Orientation of Iron Bleomycin and Porphyrin Complexes on DNA Fibers

Makoto Chikira,*,†,‡ Takamasa Iiyama,‡ Katsuyuki Sakamoto,‡ William E. Antholine,§ and David H. Petering*,∥,⊥

Department of Applied Chemistry, Chuo University, Kasuga, Bunkyo-ku, Tokyo 112-8551, Japan, Biophysics Research Institute, Medical College of Wisconsin, Milwaukee, Wisconsin 53226, and Department of Chemistry, University of Wisconsin−Milwaukee, Milwaukee, Wisconsin 53201

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Bleomycin (Blm) is an antitumor agent that requires iron and oxygen for strand cleavage of DNA. In this study, ferric bleomycin, Fe(III)Blm, or the nitric oxide adduct of ferrous bleomycin, ON−Fe(II)Blm, were bound to one-dimensionally oriented DNA fibers. Reductive nitrosylation of Fe(III) complexes took place in situ on B-form DNA fibers. Electron paramagnetic resonance (EPR) spectra were obtained as a function of the angle Φ between the magnetic field **B** and the fiber axis Z_f. For comparison, EPR spectra were acquired for ON−Fe(II)TMpyP and ON−Fe(II)TMpyP−Im on oriented DNA fibers, where TMpyP is 5,10,15,20-tetrakis(1-methyl-4-pyridino)porphyrin and Im is imidazole. EPR spectra showed both low-spin Fe(III)Blm and ON−Fe(II)Blm bound to B-form DNA in two slightly different binding orientations in the ratio of 1:0.2. With A-form DNA, a fraction of bound Fe(III)Blm was high spin. Specifically, the angle β between the fiber axis Z_f and the g axis, g_z, perpendicular to or nearly perpendicular to the equatorial plane of the iron complex was estimated as 20° and 25° for ON−Fe(II)Blm and 30° and 25° for Fe(III)Blm, respectively. The angle γ that determines the orientation of g_x and g_y axes was estimated as 90° for the two ON−Fe(II)Blm species and 10° for the two Fe(III)Blm species, respectively. The NO was held rigidly in place as the temperature increased from 123 K to room temperature for ON−Fe(II)Blm but not for ON−Fe(II)TMpyP or ON−Fe(II)TMpyP−Im. It is hypothesized that the NO is structurally oriented by hydrogen bonding like the peroxide is held in HO_2^-−Co(III)Blm (Wu et al. *J. Am. Chem. Soc.* **1996**, *118*, 1281−1294). The EPR parameters are consistent with a six-coordinate complex for ON−Fe(II)Blm, although the superhyperfine structure from the trans nitrogen was not detected. The increase in g value anisotropy upon binding ON−Fe(II)Blm to DNA fiber may be caused by an increase in the overlap of $d\pi$ and $2p\pi^*$ orbitals induced by an interaction of NO with DNA and/or by a perturbation of d orbitals due to the pyrimidine−guanine interaction. It is concluded that the EPR parameters of ON−Fe(II)Blm and Fe(III)Blm bound to oriented DNA support the hypothesis that FeBlm species bind to DNA with adduct structures similar to those formed by related CoBlm species and DNA.

Introduction

Bleomycin is a clinically useful antitumor agent that requires iron for its cytotoxic action.[1a−h] The drug causes single and double strand cleavage in cells as well as in model chemical reactions.[2a−d] Because double strand scission is correlated with inhibition of cell proliferation and is poorly repaired, this type of DNA damage is likely to be responsible for anticancer properties of this compound.[3a,b] As a natural product with a complex structure, Blm binds Fe with a set of ligand atoms to form a metal domain (Figure 1).[4a−e] A peptide linker connects this domain with another that binds to DNA through the bithiazole moiety and the positively charged R group. HO_2^-−Fe(III)Blm, formed either in the pathways 1−3,

$$\text{Fe(III)Blm} + e^- \rightarrow \text{Fe(II)Blm} \quad (1)$$

$$\text{Fe(II)Blm} + O_2 \rightarrow O_2\text{−Fe(II)Blm} \quad (2)$$

$$O_2\text{−Fe(II)Blm} + e^- + H^+ \rightarrow HO_2^-\text{−Fe(III)Blm} \quad (3)$$

or by the direct reaction of H_2O_2 with Fe(III)Blm, reacts with

* To whom correspondence should be addressed.
† E-mail: chikira@chem.chuo-u.ac.jp.
‡ Chuo University.
§ Medical College of Wisconsin.
∥ E-mail: petering@csd.uwm.edu.
⊥ University of Wisconsin−Milwaukee.

(1) (a) Umezawa, H.; Maeda, K.; Takeuchi, T.; Okami, Y. *J. Antibiot., Ser. A* **1966**, *19*, 200−209. (b) Sausville, E. A.; Peisach, J.; Horwitz, S. B. *Biochemistry* **1978,** *17*, 2740−2746. (c) Hecht, S. M. *Acc. Chem. Res.* **1986**, *19*, 383−391. (d) Stubbe, J.; Kozarich, J. *Chem. Rev.* **1987**, *87*, 1107−1136. (e) Petering, D. H.; Byrnes, R. W.; Antholine, W. E. *Chem. Biol. Interact.* **1990**, *73*, 133−182. (f) Petering, D. H.; Mao, Q.; Li, W.; DeRose, E.; Antholine, W. E. In *Metal Ions in Biological Systems*; Sigel, S., Sigel, H., Eds.; Marcel Dekker: New York, 1996; Vol. 33, pp 619−947. (g) Burger, R. M. *Chem. Rev.* **1998**, *98*, 1153−1169. (h) Claussen, C. A.; Long, E. C. *Chem. Rev.* **1999**, *99*, 2797−2816.

(2) (a) Nagai, K.; Suzuki, H.; Tanaka, N.; Umezawa, H. *J. Antibiot.* **1969**, *22*, 569−573. (b) Absalon, M. J.; Wu, W.; Kozarich, J. W.; Stubbe, J. *Biochemistry* **1995**, *34*, 2076−2086. (c) Steighner, R. J.; Povirk, L. F. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 8350−8354. (d) Sugiyama, H.; Sera, T.; Dannoue, Y.; Saito, I. *J. Am. Chem. Soc.* **1991**, *113*, 2290−2295.

(3) (a) Byrnes, R. W.; Petering, D. H. *Radiat. Res.* **1993**, *134*, 343−348. (b) Byrnes, R. W.; Petering, D. H. *Radiat. Res.* **1994**, *137*, 162−170.

