Vol. 91, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 28, 1979 Pages 528-533

REACTION OF FeBlm WITH DNA: Fe(II)Blm-NO

W. E. Antholine[1] and D. H. Petering[2]

[1]Radiation Biology and Biophysics Section, The Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226, U.S.A.

[2]Laboratory for Molecular Biomedical Research, Department of Chemistry, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin 53201, U.S.A.

Received October 8, 1979

Summary. The structure of the iron bleomycin nitric oxide complex is altered in the presence of calf thymus DNA as determined from epr studies. This altered structure predominates for one iron bleomycin nitric oxide molecule per coil of the DNA helix. In the absence of nitric oxide, as the pH is lowered, iron bleomycin dissociates in two steps, supporting the hypothesis that in-plane nitrogens may be easily perturbed.

Introduction. Bleomycin is a clinically useful antitumor agent. As a possible mechanism of action, attention has been focused on the strand scission of DNA which occurs in the presence of bleomycin and sulfhydryl reagents (1). Recently, it has been shown that ferrous bleomycin, Fe(II)Blm, is much more effective in this reaction than the metal-free ligand (2). As Fe(II)Blm oxidizes under aerobic conditions, reduced oxygen radicals such as $OH^{\cdot}$ are generated, which attack and cleave the DNA backbone (3-6). A more elaborate mechanism has now been developed in which bleomycin first binds to DNA and chelates Fe^{2+}. The bound Fe(II)Blm then binds and reacts with oxygen to produce oxygen radicals directly at the site of DNA.

To probe this mechanism in detail the catalytic interaction of Fe(II) and Fe(III)Blm with O_2 and thiols was investigated in a previous communication (7). In the present study the reaction of iron-bleomycins, with DNA is considered. Earlier Sugiura presented epr spectral evidence that Co(II)Blm is a pentacoordinate complex and that in the presence of O_2 a Co(II)Blm - O_2 adduct is formed (8). After addition of calf thymus DNA, the orientation of the axially bound oxygen changes with respect to the Co(II) plane (8). Cobalt bleomycin appears to be in-

Abbreviations. epr - electron paramagnetic resonance, Blm - bleomycin, FeBlm - iron bleomycin, FeBlm-NO$^{\cdot}$ - iron bleomycin nitric oxide complex, IHP - inositol hexaphosphate.

0006-291X/79/220528-06$01.00/0

active both in the strand scission reaction and as a cytotoxic agent (9). Thus, the present study examines the binding of the stable ferrous bleomycin-nitric oxide adduct to DNA as a more direct model for the interaction of Fe(II)Blm, DNA and O_2.

Materials and Methods.

Materials. Bleomycin, a clinical mixture containing about 70% bleomycin A_2 and significant B_2, was supplied by Bristol Laboratories. Nitric oxide gas was purchased from either Matheson or Union Carbide Gas Products. Calf thymus DNA was purchased from Sigma. Dithionite, NaCl, HCl, NaOH and $Fe(NH_4)_2(SO_4)_2 \cdot 6(H_2O)$ were reagent grade.

Methods. Calf thymus DNA and bleomycin were deoxygenated with argon before adding an appropriate aliquot of an $Fe(NH_4)_2(SO_4)_2 \cdot 6(H_2O)$ solution which was under nitrogen. The nitric oxide bound bleomycin complex, FeBlm-NO·, was prepared in the presence and absence of DNA by flushing the system anaerobically with NO·. The FeBlm-NO· solution in a tonometer was transferred anaerobically to an argon-filled glove box where samples were frozen in liquid nitrogen for epr measurements (7). Fe(2+)Blm rapidly oxidizes in air to give stock solutions of Fe(3+)Blm. All stock solutions of FeBlm and DNA were adjusted to pH 7.0 with 2N NaOH. UV-visible absorption spectra were obtained with an Acta V Spectrophotometer. A few grains of dithionite were added to curvettes to keep Fe(2+)Blm fully reduced before scanning the visible spectrum and recording the pH with a Radiometer PHM-26 meter.

Results and Discussion.

Figure 1 shows the epr spectra for the ferrous bleomycin nitric oxide complex, FeBlm-NO, in the presence and absence of DNA. In the absence of DNA the FeBlm-NO complex is similar to recently reported spectra (10-12). Upon binding to Fe(II)Blm the triplet hyperfine structure of NO· is not further resolved into nine lines by an axial nitrogen as seen with hemoglobin-nitric oxide derivatives. Nevertheless, the lack of the axial ligand in a modified FeBlm complex perturbs the spectrum of FeBlm-NO suggesting the existence of axial ligation trans to the nitric oxide (10).

