Ferric Bleomycin Catalyzed Reduction of 10-Hydroperoxy-8,12-octadecadienoic Acid: Evidence for Homolytic O–O Bond Scission†

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ABSTRACT: 10-Hydroperoxy-8,12-octadecadienoic acid (**1**) is reduced by ferric bleomycin in aqueous and methanol solutions to yield 10-oxo-8-decenoic acid (**2**) as the major product (80–90%). Trace amounts of 10-oxo-8,12-octadecadienoic acid (**3**) (5–10%) and 10-hydroxy-8,12-octadecadienoic acid (**4**) (5–10%) were also detected. The reduction product ratios remained relatively constant in the presence or absence of the reducing substrate phenol, over the pH range 6.5–8.5, in incubations from 30 s to 1 h, and over a series of ferric drug concentrations. In the presence of phenol, incubations of ferric bleomycin and **1** yielded 2,2′-biphenol and 4,4′-biphenol as oxidation products. In reactions where phenol was replaced with the drug's biological substrate DNA, **1** was found to support ferric bleomycin mediated DNA degradation. Extracts from these assays also found **2** to be the major reduction product derived from the oxidant, with trace quantities of **3** and **4** present. Control experiments demonstrated the reactions to be dependent on both **1** and ferric bleomycin. The reduction products **2** and **3** have previously been shown to originate from transient alkoxyl radicals formed by homolysis of the peroxy O–O bond. Product **4** results from heterolysis of the peroxy O–O bond [Labeque, R., & Marnett, L. J. (1987) *J. Am. Chem. Soc. 109*, 2828–2829]. The results of this investigation indicate that ferric bleomycin catalyzes the homolytic cleavage of the O–O bond of **1** almost exclusively while supporting various oxidative reactions.

The bleomycins constitute a unique family of low molecular weight glycopeptide antibiotics with demonstrated antitumor activity (Sikic et al., 1985). The therapeutic efficacy of these antineoplastic agents is presumed to be related to their ability to degrade DNA, a process for which ferrous iron chelation and reductive activation of molecular dioxygen play an important mechanistic role (Burger et al., 1986; Ajmera et al., 1986; Rabow et al., 1986; Sugiyama et al., 1985; Wu et al., 1985a,b).

By analogy to other known oxygen-dependent enzymes and chemical model systems, it is presumed that a reactive oxygen intermediate is generated by the reductive cleavage of the dioxygen O–O bond. The discovery that the binding and reduction of dioxygen by the ferrous drug complex can be circumvented by the use of hydroperoxides and ferric bleomycin provides one avenue for the design of mechanistic studies which probe the molecular details of the drug-mediated oxygen activation. In particular, one question of fundamental importance in this regard concerns the mechanism by which iron–bleomycin mediates the cleavage of the O–O bond. In general, there are two fundamental mechanisms that are reasonable for metal- or metallocomplex-mediated hydroperoxide reduction: a heterolytic or homolytic cleavage of the peroxy O–O bond. By extension then, a similar mechanism may apply to the O_2-supported reactions in which molecular dioxygen is believed to be reduced to the level of hydrogen peroxide (Burger et al., 1979a,b, 1981, 1982, 1983, 1985a). Indeed, Peisach and co-workers have reported that the Mössbauer and EPR characteristics of "activated bleomycin", the last detectable drug–iron–oxygen complex prior to substrate oxidation, are consistent with a metal–peroxide complex (Burger et al., 1981, 1983). Furthermore, Padbury and Sligar have measured equivalent substrate deuterium isotope effects on the N-demethylation of *N,N*-dimethylaniline by the ferrous bleomycin–dioxygen system and ferric bleomycin supported by hydrogen peroxide and secondary and tertiary alkyl hydroperoxides (Padbury & Sligar, 1986; Stubbe & Kozarich, 1987). Also, Hecht and co-workers have recently reported quantitative incorporation of oxidant-derived oxygen in the epoxidation of *cis*-stilbene catalyzed by both the ferrous–dioxygen and ferric–hydroperoxide systems (Heimbrook et al., 1987). These results further advocate a common intermediate is produced by all the oxidants.

Padbury and Sligar have recently reported that in the absence of reducing substrates, incubations of cumene hydroperoxide or 2-phenyl-2-butyl hydroperoxide and ferric bleomycin yielded catalytic amounts of acetophenone, the product of the β-scission of transient alkoxyl radicals formed from the homolytic cleavage of the peroxy O–O bond. Unfortunately, significant levels of the corresponding alcohols were also detected, and as a result the heterolytic O–O bond cleavage pathway cannot be excluded as a possible mechanism. Products indicative of homolytic O–O bond scission were also found to be produced from incubations of (phenylperoxy)acetic acid with ferric bleomycin (Padbury & Sligar, 1987).

Labeque and Marnett have recently developed 10-OOH-18:2[1] as a novel probe for alkoxyl radical formation during the reaction of hydroperoxides with biologically important

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[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Blm, bleomycin; HPLC, high-performance liquid chromatography; P_i, phosphate; 10-OOH-18:2, 10-hydroperoxy-8,12-octadecadienoic acid.

