BLM for 30 s was only half of the maximum observed velocity, the half-life of activated Fe·BLM at pH 5.8 must be <15 s. Although the underlying chemical step or steps that are facilitated by the presence of acid are unclear, it may be noted that if aerobic activation of Fe(II)·BLM involved scission of the O–O bond following oxygen binding to produce a perferryl species (vide supra), protonation of the dioxygenated intermediate might well accelerate O–O bond scission.[15] When the rate of product formation was measured in sodium carbonate buffer, pH 8.6, the maximum velocity observed was well below the maximum values obtained at the other pH's studied.

As noted above, after each period of aerobic (pre)incubation of Fe(II) + BLM the *initial* velocity of product formation following DNA addition was always noted to provide the maximum value. This was true even for those incubation periods (e.g., 15 s at pH 7.2) insufficient to maximize the concentration of activated Fe·BLM prior to DNA addition, in spite of the fact that measurement of velocity of product formation after DNA addition was carried out over a time period long enough (1 h) to have permitted complete activation. The implication of this analysis,

(14) (a) Takita, T.; Muraoka, Y.; Nakatani, T.; Fujii, A.; Haka, Y.; Umezawa, H. *J. Antibiot.* **1978**, *31*, 1073. (b) Nakamura, J.; Peisach, J. *J. Antibiot.* **1988**, *41*, 639. (c) Van Atta, R. B.; Long, E. C.; Hecht, S. M.; van der Marel, G. A.; van Boom, J. H. *J. Am. Chem. Soc.* **1989**, *111*, 2722.

(15) (a) Poulos, T. L.; Kraut, J. *J. Biol. Chem.* **1980**, *255*, 8199. (b) Groves, J. T.; Watanabe, Y. *J. Am. Chem. Soc.* **1986**, *108*, 7834.

Table II. Effect of d($CGCT_3A_3GCG$) Concentration of the Rate of Product Formation by Fe(II)·BLM A_2[a]

d($CGCT_3A_3GCG$), mM[b]	incubation time,[c] s	rate of product formation,[d] μM/min
2.5	0	10.2
5.0	0	4.2
2.5	30	19.2
5.0	30	23.3

[a] Carried out at 0 °C with 0.4 mM Fe(II)·BLM A_2 in 50 mM sodium cacodylate buffer, pH 7.2. [b] DNA nucleotide concentration. [c] Refers to time of incubation of Fe(II) + BLM A_2 prior to the addition of DNA. [d] Rates of formation of cytosine + cytosine propenal.

Table III. Effect of Sodium Ascorbate on the Rate of Product Formation from d($CGCT_3A_3GCG$) by Fe(II)·BLM A_2 + O_2[a]

reducing agent, mM	incubation time,[b] s	rate of product formation,[c] μM/min
none	0	7.1
5 mM sodium ascorbate	0	17.6
none	30	16.4
5 mM sodium ascorbate	15	17.6
	30	12.8

[a] Carried out at 0 °C with 0.4 mM Fe(II)·BLM A_2 + 2.5 mM d($CGCT_3A_3GCG$) (final nucleotide concentration) in 50 mM sodium cacodylate buffer, pH 7.2. [b] Refers to time of incubation of Fe(II) + BLM A_2 prior to the addition of DNA. [c] Rates of formation of cytosine propenal.

i.e., that DNA must inhibit the aerobic activation of Fe(II)·BLM, was tested directly. When the rates of product formation were measured in reactions in which d($CGCT_3A_3GCG$) was present throughout the entire reaction, an increase in the concentration of the dodecanucleotide actually resulted in a decrease in the velocity of product formation (Table II). On the other hand, when Fe(II) + BLM were incubated aerobically for 30 min prior to DNA addition, the rate of product formation was greater and increased with increasing DNA substrate concentration. These observations are consistent with the earlier suggestion that the formation of activated Fe·BLM from Fe(II)·BLM + O_2 may require an additional electron.[4,13a,16] In the absence of external reductants, activation would presumably occur by collision of two Fe(II)·BLM's in the presence of O_2,[17] a process that might prove less facile if the available Fe(II)·BLM's were largely bound to DNA.[18]