In the presence of DNA the environment of the iron is altered (Figure 1). The predominant change is the result of a large shift in the low field g-value to 2.06. In the presence of DNA, the high field g-value at 1.967 is also well resolved but comparison with the high field shoulder in the absence of DNA is difficult without further simulations. Both of these g-values may be attributed to the in-plane which is approximately perpendicular to the iron nitric oxide axis, which consists of the nitrogen from nitric oxide, the iron molecule, and possibly a trans α amino nitrogen from bleomycin. Bleomycin is thought to bind

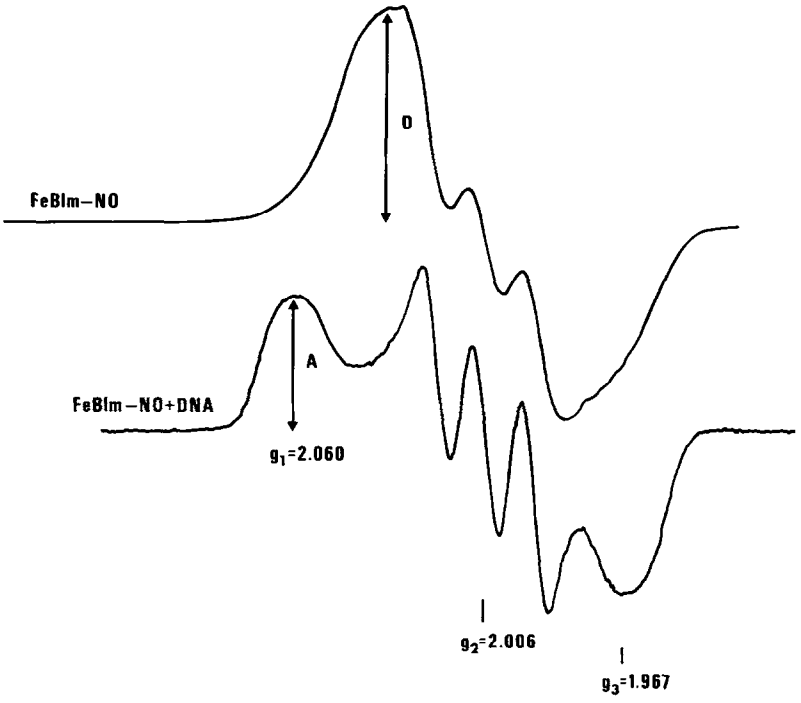

Figure 1. The epr spectrum of a frozen (-196°C) solution of 0.1 mM FeBlm-NO· in the absence (upper trace) and the presence (lower trace) of 18 mgms/ml of calf thymus DNA and .15 M phosphate buffer at pH 7.2.

to DNA through the dithiazole and terminal amine at a site distant from the metal binding site (13). The histidine imidazole nitrogen and the deprotonated peptide nitrogen, which are four peptide bonds from the dithiazole moiety, appear to be the closest direct link to the DNA bound dithiazole and terminal amine and would thus be likely sites for the in-plane perturbation of the Fe(II)Blm structure observed here.

There is little change in the triplet hyperfine parameters (a_N = 25 Gauss in the absence of DNA; a_N = 23 Gauss in the presence of DNA) suggesting little perturbation of the axial nitric oxide ferrous bond in contrast to hemoglobin nitric oxide derivatives in the presence of IHP (14-16). The spectrum of FeBlm-NO in the presence of DNA is similar to the spectra reported for NO ferrous cytochrome C, and NO ferrous myoglobin (17), and for hemoglobin nitric oxide in the presence of IHP where the interaction with the imidazole trans to nitric oxide is weakened (18).