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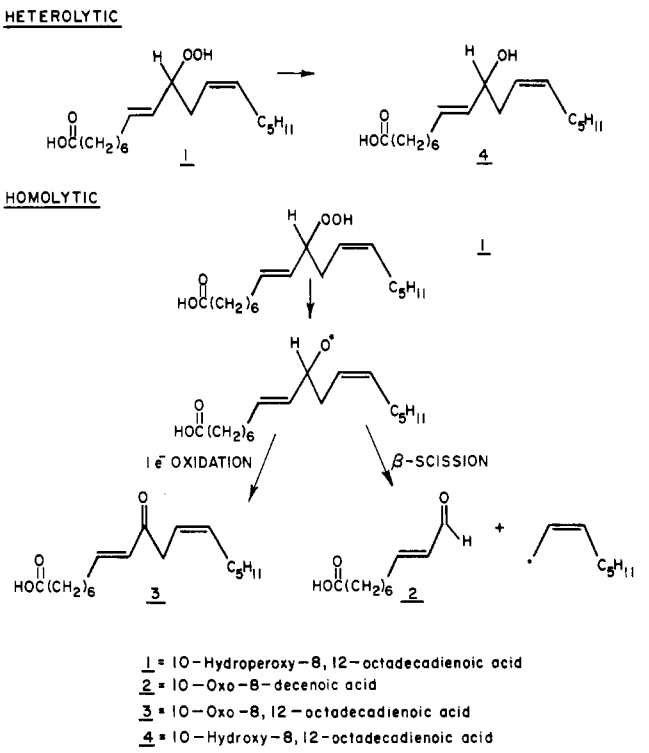


FIGURE 1: Homolytic and heterolytic breakdown pathways for 10-OOH-18:2.

metals and metallosystems (Labeque & Marnett, 1987). This oxidant offers several advantages over the simple alkyl hydroperoxides. Due to its rapid β-scission rate, a result of the extremely stable allylic radical leaving group, a single major product, 10-oxo-8-decenoic acid (**2**), is obtained from alkoxyl radical formation (Figure 1). Minor amounts of 10-oxo-8,12-octadecadienoic acid (**3**) can be formed from oxidation of the transient alkoxyl radicals. Heterolytic reduction of the hydroperoxide yields 10-hydroxy-8,12-octadecadienoic acid (**4**). Furthermore, the 10-OOH-18:2 is compatible with a wide range of solvent environments, facilitating investigations in the presence of reducing substrates.

In this investigation the results of the reduction of 10-OOH-18:2 by ferric bleomycin under a variety of reaction conditions are reported. The results indicate that ferric bleomycin catalyzes the homolytic cleavage of the O–O bond of 10-OOH-18:2 almost exclusively while supporting the oxidation of phenol or degradation of DNA.

Experimental Procedures

Bleomycin sulfate (Blenoxane) was a gift of Bristol Laboratories and was used without further purification. A molecular weight of 1550 was assumed and $E_{292} = 1.45 \times 10^4$ M^{-1} cm^{-1} for the metal-free drug (Burger et al., 1979). Ferric bleomycin was prepared as previously described (Padbury & Sligar, 1985). The [1-^{14}C]-10-hydroperoxy-8,12-octadecadienoic acid was prepared by photooxygenation of [1-^{14}C]-linoleic acid as described by Labeque and Marnett (1987). Structural confirmation of 10-oxo-8-decenoic acid, 10-oxo-8,12-octadecadienoic acid, and 10-hydroxy-8,12-octadecadienoic acid as the reduction products of 10-OOH-18:2 has previously been documented (Labeque & Marnett, 1987). All other organic and inorganic reagents were obtained from commercial sources.

Phenol Oxidation Reactions. Reactions were carried out aerobically at room temperature in 1.5 mL of the respective solvent and contained 0.75 mM phenol, 1 mM **1**, and an appropriate amount of ferric bleomycin. Reactions were initiated by addition of **1** and terminated by acidification to pH 3.5 with 3 N HCl, followed by immediate extraction with 4 × 3 mL volumes of ethyl acetate. The combined extracts were dried over $MgSO_4$ and filtered, and the extraction solvent was removed by vacuum evaporation. The resulting residue was dissolved in a minimal volume of 10% isopropyl alcohol in hexane. The reduction products were separated on a Varian Model 5000 HPLC equipped with an Altech 10-μm silica column (25 cm × 4.6 mm) and were eluted with 11:0.5:88.5 tetrahydrofuran/acetic acid/hexane at 1.4 mL/min (t_R: **1** = 8.8 min; **2** = 11.8 min; **3** = 6.6 min; **4** = 13.6 min). Radioactivity eluting from the column was quantitated in continuous fashion with a Radiomatic FloOne HP radioactivity flow detector and Scinti Verse LC premixed scintillant (Fisher). Extraction efficiency was determined to be 50–60%, 90%, and 95% from Hepes, phosphate, and methanol, respectively, on the basis of total recovered radioactivity.