(4) (a) Takita, T.; Muraoka, Y.; Nakatani, T.; Fujii, A.; Umezawa, Y.; Naganawa, H.; Umezawa, H. *J. Antibiot.* **1978**, *31*, 801−804. (b) Iitaka, Y.; Nakamura, H.; Nakatani, T.; Muraoka, Y.; Fujii, A.; Takita, T.; Umezawa, H. *J. Antibiot.* **1978**, *31*, 1070−1072. (c) Oppenheimer, N. J.; Rodriguez, L. O.; Hecht, S. M. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 5616−5620. (d) Akkerman, M. A. J.; Neijman, E. W. J. F.; Wijmenga, S. S.; Hilbers, C. W.; Bermel, W. *J. Am. Chem. Soc.* **1990**, *112*, 7462−7474. (e) Lehmann, E. W.; Ming, L.-J.; Rosen, M. E.; Que, L., Jr. *Biochemistry* **1997**, *36*, 2807−2816.

10.1021/ic991365r CCC: $19.00
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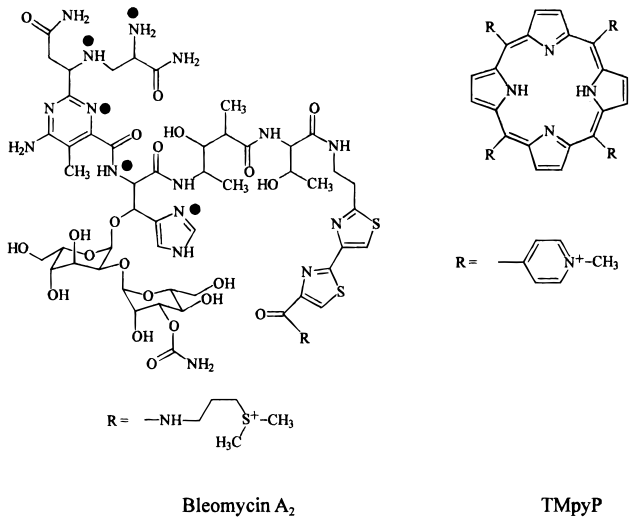

Figure 1. Structure of bleomycin A_2 (Blm) and 5,10,15,20-tetrakis-(1-methyl-4-pyridinio) porphyrin (TMpyP). Dots identify metal binding sites.

DNA without evidence of formation of iron species of higher oxidation state.[5a−c]

Information about reactions analogous to reactions 2 and 3 has emerged from studies in which Co(II) has been substituted for Fe(II).[6a−c] Dioxygenated Co(II)Blm has been observed by electron paramagnetic resonance (EPR) spectroscopy as an adduct resembling O_2−Co(III)Blm.[6a] An EPR study of the orientation of O_2−Co(II)Blm on DNA fibers showed that dioxygen is rigorously constrained to a plane approximately perpendicular to the DNA helix axis.[7] This result provided strong, direct support for the hypothesis that the metal domain interacts specifically with DNA.

Determination of the NMR structure of HO_2^-−Co(III)Blm showed that the metal domain and linker form a globular unit, that the metal domain is chirally organized about Co(III), and that the bithiazole tail is folded back over the axial binding site of peroxide.[8a,b] This structure also suggested that the metal domain as well as the DNA domain might associate with DNA. Recent analysis of HO_2^-−Co(III)Blm bound to DNA oligomers containing specific sites of binding, 5′-GC-3′ or 5′-GT-3′, demonstrated that these metallobleomycin species associate with either site through both DNA and metal domains.[9a−c] The bithiazole intercalates between the base pairs on the 3′ side of the site of specification and the pyrimidinyl ligand to Co(III) forms two hydrogen bonds to the guanine base in the minor groove.[9a,c]

A key question is whether FeBlm and CoBlm species bind to DNA in the same way. It has been shown that when ON−Fe(II)Blm binds to calf thymus DNA, the solution EPR spectrum progressively changes to a second spectrum as the base pair to drug ratio increases.[10a−c] This finding reveals that DNA perturbs the nitrosyl−iron site, suggesting that the metal domain of this species also interacts with DNA. With the use of synthetic DNA oligomers differing only in specific 5′-GC-3′ and nonspecific 5′-AT-3′ sites, it has also been demonstrated that perturbation of the ON−Fe(II)Blm spectrum only results from binding to the specific site.[10c] Similarly, the high field g value of the EPR spectrum of Fe(III)Blm shifts only when it binds to the DNA oligomer containing the specific site.[10c] Thus, the metal domain of both of these structures is altered when the drug associates with specific sites of binding.

To examine the linkage between Co- and FeBlms further, the orientation of ON−Fe(II)Blm and Fe(III)Blm on DNA fibers has been investigated in a study that parallels the one carried out with O_2−Co(II)Blm.[7] In addition, comparison of ON−Fe(II)Blm with a nitrosyl−iron porphyrin system has been carried out to demonstrate the unusual properties of ON−Fe(II)Blm when bound to oligomers of DNA. NO has long been used as a spin probe for monitoring the iron site in Fe(II)Blm.[10,11] The availability of oriented DNA fibers offers the opportunity to obtain new information on the stereospecificity of binding of iron complexes with DNA.

Experimental Section

Materials. Salmon sperm DNA was purchased from Sigma Chemical Co. Bleomycin was a gift from Nippon Kayaku Ltd. The solution of Fe(III)Blm was prepared by dissolving $Fe(III)Cl_3$ and a slight excess of Blm in water and adjusting the pH to 7.0. The Fe(III) complex of TMpyP was synthesized according to the literature.[12] The other chemicals were of reagent grade and used without further purification.

Methods. A preparation of A-form DNA fibers with a metal complex has been described previously.[7,13,14] The molar ratio of metal complex to DNA base pairs in the solution was adjusted to 1:25. To prepare nitrosylated samples under anaerobic conditions, a quartz holder with the DNA fibers was placed in a Schlenk-type EPR tube (Figure 2). The other finger was filled with a small amount of water. After the tube was evacuated by freeze-and-thaw, it was filled with NO gas and sealed. The tube was kept at room temperature until the DNA changed from A- to B-form and the Fe(III) complex underwent a reductive nitrosylation. It took about 10 days until the nitrosylation process was complete. X-band EPR spectra were measured at different angles of Φ between the DNA fiber axis (Z_f) and the static magnetic field (**B**). The EPR spectra were measured on a JEOL FE-2XG spectrometer with 100 kHz field modulation amplitude. The magnetic field was calibrated with a NMR field meter EFM-2000 (ECHO Electronics Ltd.).

Simulation of EPR Spectra. EPR spectra of paramagnetic species on DNA fibers were calculated as described previously.[13] Various

(5) (a) Kuramochi, H.; Takahashi, K.; Takita, T.; Umezawa, H. *J. Antibiot.* **1981**, *34*, 576−582. (b) Burger, R. M.; Peisach, J.; Horwitz, S. B. *J. Biol. Chem.* **1981**, *256*, 11636−11644. (c) Westre, T. E.; Loeb, K. E.; Zaleski, J. M.; Hedman, B.; Hodgson, K. O.; Solomon, E. I. *J. Am. Chem. Soc.* **1995**, *117*, 1309−1313.