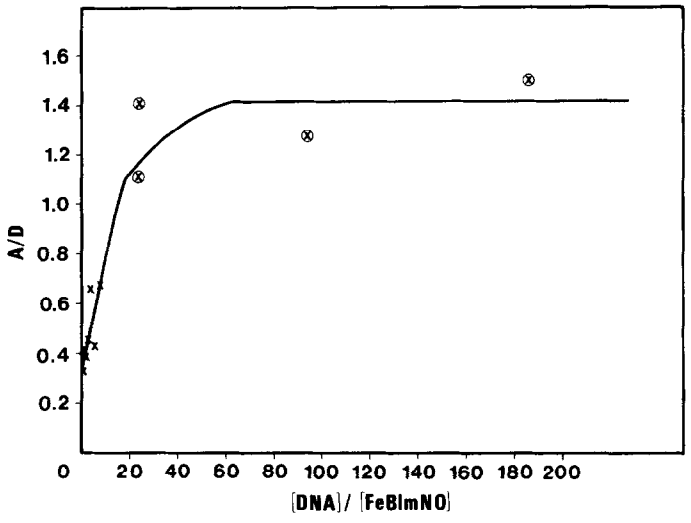

Figure 2. The epr signal intensity in the presence of DNA is plotted versus the concentration ratio of DNA bases to FeBlm-NO. The parameters A and D are defined in Figure 1 for FeBlm-NO and FeBlm-NO in the presence of DNA. The DNA concentration was measured spectrophotmetrically at 260 nms (ε=6.6 x $10^3 M^{-1}$). The FeBlm concentration was determined from the dilution of a stock solution. All solutions were made up in .05 M phosphate buffer at pH 8.0. The DNA concentration is 0.8 mM as indicated by the symbol, X; the DNA concentration is 25 mM as indicated by the circled symbol (X).

Figure 2 gives an estimate of the concentration of calf thymus DNA needed per FeBlm-NO for a major fraction of the FeBlm-NO to be in the altered structure. The ratio of the parameters A and D as defined in Figure 1 are plotted versus the ratio of DNA and FeBlm-NO concentrations. The DNA concentration was 0.8 mM when the ratios of [DNA]/[FeBlm-NO] <10 and 25 mM when the ratios of [DNA]/[FeBlm-NO] >10. Similar results were obtained with constant FeBlm-NO and variable DNA concentration. The endpoint in Figure 2 appears to be between 20 and 60 bases or 10 to 30 base pairs per molecule of FeBlm-NO. (Thirty base pairs correspond to a single coil of the DNA helix and suggest about one FeBlm-NO per coil of the helix.) Strong and Crooke report and review data with bleomycin A_2 showing a binding ratio of free ligand to DNA of 2 - 5 molecules of Blm per base pair (19). This is a substantially larger binding ratio than observed here for the FeBlm-NO· complex with DNA.

In seeking an explanation for the predominately in-plane effect of DNA on the structure, the generality of the effect is recalled. Both thiols, such as

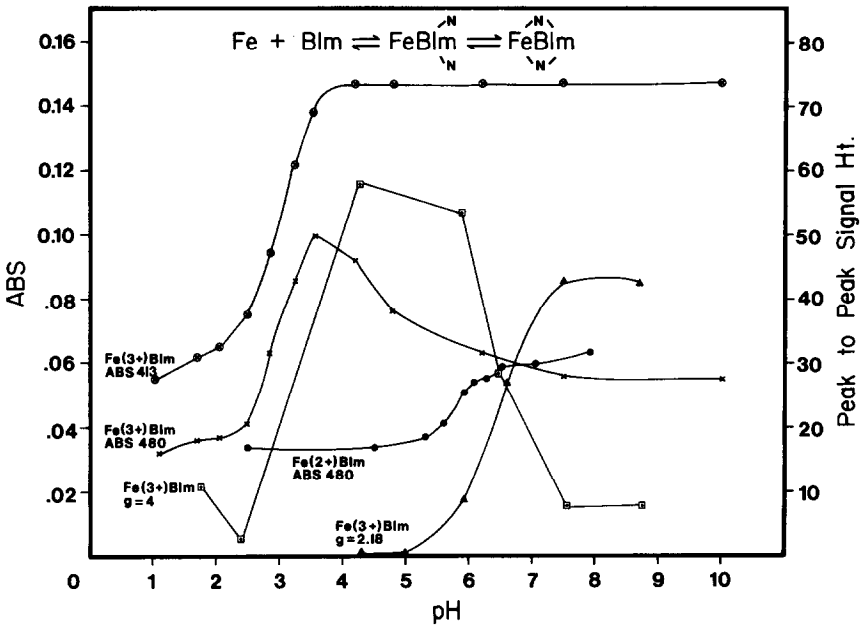

Figure 3. The pH dependence of the absorbance at 413 nm and 480 nm for Fe(3+)Blm and at 480 nm for Fe(2+)Blm (left ordinate) in addition to the pH dependence versus the peak to peak signal height (right ordinate) for high spin Fe(3+)Blm at g=4 and low spin Fe(3+)Blm at g=2.18. All solutions are made up in a .1 M NaCl. The concentration of both Fe(3+)Blm and Fe(2+)Blm is approximately 0.2 mM.

