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ON THE REACTION OF IRON BLEOMYCIN WITH THIOLS AND OXYGEN

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Summary. Iron(III)bleomycin undergoes a redox reaction with thiols. Evidence from epr and UV-visible absorbance spectra indicate that the metal complex forms an intermediate with sulfhydryl groups presumably by adding a sixth ligand. In the presence of oxygen iron bleomycin cycles between its two oxidation states to catalyze the generation of oxygen free radicals using thiols as a source of electrons.

Introduction. Horwitz and co-workers have suggested that the iron(II) chelate of the antitumor glycopeptide, bleomycin, is the active form of the drug in the DNA-strand scission reaction carried out by this compound (1-3). It was argued that the iron(II) complex is rapidly oxidized by oxygen, releasing radical species such as $OH^{\cdot}$ which attack DNA (4-6). However, since very little bleomycin or iron-bleomycin appear to be taken up by cells, the production of $OH^{\cdot}$ will be slight unless a redox cycle exists within cells in which iron bleomycin catalyzes the reduction of O_2 by cellular reductants (6). Catalytic cycles involving thiol reduction of oxygen by mono-and bis (thiosemicarbazonato) copper complexes have been described (7,8). This report examines the reaction between cellular thiols, iron, bleomycin, and oxygen.

Materials and Methods.

Materials. Bleomycin, a clinical mixture containing about 70% bleomycin A_2 and significant B_2, was supplied by Bristol Laboratories. Cysteine (CysSH) and glutathione (GSH) were purchased from Sigma Biochemical Company. All other materials were reagent grade or highest purity available.

Methods. A stock solution of iron bleomycin, FeBlm, in 1 M NaCl and 0.01 M Tris was prepared by mixing appropriately diluted stock solutions of bleomycin,

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Abbreviations: epr - electron paramagnetic resonance, Blm - bleomycin, FeBlm - iron bleomycin, CysSH - cysteine, GSH - glutathione.

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Blm, and $Fe(NH_4)_2(SO_4)_2 \cdot 6(H_2O)$ under nitrogen. FeBlm rapidly oxidizes in air to give Fe(3+)Blm. Stock solutions of cysteine and glutathione were continuously flushed with nitrogen and the thiol content was periodically checked by the thiol reagent 5,5'disulfide bis(2-nitrobenzoic acid). All stock solutions were adjusted to pH 7.0 with 2N NaOH. UV-visible absorption spectra were obtained with an Acta V Spectrophotometer. Deoxygenated samples were sealed in a tonometer. Epr spectra were obtained with a Varian E 109 Century Series spectrometer at the NIH National Biomedical ESR Center, The Medical College of Wisconsin. Samples were frozen in liquid nitrogen in quartz tubes and inserted into a standard finger dewar. Deoxygenated samples were sealed in the tonometer and transferred to an argon filled glove box before the sample was pipetted into an epr tube and frozen in liquid nitrogen. The deoxygenated procedure is the same procedure used for preparation of nitric oxide hemoglobin (9,10). A Yellow Springs Instruments Model 53 oxygen analyzer was used to measure oxygen consumption.

Results and Discussion. Figure 1A shows the time course of reduction of Fe(III) Blm to Fe(II)Blm, monitored at 580 nms. At the beginning of the reaction Fe(II)Blm, which does not absorb at this wavelength and is observed as a Yellow color is converted into an intermediate species, blue-gray in color, having an absorbance maximum at 580 nm (ε ca. 1800 $M^{-1}cm^{-1}$). A steady state is maintained for more than 200 s in figure 1A, which is followed by a first order decay of the intermediate to yield the orange-pink Fe(II)Blm with its absorbance maximum at 476 nm (ε ca. 70 $M^{-1}cm^{-1}$). Also plotted are the kinetics of oxygen consumption in the presence of cysteine and cysteine plus FeBlm (Figure 1B and 1C). It is clear that the steady state period corresponds to the aerobic