(6) (a) Sugiura, Y. *J. Am. Chem. Soc.* **1980**, *102*, 5216−5221. (b) Xu, R. X.; Antholine, W. E.; Petering, D. H. *J. Biol. Chem.* **1992**, *267*, 944−949. (c) Xu, R. X.; Antholine, W. E.; Petering, D. H. *J. Biol Chem.* **1992**, *267*, 950−955.

(7) Chikira, M.; Antholine, W. E.; Petering, D. H. *J. Biol. Chem.* **1989**, *264*, 21478−21480.

(8) (a) Xu, R. X.; Nettesheim, D.; Otvos, J. D.; Petering, D. H. *Biochemistry* **1994**, *33*, 907−916. (b) Wu, W.; Vanderwall, D. E.; Lui, S. M.; Tang, X.-J.; Turner, C. J.; Kozarich, J. W.; Stubbe, J. *J. Am. Chem. Soc.* **1996**, *118*, 1268−1280.

(9) (a) Wu, W.; Vanderwall, D. E.; Turner, C. J.; Kozarich, J. W.; Stubbe, J. *J. Am. Chem. Soc.* **1996**, *118*, 1281−1294. (b) Mao, Q.; Fulmer, P.; Li, W.; DeRose, E. F.; Petering, D. H. *J. Biol. Chem.* **1996**, *271*, 6185−6191. (c) Wanderwall, D. E.; Lui, S. M.; Wu, W.; Turner, C. J.; Kozarich, J. W.; Stubbe, J. *Chem. Biol.* **1997**, *4*, 373−387.

(10) (a) Antholine, W. E.; Petering, D. H. *Biochem. Biophys. Res. Commun.* **1979**, *91*, 528−533. (b) Kennedy, M. C.; Antholine, W. E.; Li, W.; Mao, Q.; Petering, D. H. *Inorg. Chim. Acta* **1995**, *240*, 535−540. (c) Fulmer, P.; Zhao, C.; Li, W.; DeRose, E.; Antholine, W. E.; Petering, D. H. *Biochemistry* **1997**, *36*, 4637−4374.

(11) (a) Suguira, Y. *J. Am. Chem. Soc.* **1980**, *102*, 5208−5215. (b) Suguira, Y.; Takita, T.; Umezawa, H. *J. Antibiot.* **1981**, *34*, 249−251. (c) Suguira, Y.; Suzuki, T.; Muraoka, Y.; Umezawa, Y.; Takita, T.; Umezawa, H. *J. Antibiot.* **1981**, *34*, 1232−1236.

(12) Hambright, P.; Fleisher, E. B. *Inorg. Chem.* **1970**, *9*, 1757−1761 and references therein.

(13) Chikira, M.; Suda, S.; Nakabayashi, T.; Fujiwara, Y.; Ejiri, T.; Yoshikawa, M.; Kobayashi, N.; Shindo, H. *J. Chem. Soc., Dalton Trans* **1995**, 1325−1331.

(14) (a) Harada, W.; Nojima, T.; Shibayama, A.; Ueda, H.; Shindo, H.; Chikira, M. *J. Inorg. Biochem.* **1996**, *64*, 273−285. (b) Chikira, M.; Inoue, M.; Nagane, R.; Harada, W.; Shindo, H. *J. Inorg. Biochem.* **1997**, *66*, 131−139.

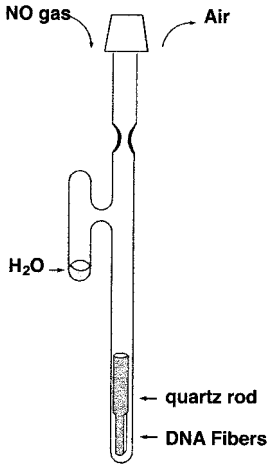

Figure 2. Schematic for apparatus for nitrosylation of an Fe(II) complex on DNA fibers.

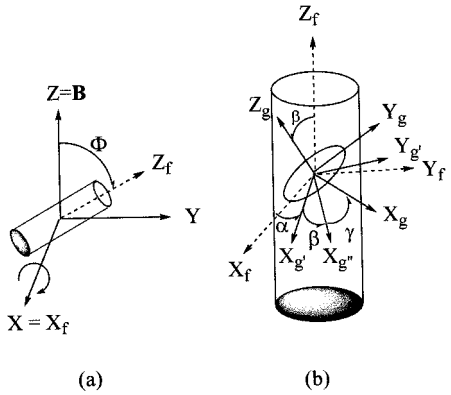

Figure 3. Coordinate axis system: (a) DNA fiber in a laboratory frame, where **B** is the static magnetic field and Φ is the angle between the fiber axis Z_f and **B**; (b) **g** tensor axes (X_g, Y_g, Z_g) in a DNA fiber axis system (X_f, Y_f, Z_f), where (α, β, γ) are Eulerian angles connecting (X_f, Y_f, Z_f) and (X_g, Y_g, Z_g).

coordinate axis systems used for the simulation are defined in Figure 3. The resonance magnetic fields for nitrosyl−Fe(III) complexes were calculated by a perturbation method to the second order for the nitrogen hyperfine interactions, assuming that the principal axes of **g** and **A** tensors are coaxial with each other. The orientation of a **g** tensor is represented by three eulerian angles α, β, and γ. It was assumed that α is totally random on the DNA fibers, whereas β and γ are distributed around specific angles. The distribution functions were represented by Gaussian functions with a standard deviation $\Delta\beta$ or $\Delta\gamma$. EPR line shapes for frozen solutions were obtained by replacing the distribution functions by unity.

Results

Orientation of Fe(III)Blm on DNA Fibers. Figure 4 shows EPR spectra of Fe(III)Blm in a frozen solution of pH 7 and in a DNA pellet. A shoulder appeared at the high field side of the g_z signals (2.45) for low-spin Fe(III)Blm in the DNA pellet (Figure 4b). The EPR spectrum for the DNA pellet was reproduced assuming the presence of two low-spin species in the ratio of 1:0.2 with $g_z = 2.45$ and 2.40, respectively (Figure 4c). The other parameters are shown in the caption of Figure 4. Primarily, g_z of the main species shifts from 2.42 for randomly oriented Fe(III)Blm in frozen solution to 2.45 upon binding DNA.