To determine whether an external reducing agent could reverse the inhibition of Fe(II)·BLM activation by DNA, the rates of product formation were measured in the presence of sodium ascorbate. As indicated in Table III, the inclusion of 5 mM sodium ascorbate in a reaction mixture containing 2.5 mM d($CGCT_3A_3GCG$) increased the rate of cytosine propenal formation[19] from 7.1 μM/min to 17.6 μM/min. The latter value was comparable to the rate of product formation noted when Fe(II) + BLM were incubated alone for 30 s in the absence of ascorbate prior to DNA addition. Although these data would seem to indicate that ascorbate completely reversed the inhibition of Fe(II)·BLM activation caused by DNA, it may be noted that in the presence of 0.4 mM Fe(II)·BLM it should be possible, in principle, to obtain 400 μM activated Fe·BLM, in comparison with an anticipated value of 200 μM in the absence of an external reductant.[13,16] Nonetheless, incubation of Fe(II) + BLM + sodium ascorbate for 15 or 30 s prior to DNA addition did not result in enhancement of the rate of C-propenal formation, relative to the rate obtained in the absence of preincubation.

Table IV. Rates of Product Formation from d($CGCT_3A_3GCG$) by Fe(III)·BLM A_2 + H_2O_2[a]

Fe(III)·BLM A_2, mM	d($CGCT_3A_3GCG$), mM[b]	H_2O_2, mM	rate of product formation,[c] μM/min
0.2	5.0	5.0	3.4
0.2	5.0	10.0	5.8
0.2	5.0	25.0	7.2
0.2	5.0	50.0	10.1
0.4	5.0	5.0	7.6
0.2	15.0	5.0	3.8
0.2	50.0	5.0	3.9

[a] Carried out at 0 °C with 0.2 mM Fe(III)·BLM A_2 in 50 mM sodium cacodylate, pH 7.2. [b] DNA nucleotide concentration. [c] Rates of formation of cytosine + cytosine propenal.

It is interesting to note that, in all of the product measurements involving Fe(II)·BLM, the maximum rate of product formation was ~20 μM/min. Since 200 μM product should be accessible with 400 μM Fe(II)·BLM in the absence of reducing agents,[13,16] this indicates that, in the fastest reactions studied, only about 10% of the total product possible was formed within 1 min.[20] In contrast, the activation of Fe·BLM was found to be much more rapid, with a $t_{1/2}$ of only several seconds. Thus BLM reacts rapidly with Fe(II) in the presence of O_2, and in the absence of DNA, to form an activated species that can effect the slower release of bases and base propenals from DNA.[21]

Fe(III)·BLM + H_2O_2. Ferric bleomycin activated with H_2O_2 has been shown to afford a species indistinguishable by EPR from activated BLM obtained by admixture of Fe(II)·BLM + O_2.[4a] Further, the two activated species produced the same products from substrate DNA oligonucleotides;[9d,11] when comparable concentrations of O_2 were present, the products were formed in the same ratios.[11] In our laboratory, we have also found that the species resulting from admixture of Fe(III)·BLM + H_2O_2 afforded the same products from d($CGCT_3A_3GCG$) as those obtained upon treatment of the oligonucleotide with Fe(II)·BLM under aerobic conditions.[22] Both reagents produced lesions primarily at $cytidine_3$ and $cytidine_1$) and afforded cytosine and cytosine propenal in the same ratio from these two sites.[13,21] At the outset of this work, we were interested in comparing the two modes of BLM activation; outlined below is the characterization of hydrogen peroxide activated Fe(III)·BLM.

Listed in Table IV are the rates of cytosine + cytosine propenal formation observed upon treatment of d($CGCT_3A_3GCG$) with Fe(III)·BLM + H_2O_2. In a typical experiment, 0.2 mM Fe(III)·BLM was incubated with d($CGCT_3A_3GCG$) in sodium cacodylate buffer, pH 7.2, at 0 °C; the reaction was initiated by addition of H_2O_2, and aliquots were removed periodically to permit measurement of product formation by HPLC analysis. The rates of product formation remained constant until two DNA lesions had been produced for each Fe(III)·BLM; as for Fe(II)·BLM + O_2, the ratio of cytosine propenal:cytosine was 3–4:1 and did not vary during the course of individual incubations.

As shown in the table, an initial experiment indicated that the rate of product formation with 0.2 mM Fe(III)·BLM A_2, 5 mM d($CGCT_3A_3GCG$), and 5 mM H_2O_2 was 3.4 μM/min. Because this value was much smaller than the maximum velocities obtained with Fe(II)·BLM (cf. Tables I–III), the effect of hydrogen per-

(16) Hecht, S. M. *Acc. Chem. Res.* **1986**, *19*, 383.