A set of parallel reaction residues were dissolved in a minimal volume of methanol and evaluated qualitatively for low molecular weight phenol oxidation products by HPLC using a Varian 5000 HPLC equipped with a Varian 10-μm Micro Pak MCH reverse-phase column (30 cm × 4 mm). Products were eluted in a mobile phase of 10:30:70 tetrahydrofuran/methanol/water at a flow of 1.0 mL/min for 10 min followed by an increase in the flow rate at 0.1 mL/min for 10 min to 2.0 mL/min and monitored at 220 nm with a Varian Vari-Chrom UV-vis detector (t_R: phenol = 9.8 min; 2,2′-biphenol = 26.8 min; 4,4′-biphenol = 20.0 min; catechol = 6.2 min; hydroquinone = 3.6 min).

DNA Oxidation Reactions. Incubations were carried out aerobically in 1.8-mL Eppendorf tubes containing 1 mL of 20 mM sodium cacodylate, pH 7.0, at room temperature. Reactions contained 3.2 μg of plasmid DNA,[2] 10.8 μM ferric bleomycin, and 0.5 mM **1**. Reactions were initiated by the addition of **1**. After a 30-min incubation, 100 μL of 2 M NaCl was added to each reaction. Each reaction mixture was divided into two equal volumes, and subsequent sample workup was carried out in parallel. To each tube, 1.1 mL of 95% ethanol was added and the DNA precipitated for 2 h at 0–4 °C. Each sample pair was centrifuged for 12 min in a Brinkmann microfuge, and the resulting supernatants were removed and combined. The combined sample supernatants were worked-up and evaluated for the hydroperoxide reduction products as described for the phenol oxidation reactions. Trace amounts of remaining liquid in the DNA pellets were removed by vacuum evaporation. For each reaction, one of the sample DNA pellets was redissolved in 10 μL of deionized water and diluted with 10 μL of gel loading buffer III (Maniatis et al., 1982). This sample was evaluated for DNA cleavage by gel electrophoresis on a 1.2% agarose gel for 2 h at 100 V and visualized by ethidium bromide staining (Maniatis et al., 1982). The second set of DNA pellets were dissolved in 10 mL of scintillation fluid (3:1 toluene/Triton X-114, 13.6 mM PPO, 0.55 mM POPOP) and counted on a Beckman Model LS3801 scintillation counter for coprecipitated radioactivity.

Results

The reaction products from incubations of 10-OOH-18:2 with the individual components of the ferric bleomycin reaction system are summarized in Table I. In the absence of a catalyst, or in the presence of unchelated ferric salts, only a small fraction of the hydroperoxide (~4–5%) degrades over the 1-h incubation. In both cases the aldehyde (**2**) is formed

[2] Plasmid DNA, pUS 207, was kindly provided by Drs. B. P. Unger and S. G. Sligar, Department of Biochemistry, University of Illinois.

Table I: Metabolism of 10-Hydroperoxy-8,12-octadecadienoic Acid by Ferric Bleomycin[a]

catalyst	1 (% metabolized)[b]	2 (%)[c]	3 (%)[c]	4 (%)[c]
none	4	75	ND[d]	25
unchelated Fe(III)	5	80	20	ND[d]
metal-free BLM	20	70	25	5
Fe^{III}–BLM	93	96	3	1
Fe^{III}–BLM (–phenol)	79	92	3	5

[a] One-hour incubations were carried out as described under Experimental Procedures in 10 mM Hepes, pH 7.5, containing 0.16 mM of the respective catalyst. [b] Values are reported as percent metabolism on the basis of recovered **1**. [c] Values are reported as percent of the total metabolized products. [d] ND, not detected.

Table II: Metabolism of 10-Hydroperoxy-8,12-octadecadienoic Acid by Ferric Bleomycin as a Function of pH and Solvent[a]

solvent	1 (% metabolized)[b]	2 (%)[c]	3 (%)[c]	4 (%)[c]
10 mM Hepes				
pH 6.5	90	82	12	6
pH 7.0	98	92	2	6
pH 7.5	99	97	2	1
pH 8.0	93	97	1	2
pH 8.5	91	97	2	1
10 mM KP_i, pH 7.5	98	85	8	7
methanol	85	76	15	9

[a] One-hour incubations containing 0.10 mM Fe^{III}–BLM were carried out as described under Experimental Procedures. [b] Values are reported as percent metabolism on the basis of recovered **1**. [c] Values are reported as percent of the total metabolized products.

as the major reduction product. Interestingly however, while the alcohol was formed as the minor product in the absence of a catalyst, the ketone (**3**) is the minor product from incubations with ferric ion. Incubations containing metal-free bleomycin demonstrated a 4-fold increase in total metabolism of the hydroperoxide as compared to the buffer and ferric ion systems. The aldehyde (**2**) was again the primary reduction product with minor amounts of the ketone (**3**) and trace quantities of the alcohol (**4**) present. The combined quantities of the aldehyde and ketone accounted for 95% of the reduction products. Although efforts were made to ensure use of metal-free reagents, it is possible that a portion of the metabolism detected with the unreconstituted drug can be attributed to trace amounts of contaminating redox active metals coordinated to bleomycin. In the presence of phenol, the ferric bleomycin complex catalyzes nearly total reduction of the hydroperoxide (~93%) with almost exclusive formation of the aldehyde (**2**). Only trace amounts of the ketone (**3**) and alcohol (**4**) were detected. In the absence of phenol, the total metabolism was reduced by approximately 14%, while the overall product profile remained relatively constant. The decrease in total metabolism products can probably be attributed to increased drug self-inactivation in the absence of the protective effects of a reducing substrate.