Fe(III)Blm bound to A-form DNA fibers fabricated from the pellet had a considerable amount of high-spin Fe(III) species as shown in Figure 5a. The line shape of the high-spin species

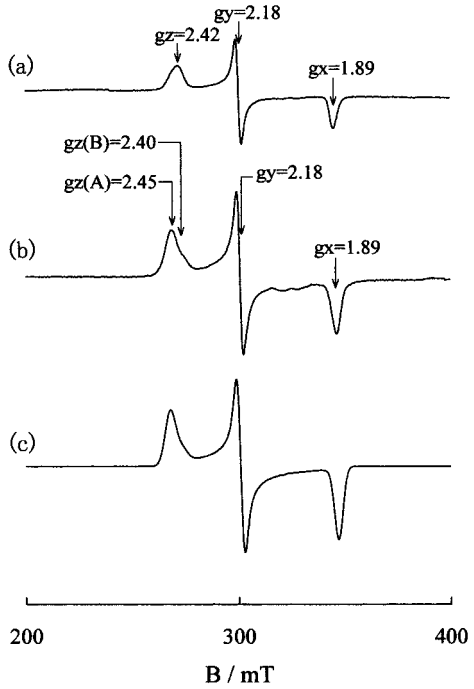

Figure 4. EPR spectra of Fe(III)Blm: (a) frozen solution at pH 7.0 and 123 K; (b) DNA pellet at 123 K; (c) calculated spectrum. Parameters for the calculated spectrum are the following. For species A, $g_x = 1.89$, $g_y = 2.18$, $g_z = 2.45$, and for species B, $g_x = 1.89$, $g_y = 2.18$, $g_z = 2.40$. The microwave frequency is 9.180 GHz. The ratio of the amount of A to B is 1:0.2.

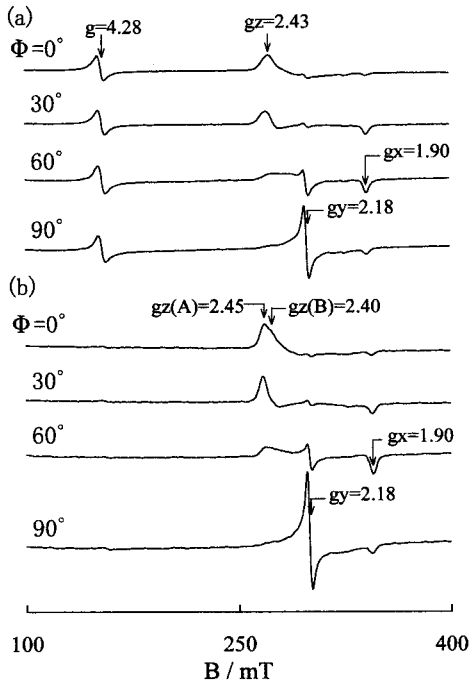

Figure 5. EPR spectra of Fe(III)Blm: (a) A-form DNA fibers at 123 K; (b) B-form DNA fibers at 123 K.

did not change with Φ, while the line shape of the low-spin species depended upon Φ. For the low-spin species, g_z predominated at $\Phi = 0°$ whereas g_y was observed at $\Phi = 90°$. Thus, the low-spin Fe(III)Blm was stereospecifically well-oriented on the DNA fiber, although there was a small amount of randomly oriented low-spin Fe(III)Blm, as indicated by the presence of signals from all three g values in a single spectrum. It should be noted that the g_z of the main low-spin species in

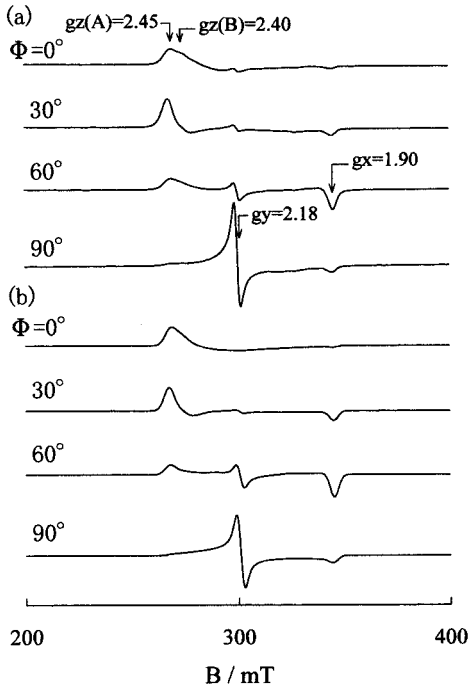

Figure 6. EPR spectra of Fe(III)Blm: (a) B-form DNA fibers at 123 K; (b) calculated spectra. Magnetic parameters used for the simulation are the following. For species A, $g_x = 1.90$, $g_y = 2.18$, $g_z = 2.45$, $\beta = 30°$, $\Delta\beta = 20°$, $\gamma = 10°$, and $\Delta\gamma = 40°$. For species B, $g_x = 1.90$, $g_y = 2.18$, $g_z = 2.40$, $\beta = 25°$, $\Delta\beta = 20°$, $\gamma = 10°$, and $\Delta\gamma = 40°$. The microwave frequency is 9.180 GHz. The ratio of A to B is 1:0.2.

the DNA pellet changed from 2.45 to 2.43 in the A-form DNA fiber. Changing the DNA fibers from A- to B-form resulted in an alteration in the EPR line shapes (Figure 5b). The high-spin signal almost disappeared and the peak of the g_z signal at $\Phi = 0°$ for the low-spin signals displayed a shoulder as in EPR spectra of the DNA pellet. The observed spectra of the low-spin species could be reproduced, assuming the presence of two species with the same pair of g_z values as those estimated for the DNA pellet ($g_z = 2.45$ and 2.40) and a concentration of 1:0.2 (Figure 6). Slightly different β values of 30° and 25° and the same γ value of 10° were chosen for the two species, respectively. Shields and McGlumphy published similar results for Fe(III)Blm on DNA fibers that were fabricated with a method different from that used in this experiment.[15] Although the second species was neglected in the previous simulations, their conclusion of the orientation of the main species of low-spin Fe(III)Blm almost coincides with the present results.

Orientation of ON−Fe(II)Blm on DNA Fibers. The EPR spectrum of Fe(III)Blm was broadened at room temperature and not detected. In contrast, the EPR spectrum of ON−Fe(II)Blm was readily detected at room temperature (Figure 7). The spectra of ON−Fe(II)Blm immobilized on DNA at room temperature are the first that have ever been recorded under physiological conditions. The EPR spectrum of ON−Fe(II)Blm on B-form DNA changes with Φ. At $\Phi = 0°$, only intensity from the middle g value, g_z, was detected. In nitrosyl−Fe(II) complexes, the g value at 2.006 is the g_z value and the low-field g value at 2.06 is g_x. The g_y value varies from greater than g_z to less than g_z, depending upon the nitrosyl−iron complex.[16] A value of 1.97 was obtained for g_y for ON−Fe(II)Blm bound on the fiber. Spectral intensities for the g_x and g_y regions were greater for Φ equal to 60°, and especially 90°.