(17) In fact, when 0.2 mM Fe(II)·BLM A_2 was incubated at 0 °C for 30 s prior to DNA addition, the velocity of C-propenal formation was 4.6 μM/min, in comparison with a value of 16.4 μM/min obtained by using 0.4 mM Fe(II)·BLM (cf. Table III).

(18) In fact, inhibition of Fe·BLM activation and consequent DNA degradation has been observed previously: (a) Burger, R. M.; Peisach, J.; Blumberg, W. E.; Horwitz, S. B. *J. Biol. Chem.* **1979**, *254*, 10906. (b) Albertini, J.-P.; Garnier-Suillerot, A.; Tosi, L. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 557. (c) Ciriolo, M. R.; Magliozzo, R. S.; Peisach, J. *J. Biol. Chem.* **1987**, *262*, 6290. (d) Reference 14c.

(19) The observation of cytosine production by HPLC was obscured by sodium ascorbate.

(20) Over the 1-h time course of DNA oligonucleotide degradation ~80% of the theoretical amount of product was formed, verifying both the presumed stoichiometry of Fe·BLM activation and DNA degradation[13] and the ability of DNA to prevent the self-inactivation of activated Fe·BLM.[14]

(21) The observation of release of bases and base propenals on a similar time scale may be noted to differ from a report that base propenal release is slow: Burger, R. M.; Projan, S. J.; Horwitz, S. B.; Peisach, J. *J. Biol. Chem.* **1986**, *261*, 15955.

(22) Sugiyama, H.; Long, E. C.; Hecht, S. M. Unpublished results.

Table V. Effect of Incubation Time of Fe(III)·BLM A_2 + H_2O_2 on the Extent of Product Formation from d($CGCT_3A_3GCG$)[a]

incubation time,[b] min	cytosine + cytosine propenal, μM	incubation time,[b] min	cytosine + cytosine propenal, μM
0	90.1	20	74.3
5	83.7	25	50.9
10	89.6	30	37.9
15	90.3	35	29.9

[a] Carried out at 0 °C with 0.2 mM Fe(III)·BLM A_2 + 5 mM H_2O_2 and 5.0 mM d($CGCT_3A_3GCG$) (final nucleotide concentration) in 50 mM sodium cacodylate, pH 7.2, over a period of 35 min. [b] Refers to time of incubation of Fe(III)·BLM A_2 + H_2O_2 prior to the addition of DNA.

Table VI. Effect of pH on the Rate of Product Formation from d($CGCT_3A_3GCG$) by Fe(III)·BLM A_2 + H_2O_2[a]

buffer	rate of formation of cytosine + cytosine propenal, μM/min
50 mM sodium cacodylate, pH 7.2	3.4
50 mM sodium carbonate, pH 8.6	7.0
50 mM sodium borate, pH 8.4	7.2
50 mM sodium cacodylate, pH 5.8	12.7
50 mM potassium acetate, pH 5.6	12.3

[a] Carried out at 0 °C with 0.2 mM Fe(III)·BLM A_2 + 5.0 mM H_2O_2 and 5.0 mM d($CGCT_3A_3GCG$) (final nucleotide concentration).

oxide concentration was studied. The rates of product formation did increase with increasing [H_2O_2], but the greatest value obtained (at 50 mM H_2O_2) was 10.1 μM/min. Thus, even at 50 mM H_2O_2 not all of the Fe(III)·BLM present could be activated within a short time. Also tested was the effect of increasing the concentration of Fe(III)·BLM. At 5 mM H_2O_2, the observed rate of product formation from DNA was found to be first order in Fe(III)·BLM. Thus, doubling the concentration of Fe(III)·BLM essentially doubled the rate of product formation. In contrast, the rate of product formation was not dependent on the concentration of d($CGCT_3A_3GCG$) over the range 5–50 mM, suggesting that those Fe(III)·BLM molecules that were activated subsequently reacted with d($CGCT_3A_3GCG$) and that excess DNA oligonucleotide could not bind any activated Fe·BLM that otherwise failed to produce cytosine + cytosine propenal. In the belief that the decreased rate of product formation with this activated Fe·BLM was due to slow reaction between Fe(III)·BLM and H_2O_2, we measured the total amount of product formed when Fe(III)·BLM and H_2O_2 were incubated together in the absence of substrate for varying lengths of time prior to incubation with 5 mM d($CGCT_3A_3GCG$) for 35 min. As shown in Table V, when Fe(III)·BLM and H_2O_2 were incubated alone for periods up to 15 min prior to DNA addition, there was little effect on the ultimate total yield of cytosine + cytosine propenal. However, longer incubations resulted in progressively lower product yields; after a 35-min (pre)incubation, the ultimate yield of cytosine + cytosine propenal was only about 30% of that obtained in the absence of an initial incubation. We attribute the progressively lower product yields to successively greater self-inactivation of H_2O_2-activated Fe·BLM prior to DNA addition. That this phenomenon did not become apparent during the first 15 min of incubation in the absence of DNA (Table V) argues strongly that little Fe(III)·BLM inactivation had occurred during this period of time, probably reflecting limited BLM activation.