The possibility that the aldehyde (**2**) and ketone (**3**) are produced by secondary metabolism of the alcohol (**4**) is ruled out by the demonstration that no aldehyde or ketone was produced in incubations where 10-OOH-18:2 is replaced with the alcohol (**4**) and H_2O_2 (data not shown). Structural confirmation of the identity of the aldehyde as the major reduction product was obtained by GC–MS and ^1H NMR as previously described (data not shown; Labeque & Marnett, 1987). The combined totals of the three reduction products cited accounted for 85–90% of the total recovered radioactivity. The formation of the aldehyde as the principal product indicates that the drug complex functions by a redox mechanism as opposed to general acid catalysis which does not produce any aldehyde (Labeque & Marnett, 1987; Gardner & Plattner, 1984; Wurzenberger & Grosh, 1986).

The oxidation of phenol by ferric bleomycin and 10-OOH-18:2 was assayed by qualitative evaluation of parallel reactions for low molecular weight oxidation products. Reaction extracts contained 2,2′-biphenol and 4,4′-biphenol, while the hydroxylated products, catechol and hydroquinone, were not detected. Control reactions demonstrated the presence of the biphenols to be dependent on both ferric bleomycin and the hydroperoxide (data not shown). The presence and identification of longer chain polymeric oxidation products were not pursued.

The iron–bleomycin complex of physiological importance has been reported to be stable over the pH range 6.5–8.5 (Burger et al., 1981; Dabrowiak, 1982). The reduction of 10-OOH-18:2 by ferric bleomycin in Hepes buffer in the presence of phenol over this pH range is reported in Table II. In essence, complete reduction of the hydroperoxide is achieved at each pH in 1-h incubations. Consistent with the control incubation results (Table I), the aldehyde (**2**) is formed as the primary product with small amounts of the ketone (**3**) and alcohol (**4**) present. The constant yield of ketone from these reactions indicates that oxidation of the alkoxyl radical remains relatively unchanged from pH 7.0 to pH 8.5, with slightly higher quantities noted at pH 6.5. Similarly, the alcohol levels remain constant at the higher pHs with slightly elevated levels noted at the lower pH values. The combined yields of the homolytic cleavage products, aldehyde (**2**) and ketone (**3**), account for >94% of the metabolized 10-OOH-18:2, over the entire pH range.

In addition to Hepes buffer systems, oxidations employing iron–bleomycin are commonly carried out in phosphate buffers (Burger et al., 1979a,b, 1981, 1982, 1983, 1985a). Furthermore, the effects of phosphate-containing compounds on the activity of the iron–bleomycin complexes is an area of active interest (Burger et al., 1985b). A 1-h incubation of 10-OOH-18:2 with ferric bleomycin in 10 mM KP_i, pH 7.5, results in virtually complete reduction of the hydroperoxide (Table II). The aldehyde (**2**) remained the predominant reduction product although increased levels of both the ketone (**3**) and alcohol (**4**) were detected as compared to those in the Hepes system. However, the combined homolytic cleavage products, aldehyde (**2**) and ketone (**3**), accounted for 93% of the metabolized hydroperoxide (**1**).

Methanol, in addition to the aqueous buffer systems, has been reported to be an effective reaction medium for bleomycin-catalyzed oxidations (Padbury & Sligar, 1985; Heimbrook et al., 1986; Murugesan & Hecht, 1985). The reduction of 10-OOH-18:2 by ferric bleomycin in methanol exhibits a slight decrease in overall metabolism as compared to that in the aqueous systems (Table II). While the aldehyde (**2**) is the predominant metabolite, marginally increased levels of both the ketone (**3**) and the alcohol (**4**) are noted in the organic solvent. The homolytic products however still account for greater than 90% of the overall metabolized hydroperoxide.

The time course of 10-OOH-18:2 reduction by ferric bleomycin is shown in Figure 2. In the presence of phenol, at 25 °C in 10 mM Hepes, pH 7.5, 90% of the hydroperoxide is reduced in the initial 10 min of the reaction. Concurrent with this reduction is the sharp increase in the formation of the aldehyde (**2**). After the first 10 min, the reduction of the remaining hydroperoxide and corresponding production of the aldehyde (**2**) proceed gradually, reaching completion by the