EPR spectra of ON−FeBlm on B-form DNA fibers at 123 K were better resolved as shown by the sharper lines for the

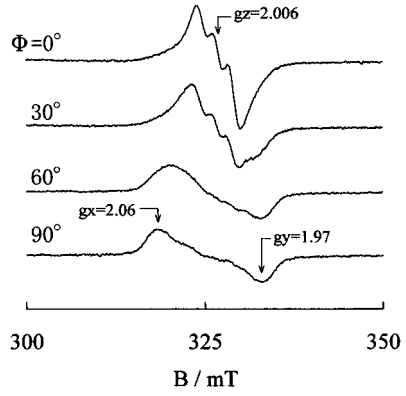

Figure 7. EPR spectra of ON−Fe(II)Blm on B-form DNA fibers at room temperature.

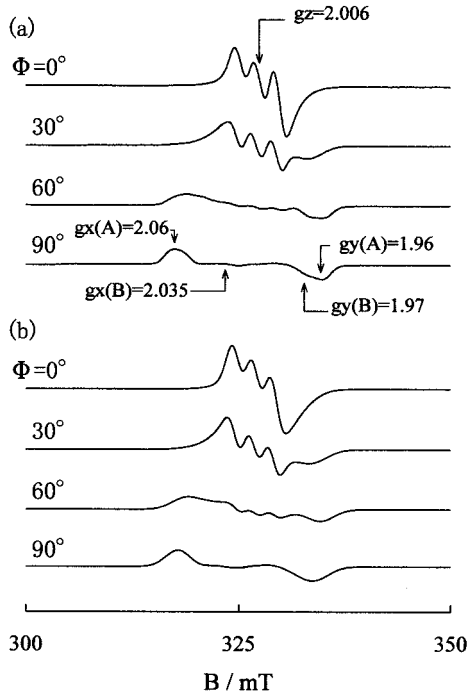

Figure 8. EPR spectra of ON−Fe(II)Blm on B-form DNA fibers: (a) 123 K; (b) calculated spectra. For the calculated spectra, the magnetic parameters are the following. For species A, $g_x = 2.06$, $g_y = 1.96$, $g_z = 2.006$, $A_x = A_y = 0.0005$ cm^{-1}, $A_z = 0.0023$ cm^{-1}, $\beta = 20°$, $\Delta\beta = 20°$, $\gamma = 90°$, and $\Delta\gamma = 30°$. For species B, $g_x = 2.035$, $g_y = 1.97$, $g_z = 2.006$, $A_x = A_y = 0.0005$ cm^{-1}, $A_z = 0.0023$ cm^{-1}, $\beta = 25°$, $\Delta\beta = 20°$, $\gamma = 90°$, and $\Delta\gamma = 30°$. The microwave frequency is 9.178 GHz. The ratio of A to B is 1:0.2.

nitrogen triplet hyperfine structure in the g_z region (Figure 8a); however, the changes in the spectra with a change in Φ value were very similar to those recorded at room temperature (Figure 7). Thus, very little change was detected in the g values and the hyperfine coupling constants as a function of temperature. The calculated spectra for ON−Fe(II)Blm on the fiber as a function of Φ are shown in Figure 8b. These spectra were obtained assuming the presence of two species in the concentration ratio of 1:0.2, as in the case of Fe(III)Blm on B-form DNA

(15) Shields, H.; McGlumphy, C. *Biochim. Biophys. Acta* **1984**, *800*, 277−281.

(16) (a) Palmer, G. Electron Paramagnetic Resonance of Hemoproteins. In *The Porphyrins*; Dolphin, D., Ed; Academic Press: New York, 1979; Vol. IV, pp 313−353. (b) Palmer, G. Electron Paramagnetic Resonance. In *Advances in Inorganic Biochemistry*; Eichorn, G. L., Marzilli, L. G., Eds.; Elsevier/North-Holland: New York, 1979; Vol. 2, pp 153−182.

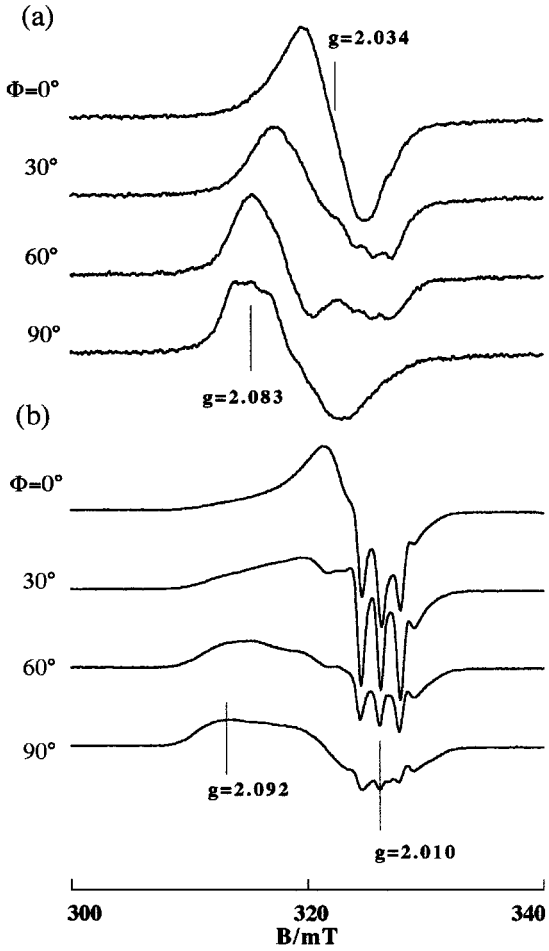

Figure 9. EPR spectra of ON−Fe(II)TMpyP on B-form DNA fiber at (a) room temperature and (b) 123 K.

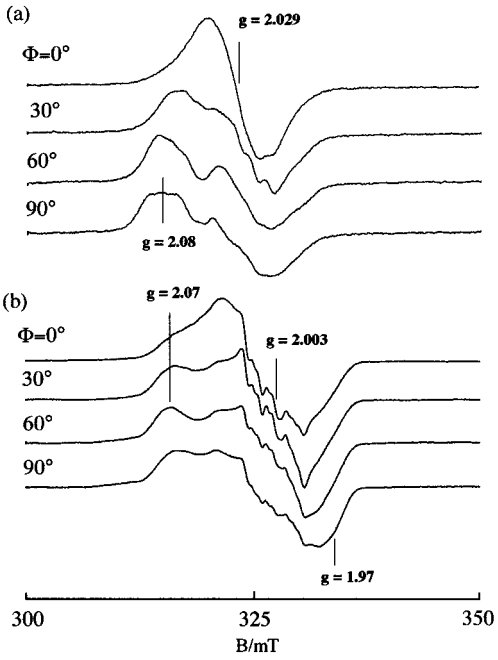

Figure 10. EPR spectra of ON−Fe(II)TMpyP−Im on B-form DNA fiber at (a) room temperature and (b) 123 K.

fibers, i.e., one with $g_x = 2.060$, $g_y = 1.960$, $g_z = 2.006$, $\beta = 20°$, and $\gamma = 90°$ and the other with $g_x = 2.035$, $g_y = 1.970$, $g_z = 2.006$, $\beta = 25°$, and $\gamma = 90°$. The other parameters are shown in the caption of Figure 8.