cysteine and glutatione (7), and oxygen during the oxidative process (3) also perturb in-plane structural features of Fe(II)Blm and Fe(III)Blm. Thus we looked at the pH dependence of binding of iron to these two oxidation states of FeBlm (Figure 3). Fe(III)Blm dissociates in two steps as interpreted from epr spectra and corresponding absorbance changes. The low spin form at pH 7 is converted to an intermediate high spin form and then to Fe(III) which is not detected by epr spectroscopy due to its fast relaxation. Other authors have reported a high spin form of FeBlm (11, 20). We show here it is linked by reversible equilibrium changes to the native structure.

Similarly, Fe(II)Blm in the presence of dithionite shows the same first step (Figure 3). However, the dithionite prevents spectral observation at lower wavelength to observe the second step. Nevertheless, Gupta has presented recent ^{13}C nmr evidence that the iron center of Fe(II)Blm at pH 5.2 has lost both imidazole and amine nitrogen ligands (20). The reduced number of ligands leads to the spin

state change. Their data also support the binding of Fe(II) and Blm to the other three ligands of Blm, the pyrimidine and the primary and secondary amine groups. Gupta et al., however, interpret these data only in terms of the binding of two groups to the iron; the pyrimidine and the primary amine (20). The pHs at which Fe(II) and Fe(III)Blm are half dissociated in this first dissociation step are 5.7 and 4.8 respectively. That is the imidazole and peptide nitrogen are only weakly bound to the iron and are readily displaced by H^+. Thus, it is proposed that in the interaction of FeBlm with DNA and with thiols this part of the in-plane metal-ligand structure is conformationally perturbed.

Acknowledgement. This research was carried out with funds from The National Cancer Institute Grant CA-22184. The ESR work was done at a facility supported by NIH Grant RR-01008.

References.

1. Sausville, E. A., Peisach, J., and Horwitz, S. G. (1978) Biochem. 17, 2740-2746.
2. Sausville, E. A. Stein, R. W., Peisach, J., and Horwitz, S. B. (1978) Biochem. 17, 2746-2754.
3. Sugiura, Y., and Kikuchi, T. (1978) J. Antibiot. 31, 1310-1312.
4. Oberley, L. W., and Buettner, G. R. (1979) FEBS Lett. 97, 47-49.
5. Sugiura, Y. (1979) Biochem. Biophys. Res. Commun. 82, 649-653.
6. Solaiman, D., Rao, E. A., Petering, D. H., Sealy, R. C., and Antholine, W. E. Int. J. Rad. Oncol. Biophys. (in press).
7. Antholine, W. E., and Petering, D. H. Biochem. Biophys. Res. Commun. (in press).
8. Sugiura, Y. (1978) J. Antibiot. 31, 1206-1208.
9. Nunn, A. D., and Lunec, J. (1978) European J. of Cancer 14, 857-900.
10. Sugiura, Y. (1979) Biochem. Biophys. Res. Commun. 88, 913-918.
11. Burger, R. M., Peisach, J., Blumberg, W. E., and Horwitz, S. B. (1979) Abs. Biophys. J. (2, part 2): 37a.
12. Antholine, W. E., and Petering, D. H. (1979) Abs. Am. Chem. Soc., Sept. Meeting.
13. Taketa, T., Muraoka, Y., and Nakatani, T. (1978) J. Antibiot 31, 1073-1077.
14. Antholine, W. E., Mauk, A. G., and Taketa, F. (1973) FEBS Letts 36, 199-202.
15. Rein, H., Ristau, D., and Scheler, W. (1972) FEBS Letts. 24, 24.
16. Trittelvitz, E., Sick, H., and Gersonde, K. (1972) European J. Biochem. 31, 578.
17. Yonetani, T., Yamamoto, H., Erman, J. E., Leigh, J. S., and Reed, G. H. (1972) J. Biol. Chem. 247, 2447-2455.
18. Perutz, M. F., Kimartin, J. V., Nagai, K., Szabo, A., and Simon, S. R. (1976) Biochem. 15, 378-387.
19. Strong, J. E. and Crooke, S. T. in Current Status and New Developments (1978) (edit. by Carter, S. K., Crooke, S. T., and Umezawa, H.) Academic Press.
20. Grupta, R. K., Ferretti, J. A., and Caspary, W. J. (1979) Biochem. Biophys. Res. Commun. 89, 534-541.