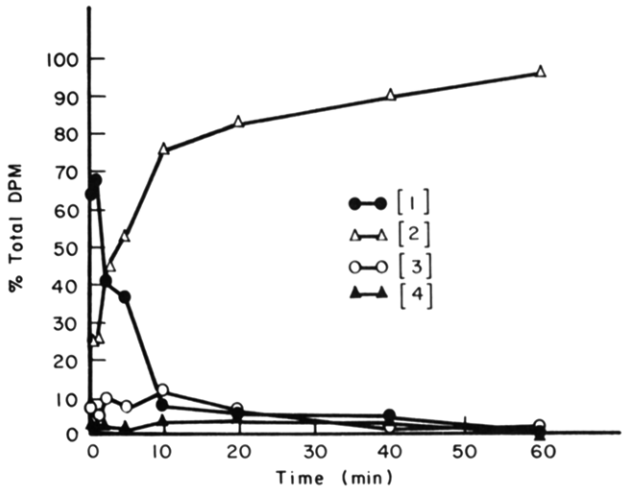


FIGURE 2: Time dependence of the reduction of 10-OOH-18:2 by ferric bleomycin. Incubations performed as described under Experimental Procedures in 10 mM Hepes, pH 7.5, containing 0.1 mM ferric bleomycin. Metabolite levels reported as percent of total recovered radioactivity.

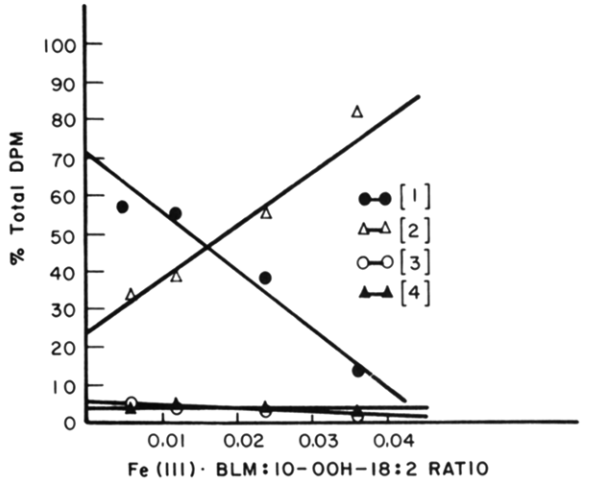


FIGURE 3: Ferric bleomycin dependence of the reduction of 10-OOH-18:2. Incubations performed as described under Experimental Procedures in 10 mM Hepes, pH 7.5. Metabolite levels reported as percent of total recovered radioactivity.

1-h time point. The alcohol (**4**) remains at a basal level of 2–3% of the total recovered radioactivity over the entire time course. The ketone (**3**) however exhibits a slight decrease from approximately 10% at the initial time points to 2–3% at the longer incubation times.

Figure 3 exhibits the dependence of the 10-OOH-18:2 reduction on the ferric bleomycin level. Increasing quantities of the drug result in decreasing levels of hydroperoxide (**1**) and corresponding increasing amounts of aldehyde (**2**) in a relatively linear fashion in the 15-min incubations employed. The levels of ketone (**3**) and alcohol (**4**) maintain a constant background level over the range of bleomycin to hydroperoxide ratios.

The result of substituting DNA, bleomycin's native substrate, for phenol is presented in Figure 4 and Table III. Lanes 1 and 6 (Figure 4) contain the substrate plasmid DNA in the absence of the reaction components. The plasmid sample is comprised of two major higher molecular weight subpopulations. Addition of ferric bleomycin to the DNA (lane 2) results in some background degradation of the two DNA subpopulations, resulting in a single lower molecular weight band. This background activity may be due to trace amounts of contaminating metals or reducing agents in the

Table III: Metabolism of 10-Hydroperoxy-8,12-octadecadienoic Acid by Ferric Bleomycin in the Presence of DNA[a]

lane	**1** (% metabolized)[b]	**2** (%)[c]	**3** (%)[c]	**4** (%)[c]
1				
2				
3	85	86	5	9
4	60	17	3	80
5	90	86	7	7
6				

[a] Reaction conditions are described under Experimental Procedures. [b] Values are reported as percent metabolism on the basis of recovered **1**. [c] Values are reported as percent of the total metabolized products.

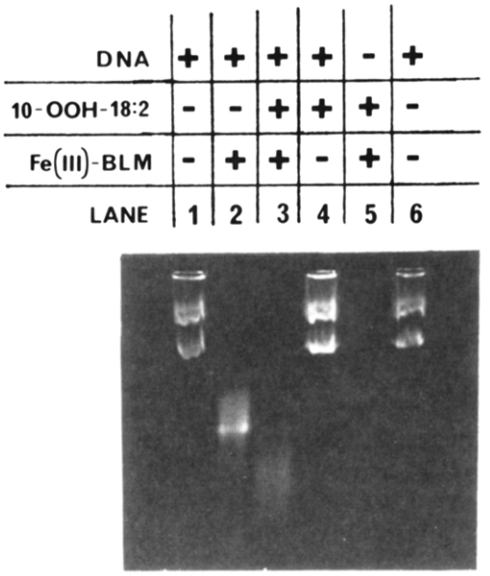


FIGURE 4: DNA cleavage by ferric bleomycin and 10-OOH-18:2. Reaction mixtures were as described under Experimental Procedures.