Orientation of ON−Fe(II)TMpyP and ON−Fe(II)TMpyP−(Im) on DNA Fibers. We have reported that Fe(III)TMpyP and Fe(III)TMpyP−$(Im)_2$ on A-form DNA fibers are oriented as determined by changes in the EPR spectrum at 123 and 25 K, respectively, as the fibers rotated.[13] Simulations of the high-spin EPR signal for Fe(III)TMpyP on the fiber were consistent with an angle of 45° formed by the fiber axis and the axis perpendicular to the heme, g_z. The EPR spectrum for Fe(III)TMpyP on the fiber switched to a low-spin signal, Fe(III)TMpyP−$(Im)_2$, in the presence of excess imidazole. Fe(III)TMpyP−$(Im)_2$ was also oriented on the fiber, but the angle formed by the fiber axis and the axis perpendicular to the heme was 90°. By contrast to ON−Fe(II)Blm, the EPR spectra for ON−Fe(II)TMpyP in the presence and absence of excess imidazole varied substantially as the temperature was dropped from room temperature to 123 K (Figures 9 and 10). At both temperatures there was a change in the EPR spectrum as Φ varied, indicating that the nitrosyl heme is oriented on B-form DNA fibers at each temperature. The EPR line shapes of ON−Fe(II)TMpyP and ON−Fe(II)TMpyP−Im observed at room temperature were rather similar in that only a broad absorption was observed in the g_y region at $\Phi = 0°$ and the three-line hyperfine structure from NO nitrogen was resolved in the g_x absorption at $\Phi = 90°$. However, those at 123 K were quite different from each other and the resolution of the hyperfine structure was better. The nine lines from the triplet of triplet pattern due to a 2.1 mT coupling from the nitrogen of NO and a 0.6 mT splitting from the nitrogen in the proximal imidazole were recognizable in the g_z region for ON−Fe(III)-TMpyP−Im at 123 K but not at room temperature. Moreover, the g values changed as the temperature was altered. This was most easily recognized for ON−Fe(II)TMpyP−Im in the high-field region where the spectral intensity was greater and the high-field g value was partially resolved from the g_z value.

Discussion

The present experiments have examined the orientation of iron bleomycin and iron porphyrin complexes on salmon sperm DNA fibers aligned in the magnetic field of an EPR spectrometer. Besides providing new information about the geometry of these species when associated with DNA, the results with the FeBlm structures can be compared with previous findings about CoBlmDNA adducts to determine whether all of the metal domains in these adducts display similar binding geometries.

A previous EPR study of the orientation of O_2−Co(II)Blm bound to salmon sperm B-DNA revealed that the dioxygen ligand is rigorously constrained to a plane nearly perpendicular to the DNA helix axis.[7] Approximating the Co−O−O angle as 125°, the equatorial plane of the octahedral Co(II) center makes an angle of about 55° with the helix axis.

A similar picture emerges from unpublished NMR structural work on HO_2−Co(III)BlmA_2 bound to d(GGAAGCTTCC)$_2$ (DNAa) as well as from the published structures.[9a,17] The in-plane ligands make an angle of about 60° with the DNA helix axis. According to this calculation, the angle between the normal to the metal coordination plane and the double helix axis is 30−35° in both structures. This strongly suggests that the geometric orientation of the metal domain in the two CoBlm structures is similar. The metal domain and peptide linker form a globular unit in HO_2−Co(III)Blm that is flexibly tethered to the bithiazole tail domain according to a comparison of the free

(17) Zhao, C.; Mao, Q.; DeRose, E.; Blumenfeld, A.; Petering, D. H. *J. Inorg. Biochem.* **1999**, *74*, 349−349.

and DNA-bound structures.[8,9] As such, the conformational relationship between the metal domain and DNA is not fixed by the intercalative interaction between the bithiazole group and its base pair neighbors. Thus, it is hypothesized that both the dioxygen and peroxy adducts of CoBlm utilize the same hydrogen-bonding interactions between the pyrimidinyl ligand of the drug and guanine at 5′-G-pyrimidine-3′ sites of DNA to constrain the conformation of the metal domain with respect to DNA.

Stereospecificity of Fe(III)Blm on DNA Fibers. Free Fe(III)Blm is a mixture of high- and low-spin forms.[18] Previous experiments with Fe(III)Blm interacting with DNAa and a related structure that does not contain a specific binding site, $d(GGAAATTTCC)_2$ (DNAb), demonstrated that only low-spin Fe(III)Blm is observed when it is bound to the DNA oligomer bearing the specific site.[10c] When associated with DNAb, the iron center is almost exclusively high-spin.[10c] On the basis of this and other evidence, it was concluded that the low-spin form of Fe(III)Blm bound to natural DNA samples is a signature for the drug bound to specific 5′-G-pyrimidine-3′ sites. Low-spin Fe(III)BlmDNA is also considered as an indication of site-specific binding in the present study.

Low-spin Fe(III)Blm was detected when bound to B-form DNA, whereas the high-spin drug was also observed on A-form DNA fibers. By analogy to low-spin heme proteins,[16a,b] g_z of the low-spin Fe(III)Blm is assumed to be the axial direction and g_y and g_x are the in-plane values. Then the unpaired electron is primarily in d_{yz} and mixing of d_{xz} and d_{xy} accounts for the shift from the spin-only value of 2.00. The angle β of about 25−30° between the fiber axis and g_z of the two low-spin species was almost equal to the angle between the DNA fiber axis and the axial direction of O_2−Co(II)Blm bound to the same type of DNA.[7] In our definition of Eulerian angles, the γ value of 0° corresponds to the orientation of the g_x axis in the plane defined by the fiber axis and g_z axis. An estimated γ value of 10° suggests that the rhombic axes are also fixed on the DNA near the plane defined by the fiber axis and the g_z axis, although $\Delta\gamma$ of 40° is larger than $\Delta\beta$ of 20°. From this analysis, the metal domain of low-spin Fe(III)Blm achieves the same orientation that is found in O_2−Co(II)Blm−DNA and HO_2−Co(III)Blm−DNAa. These results suggest that all three of these metallodrug species bind to 5′-G-pyrimidine-3′ sites on DNA in the same way. If β does not change upon binding of hydrogen peroxide in the sixth axial coordination position of Fe(III)Blm and if the Fe−O_2−H bond angle is about 125° as in HO_2−Co(III)−Blm, the peroxide group will be aligned approximately perpendicular to the helix axis in the same orientation as seen in the analogous cobalt complex.