In an effort to accelerate the reaction between Fe(III)·BLM and H_2O_2, rates of product formation were measured at acidic and basic pH's; the results are summarized in Table VI. At basic pH, in either carbonate or borate buffer, the total product formed per unit time was approximately twice as great as the amount obtained at pH 7.2. Even more striking were the results obtained at lower pH in acetate or cacodylate buffers, where product formation was enhanced almost 4-fold relative to the rate at neutral pH. Optimization of the activation of Fe(III)·BLM with H_2O_2 was investigated in more detail at pH 5.8 (Table VII). When 0.2 mM Fe(III)·BLM was employed for activation, increasing the

Table VII. Effect of d($CGCT_3A_3GCG$) and H_2O_2 Concentrations on the Rate of Product Formation by Fe(III)·BLM A_2[a]

d($CGCT_3A_3GCG$), mM[b]	H_2O_2, mM	rate of formation of cytosine + cytosine propenal, μM/min
5.0	5.0	12.7
5.0	10.0	21.6
5.0	25.0	20.6
2.5	10.0	20.5

[a] Carried out at 0 °C with 0.2 mM Fe(III)·BLM A_2 in 50 mM sodium cacodylate, pH 5.8. [b] DNA nucleotide concentration.

Table VIII. Effect of Incubation Time of Fe(III)·BLM A_2 + H_2O_2 on the Rate of Product Formation from d($CGCT_3A_3GCG$)[a]

incubation time,[b] s	rate of formation of cytosine + cytosine propenal, μM/min
0	21.6
15	20.7
30	17.2
60	10.7
120	4.1
180	2.5

[a] Carried out at 0 °C with 0.2 mM Fe(III)·BLM A_2 + 10 mM H_2O_2 and 5.0 mM d($CGCT_3A_3GCG$) (final nucleotide concentration) in 50 mM sodium cacodylate, pH 5.8. [b] Refers to time of incubation of Fe(III)·BLM A_2 + H_2O_2 prior to the addition of DNA.

concentration of H_2O_2 from 5 to 10 mM further enhanced the rate of product formation to 21.6 μM/min, thus approximating the rates obtained by the use of aerobically activated Fe(II)·BLM (cf. Tables I–III). That this rate of product formation may be the maximum rate achievable in the present system, reflecting facile activation of the majority of the Fe·BLM present, was consistent with the observations that no further rate enhancement could be obtained at 25 mM H_2O_2 concentration and that, as noted at neutral pH, the rate of product formation was not affected by a change in substrate concentration. More direct evidence demonstrating rapid activation of Fe(III)·BLM by H_2O_2 at pH 5.8 is presented in Table VIII. Here, rates of product formation were measured by adding the DNA substrate after Fe(III)·BLM and H_2O_2 had been incubated in the absence of DNA for varying lengths of time. As shown in the table, maximum velocity of product release was achieved after 15 s; when the lengths of the incubation prior to DNA addition were increased, the velocity of product formation decreased steadily. As noted above for Fe(III)·BLM activation at neutral pH, the diminution of product formation at longer incubation times undoubtedly reflects self-inactivation of activated Fe-BLM in the absence of a DNA substrate[14] and indicates that the $t_{1/2}$ for Fe(III)·BLM activation at pH 5.8 was <8 s. In comparison, the analogous activation process required several minutes at neutral pH (cf. Table V). Also apparent from Table VIII was the instability of the activated Fe·BLM derived from Fe(III)·BLM + H_2O_2. Incubation in the absence of substrate for 60 and 180 s prior to DNA addition reduced the velocity of product formation by ~50% and 90%, respectively. As before, if it is assumed that the extent of product formation is proportional to the amount of activated Fe·BLM present, then $t_{1/2}$ for the activated Fe·BLM derived from Fe(III)·BLM + H_2O_2 was ~1 min at pH 5.8.