plasmid preparation. However, addition of both ferric bleomycin and 10-OOH-18:2 to the plasmid DNA results in extensive degradation of the substrate as evidenced by the smear of low molecular weight DNA fragments in lane 3. Control incubations of 10-OOH-18:2 with DNA in the absence of the metallodrug complex demonstrated no detectable DNA damage (lane 4). Lane 5 contains no DNA. The hydroperoxide-derived products obtained from these reactions are summarized in Table III. In the full reaction system (lane 3), near complete reduction of the hydroperoxide (**1**) is achieved with the aldehyde (**2**) as the major product. Background amounts of the ketone (**3**) and the alcohol (**4**) were also detected. In the absence of bleomycin (lane 5), a significant amount (~60%) of the hydroperoxide is reduced. As noted above, this reduction was not accompanied by extensive DNA destruction as judged by the electrophoretic gels. Furthermore, while the bleomycin-containing reactions gave primarily homolytic breakdown products (**2** and **3**), the alcohol (**4**), the heterolytic reduction product, predominates in reactions in which the iron–drug complex is excluded. Incubation of 10-OOH-18:2 and ferric bleomycin in the absence of DNA (lane 5) yields results consistent with those observed for the Hepes and phosphate buffer systems used in the phenol oxidation reactions. No radioactivity was found to coprecipitate with the DNA pellet in any of the reaction mixtures.

Discussion

Incubation of 10-OOH-18:2 with ferric bleomycin results in almost exclusive metabolism (>90%) of the hydroperoxide by a homolytic pathway while supporting oxidation of the small organic substrate phenol or degradation of DNA, the drug's

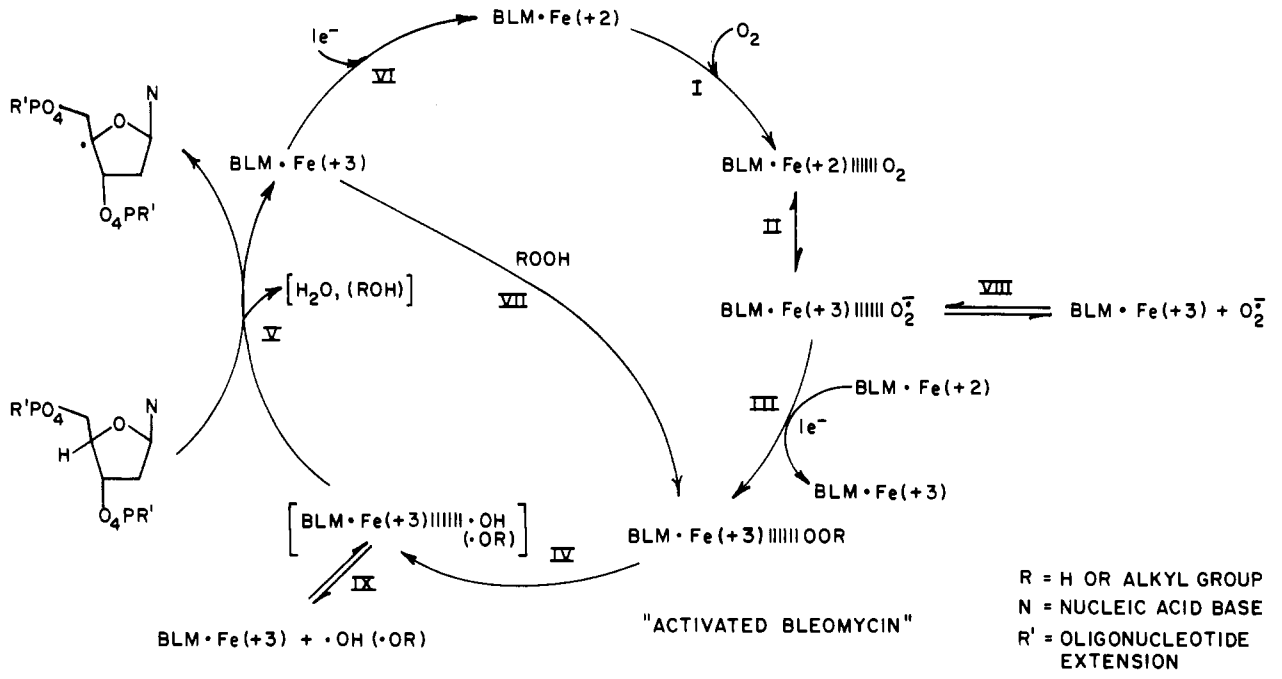


FIGURE 5: Proposed minimal reaction cycle for iron–bleomycin.

presumed biological substrate. This mode of reduction is demonstrated as the primary pathway in several different aqueous buffer systems, over the pH range 6.5–8.5, in methanol, and over a series of drug concentrations. The results obtained with 10-OOH-18:2 reinforce our previous observation of products indicative of homolytic O–O bond cleavage from incubations of cumene hydroperoxide, 2-phenyl-2-butyl hydroperoxide, or (phenylperoxy)acetic acid with ferric bleomycin (Padbury & Sligar, 1987) and identification of oxygen-free radical species from incubations of cumene or *tert*-butyl hydroperoxide with the ferric drug complex.[3] These combined observations permit us to propose several modifications to the cyclic reaction sequence describing the interaction of bleomycin with iron, electrons, dioxygen, hydroperoxides, and DNA previously formulated by Peisach and co-workers (Figure 5; Burger et al., 1979a,b, 1981, 1982, 1983, 1985a).