The similar Φ dependence of the EPR spectra of low-spin Fe(III)Blm on A- and B-form DNA shown in Figure 5 suggests that the orientation of the low-spin Fe(III)Blm binding site does not change despite the conformational differences in these DNA samples. A- and B-form DNA differ substantially in the depth of their minor grooves, where FeBlm is expected to bind, and in the angle that the base pairs make with the helix axis.[19] The approximate 20° angle that both the base pairs of A-form DNA and the in-plane ligands in Fe(III)Blm makes with the helix axis suggests that hypothetical hydrogen bonding between the drug pyrimidinyl group and the site-specifying guanine residue can be established without the angular distortion seen in this interaction in the structure of HO_2−Co(III)Blm with B-form DNA.[9,17] It is anticipated that these results with A-form DNA can be transferred to the interaction of FeBlm with A-form RNA.[20]

Significant amounts of high-spin Fe(III)Blm were detected only when Fe(III)Blm was on A-form DNA fibers. The high-spin species on A-form DNA fibers reversibly changed to a low-spin species on B-form DNA fibers, indicating conformational changes at the metal binding site by the structural shift from A- to B-DNA. Possibly, when the DNA is changed from the B to A conformation, Fe(III)Blm binds less specifically to 5′-G-pyrimidine-3′ sites because of difficulty in intercalating between base pairs adjacent to these sites with the bithiazole tail and at the same time stably binding to the dinucleotide site through the metal domain. The facile change in spin state of free Fe(III)Blm as it binds to DNAa or DNAb or to A- or B-form DNA cannot be explained at present.[10c] A simple explanation is that the coordination sphere of Fe(III)Blm rearranges in the transition from high to low spin.

The g value of 4.28 for high-spin Fe(III)Blm corresponds to the case of maximal rhombic distortion of the ligand field around the metal ion, $E/D = {}^1/_3$, where D and E are the spin Hamiltonian parameters representing anisotropic spin−spin interaction.[16b] Because the $g = 4.28$ signal is comprised of all g_x, g_y, and g_z transitions, the apparent independence of its intensity on Φ does not necessarily mean that the high-spin species was randomly oriented on the fibers.

EPR Spectroscopy of ON−Fe(II)Blm and ON−Fe(II)-TMpyP in the Absence and Presence of DNA. There is an abundance of literature on the EPR spectra of nitrosyl−Fe(II) porphyrins in frozen solutions and on the use of nitric oxide as a probe of hemoprotein structure.[21a−g] Since one of the g values in these complexes is close to 2.00 (the free electron value), the unpaired electron is assumed to occupy an orbital with d_{z^2} character, and this orbital is likely to be involved in binding a ligand trans to NO.[16a,21a] The observed in-plane anisotropy of the EPR spectra has been attributed to the difference in the interaction between d_{yz} and d_{zx} with the $p\pi$ orbital of a ligand trans to NO or with π^* orbitals of NO. This model is also used to explain the observed superhyperfine splitting due to an imidazole nitrogen-coordinated trans to the NO group. On the other hand, Waleh et al. calculated the ground electronic structure of nitrosyl ferrous heme by a semiempirical INDO-SCF procedure and proposed that two electronic states corresponding to the formal assignment of the unpaired electron to either NO or iron $d\pi$ atomic orbitals are nearly degenerate.[21e] They attributed the observed temperature dependence of the EPR spectra to these two low-lying electronic states, the energy levels of which are modulated sensitively by the environment of the nitrosyl heme group.

Despite the different explanations for the assignment of the unpaired electron spin orbitals, many experimental results have been accumulated on the correlations between the g or A values and the heme environment. If the axial position is vacant or

(18) Albertini, J. P.; Garnier-Suillerot, A. *Biochemistry* **1984**, *23*, 47−53.
(19) Cantor, C. R.; Schimmel, P. R. *Biophysical Chemistry*; W. H. Freeman and Co.: San Francisco, CA, 1980; Part 1, p 179.
(20) Carter, B.; DeVroom, E.; van der Marel, G. A.; van Boom, J. H.; Hecht, S. M. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9373−9377.
(21) (a) Kon, H.; Kataoka, N. *Biochemistry* **1969**, *8*, 4757−4762. (b) Yoshimura, T.; Ozaki, T.; Shintani, Y.; Watanabe, H. *Arch. Biochem. Biophys.* **1979**, *193*, 301−313. (c) Hori, H.; Ikeda-Saito, M.; Yonetani, T. *J. Biol. Chem.* **1981**, *256*, 7849−7855. (d) Stone, J. R.; Sands, R. H.; Dunham, W. R.; Marletta, M. A. *Biochem. Biophys. Res. Commun.* **1995**, *207*, 572−577. (e) Waleh, A.; Ho, N.; Chantranupong, L.; Loew, G. H. *J. Am. Chem. Soc.* **1989**, *111*, 2767−2772. (f) Decatur, S. M.; Franzen, S.; DePillis, G. D.; Dyer, R. B.; Woodruff, W. H.; Boxer, S. G. *Biochemistry* **1996**, *35*, 4939−4944. (g) Migita, C. T.; Salerno, J. C.; Masters, B. S. S.; Martasek, P.; McMillan, K.; Ikeda-Saito, M. *Biochemistry* **1997**, *36*, 10987−10992.

occupied by a weakly coordinating group, the three g values are $g_x \approx 2.10$, $g_y \approx 2.03$, and $g_z \approx 2.01$. When a strong basic group coordinates to the axial position, g_x, g_y, and g_z change in the ranges 2.06−2.09, 1.96−1.99, and 2.002−2.009, respectively.[21a−c] The three g values of 2.06, 2.006, and 1.98 observed for the main component of ON−Fe(II)Blm and 2.07, 2.002, and 1.97 for ON−Fe(II)(TMpyP)−Im at 123 K on the B-form DNA fibers correspond to the latter case. An A_z^N(NO) value of 2.3 mT for ON−Fe(II)Blm at 123 K also implies the presence of an axial ligand if the parameters behave as those for ON−Fe(II)(TMpyP)−Im. An A_z^N(NO) value of 1.7 mT observed for ON−Fe(II)(TMpyP) is expected in the absence of an axial ligand.[21a] It should be noted that these hyperfine parameters for nitrosylated Fe(II) porphyrins and many hemoproteins change with temperature.[21b] These changes have been attributed to the change in the geometry of NO on the porphyrins and/or to the presence of two distinct nearly degenerate, low-energy electronic states.[21b,e]