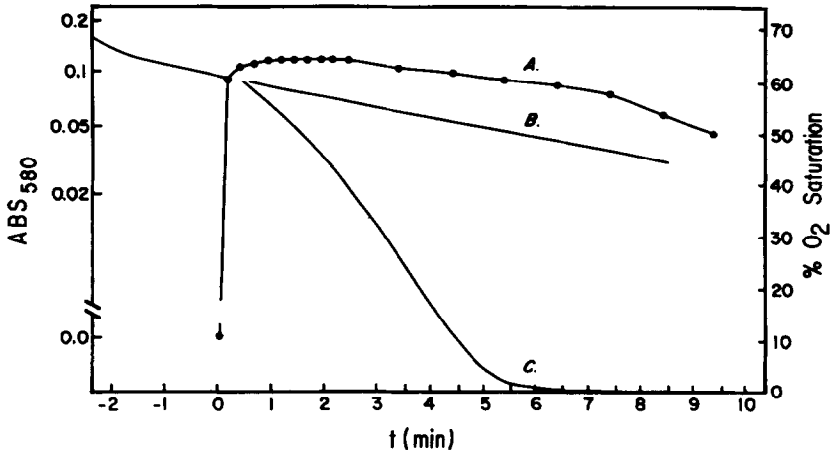

Fig. 1. The time course of oxygen uptake (right ordinate) is compared with the decrease in the absorbance (left ordinate) of the transient for FeBlm in the presence of cysteine. A. Decrease in absorbance at 580 nm for 0.09 mM Fe(III)Blm plus 0.01 M cysteine. B. O_2 uptake for 0.06 M cysteine in the absence of FeBlm. C. O_2 uptake for 0.09 mM Fe(III)Blm plus 0.01 M cysteine. All solutions were made up in 0.1 M Tris. The final pH was 7.0.

Table 1. Reduction of Fe(III)Blm by Cysteine* and Glutathione*

[Cys]	Pseudo rate constant k_{obs}	Correlation coefficient in obtaining k_{obs}	Second order rate constant k^+
.0019 M	.00082 sec^{-1}	.993	26 $sec^{-1}M^{-1}$
.0023 M	.0013 sec^{-1}	.9997	34 $sec^{-1}M^{-1}$
.0026 M	.0021 sec^{-1}	.995	48 $sec^{-1}M^{-1}$
.0076 M	.0037 sec^{-1}	.999	29 $sec^{-1}M^{-1}$
.0104 M	.0043 sec^{-1}	.994	25 $sec^{-1}M^{-1}$
.0260 M	.0135 sec^{-1}	.998	31 $sec^{-1}M^{-1}$
[Glu]			
.22 M	.00082 sec^{-1}	.995	0.23 $sec^{-1}M^{-1}$

*In 0.01 M Tris-(hydroxymethyl)-aminomethane and 0.1 M NaCl at 22°C and a final pH of 7.0 after flushing with N_2 for 5 min. before adding Fe(III)Blm to a final concentration of 0.90×10^{-4} M and sealing the tonometer.

+The calculated second order rate constants for V = k[FeBlm] [thiol].

portion of the reaction in which O_2 is undergoing reduction. As the solution is depleted of O_2, cysteine reduces the complex without its attendant re-oxidation by oxygen. The rate constant for the second portion of the reaction is the same as observed in the reduction of Fe(III)Blm by cysteine carried out totally under anaerobic conditions (Table 1). A second order rate constant, k, of 30 $min^{-1}M^{-1}$ for a reaction first order in both Fe(III)Blm and CysSH is obtained from a plot of lnk vs. ln[CysSH] in which the slope is 0.93 and the correlation coefficient of the data is 0.97. Assuming a similar form for the rate expression of the reaction of Fe(III)Blm with glutathione, a second order constant of 0.23 $min^{-1}M^{-1}$ is calculated from data in Table 1. The 100 fold difference is much larger than the difference observed when Cu(II)Blm reacts with cysteine (7.2 $min^{-1}M^{-1}$) and glutathione (0.72 $min^{-1}M^{-1}$) (6).

The nature of the steady-state intermediate in the reaction of FeBlm with thiols and O_2 has been investigated with epr spectroscopy. Figure 2 shows the epr spectra of Fe(III)Blm in the presence and absence of CysSH or GSH. Also shown are the spectra of Fe(III)Blm + CysSH and Fe(III)Blm + GSH before and after flushing the sample with argon to remove O_2. A large shift in g-values from Fe(III)Blm to Fe(III)Blm in the presence of thiols is seen. On the basis of the following arguments this is interpreted as due to the formation of an