The high-spin ferrous bleomycin complex (top center, Figure 5) reversibly binds molecular dioxygen at a rate which is proportional to the O_2 concentration to form an EPR-silent ferrous dioxygen complex (I) (Burger et al., 1979a, 1981). Evaluation of the oxygenated bleomycin complex by Mössbauer, ^{57}Fe EPR, and ^{1}H NMR spectroscopies has led to the proposal that the electronic structure of the oxygenated drug complex is best described as a superoxide anion bound to a low-spin ferric ion (II) (Burger et al., 1979a, 1981; Oppenheimer et al., 1979). Autoxidation of such an intermediate is one possible explanation of low levels of superoxide anion detected in solution by EPR spin-trapping techniques (VIII) (Sugiura & Kikuchi, 1978; O'Berley & Beuttner, 1979; Lown & Joshua, 1980; Lown, 1979; Dabrowiak et al., 1979; Kubota et al., 1987; Sugiura, 1978, 1979, 1980). A single electron, supplied from a second ferrous bleomycin molecule, reduces the oxygenated complex to an unstable transient ferric intermediate which Peisach and co-workers have termed "activated bleomycin" in a one-to-one mixture with ferric bleomycin (III) (Burger et al., 1981). The activated bleomycin species is the last detectable drug–iron–oxygen intermediate prior to DNA degradation (Burger et al., 1981). Activated bleomycin and ferric bleomycin demonstrate identical optical spectra but can be distinguished quantitatively by EPR (Burger et al., 1981).

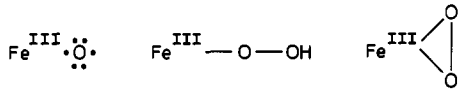
The binding and reduction of dioxygen can be circumvented by the activation of ferric bleomycin with H_2O_2 and alkyl hydroperoxides (VIII) (Burger et al., 1981; Padbury & Sligar, 1985, 1986). The EPR spectra for the activated intermediate formed from H_2O_2 or ethyl hydroperoxide and ferric bleomycin are indistinguishable from that obtained aerobically (Burger et al., 1981). Investigations using chemical methods to probe the reactive nature of iron–bleomycin provide additional evidence to support that a common intermediate is generated from the dioxygen and hydroperoxide systems. Padbury and Sligar have measured equivalent substrate deuterium isotope effects on the N-demethylation of *N*,*N*-dimethylaniline by the ferrous bleomycin–dioxygen system and ferric bleomycin supported by several different hydroperoxy compounds (Padbury & Sligar, 1986; Stubbe & Kozarich, 1987). Furthermore, quantitative incorporation of oxidant-derived oxygen in the epoxidation of *cis*-stilbene catalyzed by both the ferrous–dioxygen and ferric–hydroperoxide systems has recently been reported (Heimbrook et al., 1987).

In addition to activation by hydroperoxy compounds, catalytically active bleomycins have also been generated by several different single oxygen atom donors with the ferric drug complex. These include $NaIO_4$ (Murugesan & Hecht, 1985), $KHSO_5$ (Pratviel et al., 1986), and iodosobenzene (Murugesan & Hecht, 1985; Heimbrook et al., 1986, 1987), although the latter has received the greatest attention. Delivery by iodosobenzene of a single oxygen atom to the ferric ion has been proposed to generate a catalytically active iron–oxo intermediate formally equivalent to compound I of the peroxidases (Dunford & Stillman, 1976). However, the utility of iodosobenzene as a model for the O_2-driven reactions is compromised by increasing evidence which argues that such systems act by Lewis acid behavior of metal–iodosobenzene complexes as opposed to an "oxygen-transfer" mechanism with the formation of a discrete metal–oxo complex (Heimbrook et al., 1986; Murugesan & Hecht, 1985; Moriarty et al., 1985). Additionally, iron systems utilizing iodosobenzene activation,

[3] G. Padbury, S. A. Martinis, and S. G. Sligar, unpublished results.

including bleomycin, exhibit high levels of incorporation of solvent-derived oxygen into oxygenated products (i.e., epoxides) not seen with hydroperoxide- or dioxygen-supported reactions, perhaps indicating a mechanistic dichotomy between the single and double oxygen oxidants (Murugesan & Hecht, 1985; Heimbrook et al., 1986, 1987; Heimbrook & Sligar, 1981; White & McCarthy, 1984; MacDonald et al., 1982). For these reasons, single oxygen donors have been excluded from the scheme presented in Figure 5.