The observed g and A^N(NO) values for the main species of ON−Fe(II)Blm are consistent with the presence of a strong axial ligand trans to the NO. Nevertheless, a nitrogen coupling for the axial ligand is not resolved either in the absence or in the presence of DNA. This splitting can be seen in ON−Fe(II)(TMpyP)−Im bound to DNA or in hemoproteins in which the trans ligand is a nitrogen from an imidazole. One explanation for the absence of superhyperfine structure is that the specific molecular configuration increases the distribution of the unpaired electron in the d_{yz} orbital, which scarcely overlaps with a σ orbital of the axially coordinating nitrogen that has been proposed to be the primary amino nitrogen of the terminal β-aminoalanine.[4b,e,8]

The nonequivalent values for g_x and g_y for ON−Fe(II)Blm may be attributed to an in-plane anisotropy caused by iron coordination to nitrogens from four different groups (Figure 1). The spreading of these g values upon binding of ON−Fe(II)Blm to the DNA fiber suggests a further rhombic distortion due to an interaction of DNA with the in-plane ligand set. Such a perturbation might arise from a drug pyrimidinyl−guanine hydrogen bonding interaction as seen in HO_2−Co(III)BlmDNA.[9a,c] Indeed, in studies of the binding of ON−Fe(II)Blm to DNAa and DNAb, the rhombic distortion is primarily observed when site-specific binding is possible with DNAa.[10c] As a result, in present and previous experiments with natural DNA samples, the appearance of the perturbed EPR signal when ON−Fe(II)Blm binds to DNA is taken to indicate that the drug has formed a site-specific adduct. The presence of a minor g_x component at 2.035 with an estimated A^N(NO) of 2.3 mT of ON−Fe(II)BlmDNA (Figure 8) suggests that a small fraction of bound drug is not bound at specific sites.

Geometry of NO Complexes Bound to Oriented DNA Fibers. To examine the geometry of the NO complexes associated with DNA, NO was used to reduce DNA adducts of Fe(III)Blm, Fe(III)TMpyP, or Fe(III)(TMpyP)−$(Im)_2$. Then NO became bound to the Fe(II) complexes, either directly with the first two complexes or after displacement of one imidazole ligand in the case of the third complex. It should be noted that all these reactions took place in the B-form DNA fiber, indicating that the DNA fiber is a medium well-suited for some reactants to diffuse, reorient, and transfer electrons.

A β value of 20−25° for DNA-bound ON−Fe(II)Blm was estimated from the simulation of the EPR spectra in Figure 8. In turn, this value suggested that the conformational relationship between the metal domain and DNA remains nearly the same as that in Fe(III)BlmDNA. If the Fe−N−O bond angle is 125° (120° for sp^2 hybrids,[16] 135° for nitrosylcytochrome c oxidase,[22a] 142° for Fe(TPP)(1-MeIm)(NO)[22b]), then the bond axis for N−O can be nearly perpendicular to the fiber axis, making an angle of 75−80°.[22] According to this calculation, ON−Fe(II)Blm shares a similar conformation when binding to 5′-G-pyrimidine-3′ sites as Fe(III)Blm, O_2−Co(II)Blm, and HO_2−Co(III)Blm.

The estimated γ value of 90° for ON−Fe(II)Blm implies that the g_x axis is perpendicular to the plane defined by the fiber axis and the g_z axis. The difference in the estimated γ values between Fe(III)Blm and ON−Fe(II)Blm suggests that the in-plane anisotropy of the d orbitals in ON−Fe(II)Blm is caused not only by the coordination of the nitrogens from four different groups but also by a bent configuration of the Fe−N−O angle. The two factors that may contribute to the in-plane anisotropy make it difficult to assess the relationship between the γ value and the orientation of the NO. However, the $\Delta\gamma$ value of 30° indicates that a rotation of NO around the Fe−N(O) axis is restricted even at room temperature. How the EPR line shapes depends on γ is shown in Supporting Information.

This coincides with the experimental result that a large change in temperature did not affect the g and A values for ON−Fe(II)Blm bound to DNA fibers. Thus, there are no distinguishable high- and low-temperature Fe−NO geometries or two distinct nearly degenerate, low-energy electronic states as proposed for nitrosyl ferrous heme and for various ON−Fe(II)porphyrin complexes.[21b,e] This implies not only that the general conformation of the metal domain is determined upon binding to DNA but that the NO ligand is rigorously constrained in the adduct. In turn, this suggests that a cavity comprised of DNA and a vacant coordination site of Fe(II)Blm exerts a specific, potential field on NO that strongly restricts the orientation of the NO bond axes, even at room temperature. Molecular modeling of HO_2−Co(III)Blm bound to a specific DNA oligomer showed that the peroxide is held in place by hydrogen bonds with the penultimate oxygen.[9a] It is likely that these same hydrogen bonds are formed with nitrogen from NO to fix the geometry of the nitrosyl group in the ON−Fe(II)BlmDNA adduct.

The results with ON−Fe(II)Blm stand in contrast to the findings with ON−Fe(II)TMpyP bound to DNA in the absence and presence of imidazole shown in Figures 9 and 10. EPR spectra of DNA adducts of ON−Fe(II)TMpyP and ON−Fe(II)TMpyP−Im at room temperature are similar in line shape and Φ dependence. This indicates that the imidazole had little effect on the g or A values of ON−Fe(II)TMpyP−Im at room temperature. A considerable change in these values with temperature revealed a variation of the conformational orientation of these complexes.[21b,e] The porphyrin ligand is highly symmetrical in comparison with the metal domain of bleomycin and may be able to find alternative conformations with different temperature-dependent stabilities that permit oriented binding of the molecule to DNA.[23] Although low-temperature EPR spectra have not yet been simulated successfully, it is evident that the **g** tensor axis of ON−Fe(II)(TMpyP) on B-form DNA fibers is not randomly oriented at low temperature because not only the relative intensity of g_z but also those of g_x and g_y strongly depend on Φ as shown in Figures 9 and 10.

The different behavior of porphyrin and bleomycin molecules may result from the restrictive effect that DNA intercalation of the bithiazole tail has on the conformational freedom of the rest

(22) (a) Barlow, C.; Erecinska, M. *FEBS Lett*. **1979**, *98*, 9−12. (b) Piciulo, P. L.; Rupprecht, G.; Scheid, W. R. *J. Am. Chem. Soc*. **1974**, *96*, 5293−5295.
(23) Barnes, N. R.; Schreiner, A. F. *Inorg. Chem.* **1998**, *37*, 6935−6939.

of the bleomycin structure. Because the metal domain peptide linker behaves as a large, asymmetric structural unit anchored to the thermodynamically stronger intercalation site, it may have little freedom to adopt different conformations as the metal domain binds to specific sites in DNA.

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Supporting Information Available: Calculated EPR spectra of NO−Fe−(II)Blm on B-form DNA fibers for different γ values. This material is available free of charge via the Internet at http://pubs.acs.org.

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