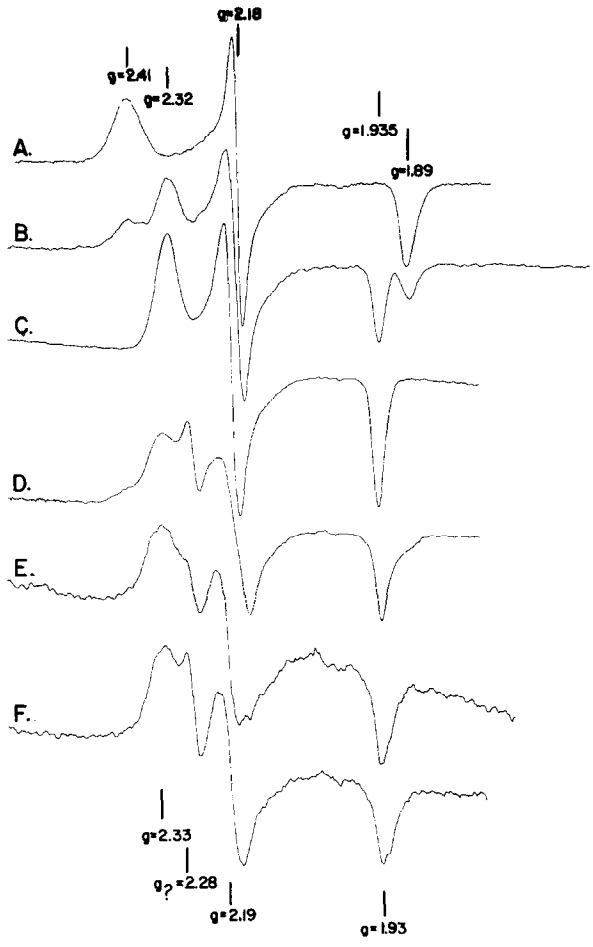

Fig. 2. The ESR spectrum of a frozen (-196°C) solution of 0.09 mM Fe(III)Blm containing 0.01 M Tris and 0.01 M NaCl.
A. No addition.
B. Plus 9 mM glutathione and frozen immediately after mixing;
C. Plus 9 mM glutathione flushed with argon before freezing;
D. Plus 4 mM cysteine frozen immediately after mixing;
E. Plus 4 mM cysteine flushed with argon before freezing, gain x 4;
F. Plus 1 mM cysteine, gain x 2. The final pH was 7.0 for above solutions.

intermediate Fe(III)Blm-thiol adduct. A decrease in the low field g-value with increasing crystal field has been described for ferric low-spin compounds of Hemoglobin A (11). Considering a variety of heme ligands, a large decrease in g-value correlates with the major crystal field contribution from mercaptide, which easily overrides the contribution of non-sulfur ligands (11). In another example, the g-values for bis(2-formylpyridine thiosemicarbaznato) iron(III), where the iron is in an environment, containing two sulfur and four nitrogen

atoms are 2.18, 2.135, and 1.998 (12). Here the g-values deviate even less from the spin only g-value of 2.0023.

As described above the anaerobic reduction of Fe(III)Blm by GSH is slow, so that an undiminished signal for the intermediate involving Fe(III)Blm and GSH can easily be obtained despite the time needed to deoxygenate and free the sample. Thus, the FeBlm-GSH adduct is reasonably stable. This is the first indication that the Fe(III), which is presumably 5-coordinated to the bleomycin, can add a sixth ligand using Lewis bases from solution. With CysSH, the reaction is faster and some reduction to the diamagnetic Fe(II)Blm occurs during this procedure. However at concentrations of Fe(III)Blm and CysSH of 9×10^{-5}M and 9.9×10^{-4}M, respectively, a signal about one-half the intensity of that of the starting complex can be observed.

The significance of the portion of the spectrum described by $g_?$ for Fe(III) Blm-CysSH is unclear. The intensity of the $g_?$ peak diminished, with decreasing O_2 (Figure 1E) or decreasing thiol concentration (Figure 1F). This feature of the spectrum may be attributed to an O_2 dependent intermediate. The epr spectra of the intermediate reported here look similar though not identical to the transient ($g_1 = 2.254$, $g_2 = 2.171$, $g_3 = 1.937$) reported by Sigiura and Kikuchi to develop 90 sec after Fe(II)Blm reactions with O_2 (4). Yonetani et al. have described a similar feature, $g_?$, for NO ferrous cytochrome c peroxidase, NO ferrous catalase, NO ferrous cytochrome c, and NO ferrous myoglobin (13). Since this feature is not observed in the Fe(III)Blm + GSH adduct, the question remains whether it is linked with the catalytic reactivity of the complex with CysSH and oxygen.

The experiments described here may be summarized by the following mechanism:

$$\underset{\text{(thiol)}}{\text{Fe(III)Blm + RSH}} \rightleftharpoons \underset{\text{(intermediate)}}{\text{Fe(III)Blm - SR}} + H^+ \qquad (1)$$

$$\text{Fe(III)Blm - SR} \longrightarrow \text{Fe(II)Blm} + RS^{\cdot} \qquad (2)$$

$$\text{Fe(II)Blm} + O_2 \xrightarrow{\text{fast}} \text{Fe(III)Blm} + O_2^{\cdot-} \qquad (3)$$

The radical species react rapidly to produce $OH^{\cdot}$ which is the sole reduced radical from O_2 which is spin trapped under these conditions in the study of reaction 3 (4-6). Because reduction of Fe(III)Blm is much slower than the oxidation of Fe(II)Blm by O_2, net reduction of the complex does not occur until oxygen becomes depleted. Thus, the complex cycles between its two oxidation states to catalyze the generation of oxygen free radicals using thiols as a source of electrons. Therefore, these studies confirm the proposal made earlier that FeBlm may react with common cellular reducing agents and O_2 to produce free radical species (6). Such amplification of the effects of the reaction between Fe(II)Blm and O_2 provides a plausible mechanism for the production of cytotoxic amounts of free radical species from the small amounts of FeBlm taken into cells.

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