The exact structure of the activated complex is not known with certainty, but ^{57}Fe EPR and studies using $^{17}O_2$ for activation indicate it is a ferric complex containing at least one bound oxygen normally derived from O_2 as one of the metal ligands with an overall oxidation state of V (Burger et al., 1981, 1983, 1985a). With these considerations in mind, Peisach and co-workers proposed three plausible iron–oxygen structures for the activated complex (Burger et al., 1981):



A B C

In structure A the oxygen is at the state of atomic oxygen. The formation of such an intermediate from incubations of hydroperoxides and ferric bleomycin would require the heterolytic cleavage of the peroxy O–O bond. As demonstrated by this investigation, and our previous studies with the simple alkyl hydroperoxides (Padbury & Sligar, 1987), the chemical evidence available provides compelling testimony to suggest that homolytic cleavage is the primary mechanism by which ferric bleomycin reduces hydroperoxides. One might suggest that the ability of the single oxygen donors, such as iodosobenzene, to activate ferric bleomycin might argue for an intermediate as depicted by structure A. However, the complications with these oxidants noted previously serve to discount this notion. Furthermore, while hydroperoxy compounds have been demonstrated to generate EPR signals characteristic of activated bleomycin (Burger et al., 1981), a recent investigation by our laboratory failed to detect the activated bleomycin EPR signature from incubations of iodosobenzene and ferric bleomycin under a wide variety of experimental conditions.[3]

In structures B and C the bound oxygen is at the level of peroxide. In Figure 5, activated bleomycin is represented as the metal–hydroperoxide complex (B) as opposed to the metal–peroxo complex (C) for several reasons. Formation of the peroxo complex, as represented in structure C, would require cleavage of the R–OOH bond. To our knowledge, this type of cleavage is unprecedented in biologically relevant iron- and iron–chelate-catalyzed decompositions of hydroperoxy compounds. This is presumably due to the thermodynamic barrier, where the R–OOH bond is significantly stronger (90 kcal/mol; Benson & Snow, 1971) than the adjacent RO–OH bond (44 kcal/mol; Benson & Snow, 1971). Furthermore, the hydroperoxide reduction products observed from the 10-OOH-18:2, and from the simpler alkyl hydroperoxides (Padbury & Sligar, 1987), are inconsistent with cleavage of the R–O bond and support lysis of the peroxy O–O bond. It should be noted, however, that a structure such as C cannot unequivocably be ruled out, especially in the dioxygen-supported reaction.

The ternary activated bleomycin complex breaks down concurrently with formation of oxidized product. On the basis of the chemical evidence reported herein, it is proposed that the disappearance of the activated complex corresponds to the homolytic cleavage of the O–O bond of the iron–hydroperoxide complex (IV) to yield the equivalent of ferric bleomycin and two hydroxyl, or a hydroxyl and alkoxyl radical, for the dioxygen-supported and alkyl hydroperoxide supported reactions, respectively. It is possible that one of these radicals is coordinated to the metal in a transient higher valent intermediate analogous to compound II of the peroxidases (Fe^{IV}=O; Dunford & Stillman, 1976). Such an intermediate however is purely speculative, and for the current purposes, no formal iron–oxygen complex is specified in Figure 5. Leakage of such radical species from the iron–drug–DNA complex (IX) provides one explanation for the low levels of hydroxyl radicals detected by EPR spin-trapping methods (Sugiura & Kikuchi, 1978; O'Berley & Beuttner, 1979; Lown & Joshua, 1980; Dabrowiak et al., 1979; Lown, 1979; Kubota et al., 1987; Sugiura, 1978, 1979, 1980). Experiments using various radical scavengers as inhibitors have provided contradictory results (Padbury & Sligar, 1986; Lown & Sim, 1977; Rodriguez & Hecht, 1982; Cunningham et al., 1983, 1984; Bartkowiak et al., 1982; Gutteridge et al., 1985). However, the failure of many of these radical scavengers and inhibitors to provide significant protection of substrates from drug-catalyzed oxidation may suggest that these reactive species are not released as dissociable intermediates to the solvent in significant quantities. Hydroxyl radicals, or an equivalent species, generated at the iron center are then able to hydrogen abstract from the deoxyribose 4′-carbon of DNA yielding a 4′-carbon radical (V), the initial lesion responsible for the nucleic acid free base and base propenal degradation products observed from bleomycin-treated DNA (Burger et al., 1986; Ajmera et al., 1986; Rabow et al., 1986; Sugiyama et al., 1985; Wu et al., 1985a,b).

Finally, the reaction cycle is completed by regeneration of the ferrous complex by a one-electron reduction of the ferric species ($E^{\circ\prime}$ = 129 mV vs SHE; Melnyk et al., 1981). Numerous reductants have been reported to mediate this step including ascorbate, dithiothreitol, 2-mercaptoethanol, cysteine, glutathione, and NADPH–cytochrome P-450 reductase (Sausville et al., 1976; Antholine & Petering, 1979; Scheulen et al., 1982; Kilkuskie et al., 1984).

Summary

This investigation has documented chemical evidence which indicates that ferric bleomycin cleaves the O–O bond of 10-OOH-18:2 predominantly by a homolytic mechanism while supporting substrate oxidation under a variety of reaction conditions commonly employed in in vitro iron–bleomycin-mediated oxidations.

The results reported herein, when considered with previous results from iron–bleomycin-catalyzed hydroperoxide metabolism investigations from our laboratory[3] (Padbury & Sligar, 1987), lend credibility to the proposal that a homolytic cleavage mechanism is operative in the ferrous iron–dioxygen supported reactions in which molecular oxygen is believed to be reduced to the level of hydrogen peroxide prior to O–O bond scission.

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