While it is not possible on the basis of the available data to provide a detailed mechanistic rationale for the observed acceleration of Fe(III)·BLM activation in acidic and basic solution, it may be noted that analogous observations have been made for the reaction of Fe(III)·EDTA with H_2O_2.[23] Detailed study of the latter system suggested that the Fe(III)·EDTA complex exists in solution with a bound hydroxide ion. Under general acid/base catalysis, protonation of the hydroxide ion may facilitate its departure; enhanced formation of the nucleophilic hydroperoxide anion may result in faster displacement of the existing metal

(23) (a) Bull, C.; McClune, G. J.; Fee, J. A. *J. Am. Chem. Soc.* **1983**, *105*, 5290. (b) Ahmad, S.; McCallum, J. D.; Shiemke, A. K.; Appelman, A. H.; Loehr, T. M.; Sanders-Loehr, J. *Inorg. Chem.* **1988**, *27*, 2230.

ligand. Conceivably, these principles may also apply to the reaction of Fe(III)·BLM and H_2O_2.

One other interesting facet of Fe·BLM activation is apparent from Table VIII. Unlike aerobic activation of Fe(II)·BLM, which was found to be suppressed by added DNA (Table II), d-($CGCT_3A_3GCG$) had no measurable effect on H_2O_2-mediated activation of Fe(III)·BLM. Thus, following admixture of Fe(III)·BLM + H_2O_2, the same (maximal) rate of product formation was noted whether d($CGCT_3A_3GCG$) was present from the start of incubation or was added after 15 s. Presumably, this observation reflects the fact that no reductive transformation of Fe(III)·BLM (via collision of two Fe·BLM's) is required to achieve activation.

Experimental Section

Bleomycin (blenoxane) was obtained from Bristol Laboratories through the courtesy of Dr. William Bradner and was fractionated as described.[24] Ferrous and ferric ammonium sulfate were obtained from Alfa Products and were used without further purification. HPLC analysis was performed by using a Varian HPLC that included a Model 2050 variable wavelength detector. The analysis of degradation products arising from the dodecanucleotide was carried out on a Rainin C_{18} column (4.6 mm × 10 cm; 3-μm particle size), which was washed with 0.1 M NH_4OAc at a flow rate of 1.4 mL/min. Quantitation of products against an internal standard (7-methylguanine) was done with a Hewlett-Packard Model HP3394A integrator.

General Procedure for Kinetic Studies of d($CGCT_3A_3GCG$) Degradation by Fe(III)·BLM. The following procedure is typical. A reaction mixture (100-μL total volume, in a 0.5-mL conical vial) containing 200 μM Fe(III)·BLM A_2 and 5 mM d($CGCT_3A_3GCG$) (DNA nucleotide concentration) in 50 mM sodium cacodylate buffer, pH 7.2, was cooled to 0 °C and treated with H_2O_2 to a final concentration of 5.0 mM. The reaction mixture was stirred open to air at 0 °C; 10-μL aliquots were withdrawn at predetermined time intervals and quenched with 0.1 M sodium thiosulfate (which was shown in separate experiments to rapidly consume available H_2O_2). Each aliquot was frozen promptly in dry ice and thawed immediately before HPLC analysis. The following HPLC retention times were observed: cytosine, 1.4 min; 7-methylguanine, 11.5 min; 3-(cytidin-1′-yl)propenal, 17.5 min. Reactions carried out in other buffers employed the same procedure.

General Procedure for Kinetic Studies of d($CGCT_3A_3CCG$) Degradation by Fe(II)·BLM. The following procedure is typical. A reaction mixture (100-μL total volume, in a 0.5-mL conical vial) containing 400 μM BLM A_2 and 2.5 mM d($CGCT_3A_3GCG$) in 50 mM sodium cacodylate, pH 7.2, was cooled to 0 °C and treated with cold $Fe^{II}(NH_4)_2(SO_4)_2$ solution to a final concentration of 400 μM. The reaction was stirred open to air at 0 °C; 10-μL aliquots were withdrawn at predetermined time intervals and quenched with 10 μL of 0.1 M trichloroacetic acid. Each aliquot was frozen promptly in dry ice and thawed immediately before HPLC analysis, which was carried out as described above.

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(24) Chien, M.; Grollman, A. P.; Horwitz, S. B. *Biochemistry* **1977**, *16*, 3641.